PROPERTIES OF MARKETED FERTILIZERS

Physical and Chemical Characteristics of Mixed Fertilizers and Superphosphates Marketed in 1955–56

Part I. Forms and Solubility of Phosphorus

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Part II. Reaction, Particle Size, and Inert Matter Contents

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The distribution of phosphorus content among the water-soluble, available, and citrate-insoluble forms and information relative to the reaction, particle size, moisture, acid-insoluble ash and carbonate contents, and the acid- or non-acid-forming qualities of mixed fertilizers and superphosphates marketed in the United States during the 1955–56 fertilizer season were obtained and compared with data derived from a similar survey of fertilizers marketed in 1949–50. The portion of the total phosphorus in water-soluble form decreased from 46.9% in 1949–50 to 43.4% in 1955–56; the water-soluble portion of the available phosphorus decreased from 50.2 to 45.8%. In both periods the acid-insoluble ash and the calcium carbonate equivalent added as make-weight material or partially to offset the acid-forming character of mixed fertilizers amounted to approximately one eighth of the average fertilizer.

SOLID MIXED FERTILIZERS and superphosphates marketed in the United States during the 1955–56 fertilizer year were surveyed for the purpose of obtaining information on some of their physical and chemical characteristics. These papers present data on the distribution of the phosphorus content among the water-soluble, available, and citrate-insoluble forms and information relative to the reaction, particle size and moisture, acid-insoluble ash and carbonate contents, and the acid- or non-acid-forming qualities of mixed fertilizers and superphosphates by regions. They compare the results with data obtained in a similar survey of fertilizers marketed in 1949–50 (*4, 5*).

Design of Survey

To make the survey as representative as possible, the selection of mixed fertilizer samples from each major geographical region was based on the tonnage of the several grades marketed and the production of individual manufacturers. Scholl, Wallace, and Fox (*7*) reported that 97% of the 15,258,053 tons of mixtures consumed in 1953–54, the latest season for which data then were available, represented 176 different grades marketed in excess of 2500 tons each. In general, the 15 principal grades in each region were sampled in proportion to their marketed tonnage on the basis of one sample for each 30,000 tons. At least one sample was selected, however, for each such grade marketed in excess of 15,000 tons. Where additional samples were required, random selections were made from among grades marketed in excess of 2500 tons each.

Owing to the limited number of samples required for the West, South Central, Mountain, and Pacific regions, random selection of samples was made from among the principal grades on the basis of one sample for each 30,000 tons of mixtures marketed.

Estimates of the relative marketings of the several manufacturers in each region were derived largely from the number of official inspection samples drawn for each manufacturer as indicated by state fertilizer reports. The number of samples selected to represent each manufacturer then was based on one sample for each 30,000 tons of estimated marketings. Random selection of samples from additional manufacturers was made to provide the desired number of samples from the particular region.

On the basis of the above consideration state control officials were requested to supply, where possible, unground official inspection samples of specified grades of mixtures marketed by specified manufacturers. In some instances unground samples were not available, and in others the desired grade either could not be supplied as the product of the specified manufacturer or could not be supplied at all. In such cases the control official was asked to supply ground samples representing the desired grade and manufacturer, the desired grade marketed by another manufacturer, a randomly selected grade marketed by the initially selected manufacturer, or a grade representing both random selection of grade and manufacturer.

In addition, each control official was asked to supply for comparison three or four samples of superphosphate randomly selected as to grade and manufacturer.

State fertilizer control officials of 35 states cooperated by supplying 491 official inspection samples of mixed fertilizers representing 90 grades, 60 plant-nutrient ratios, and 160 manu-

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facturers. Two hundred thirty-nine or nearly 49% of the samples represented products marketed by 10 manufacturers. Five of the 491 samples represented N-K mixtures marketed in four grades and three plant-nutrient ratios by five manufacturers in four states of the South Atlantic region. The products of one of these manufacturers were not otherwise represented in the survey. Seventeen of the plant-nutrient ratios were represented by 47 grades and a total of 356 or 72.5% of the samples.

Thirty states supplied 103 official inspection samples of nine grades of superphosphate marketed by 54 manufacturers. Seventy-five samples represented three grades of normal superphosphate (18 to 20%) marketed in 26 states by 41 manufacturers and 28 represented six grades of concentrated superphosphate (42 to 49%) marketed in 12 states by 17 manufacturers. Thirty-one superphosphates represented products of 22 manufacturers not otherwise represented in the survey.

Distribution of both superphosphates and mixed fertilizers by regions in relation to the numbers of states, samples, grades, plant-nutrient ratios, and manufacturers is presented in Table I. Table II shows the distribution of samples among the P, N-P, N-K, P-K, and N-P-K classifications in relation to the number of manufacturers and the number of samples representing each manufacturer. Approximately one third of the superphosphates and one third of the mixed fertilizers collected were the products of five manufacturers. Sixteen grades—0-20-20, 2-12-12, 3-9-6, 3-9-9, 3-12-12, 4-12-12, 4-8-6, 4-8-8, 5-10-10, 6-12-12, 4-10-6, 4-10-7, 4-16-16, 5-20-20, 5-10-5, and 10-10-10 representing 11 nutrient ratios—were represented by at least 10 samples each for a total of 322 or nearly two thirds of the 486 phosphorus-containing mixtures.

PART I. FORMS AND SOLUBILITY OF PHOSPHORUS

Analytical Methods

Total, water-soluble, and citrate-insoluble P_2O_5 contents of the superphosphates and mixtures were determined in accordance with official methods of analysis adopted by the Association of Official Agricultural Chemists (*2*). As requested, nearly 80% of the mixtures were supplied in the unground condition. In these cases analyses were conducted on freshly ground portions of the samples.

Results. In agreement with earlier surveys, wider variations and lower mean values were observed in the water-soluble and in the available (total minus citrate-insoluble) portions of the total phosphorus in mixtures than in superphosphates. Analytical data for each region are summarized in Table III in relation to superphosphates and to classes of mixtures.

Water-Soluble Portion of Total Phosphorus. The average value for the water-soluble portion of the total P_2O_5 content of the superphosphates is substantially the same as in the 1949–50 survey (*5*). The averages for the N-P, N-P-K, and all mixtures are 3.1 to 4.2 percentage units lower and the average for the P-K mixtures 17.1 units higher.

Available Portion of Total Phosphorus. In comparison with the 1949–50 survey, the average values for superphosphates and N-P mixtures are substantially the same and those for P-K, N-P-K, and all mixtures from 1.3 to 2.0 percentage units higher.

Water-Soluble Portion of Available Phosphorus. In relation to the 1949–

Table I. Distribution of Samples in Relation to Region and Numbers of States, Grades, Plant-Nutrient Ratios, and Manufacturers

	Superphosphates					Mixed Fertilizers							
		Grades					Grades						
Region	States	Normal	Concd.	Manufacturers	Samples	States	N–P	N–K	P–K	N–P–K	Plant-nutrient ratios	Manufacturers	Samples
	Number												
New England	3	2	1	9	12	3	...	...	2	12	10	13	16
Middle Atlantic	6	2	...	14	21	5	...	...	2	17	13	26	59
South Atlantic	4	2	1	9	12	5	...	4	3	36	30	64	168
East North Central	3	2	...	10	12	5	...	...	3	13	10	31	111
West North Cent.	5	2	3	16	19	5	4	...	2	19	14	29	47
East South Cent.	3	1	2	8	10	4	...	...	1	15	13	28	62
West South Cent.	1	1	2	4	4	3	...	...	...	10	7	12	14
Mountain	4	2	4	6	12	4	3	...	...	3	6	8	10
Pacific	1	1	...	1	1	1	2	...	...	2	3	3	4
U. S.	30	3	6	54	103	35	9	4	6	71	60	160	491

Table II. Distribution of Samples in Relation to Manufacturers

	Superphosphates						Mixed Fertilizers									
	Normal		Concentrated		All		N–P		N–K		P–K		N–P–K		All	
No. of Samples per Manufacturer	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples	Manufacturers	Samples
	Number															
1	27	27	8	8	35	35	13	13	5	5	12	12	94	94	102	102
2	7	13	5	9	11	22	2	4	...	...	5	10	26	52	30	60
3	1	3	1	3	2	6	..	..	...	...	..	..	5	15	6	18
4	..	..	..	..	...	...	..	..	...	...	1	4	3	12	1	4
5	1	3	1	2	1	5	..	..	...	...	..	..	4	20	6	30
6–10	5	29	2	6	5	35	..	..	...	...	..	..	5	40	5	38
11–20	..	..	..	..	...	...	..	..	...	...	..	..	3	37	4	49
>20	..	..	..	..	...	...	..	..	...	...	..	..	6	173	6	190
Total	41	75	17	28	54[a]	103	15	17	5	5	18	26	146	443	160[b]	491

[a] 32 manufacturers marketed 72 superphosphates and 282 mixtures; 22 marketed 31 superphosphates but no mixtures.
[b] 128 manufacturers marketed 209 mixtures but no superphosphates.

50 survey the average value for the water-soluble portion of the available P_2O_5 of superphosphates is substantially identical. The averages for the N-P, N-P-K, and all mixtures are 3.0 to 5.2 percentage units lower, and the average for the P-K mixtures 16.9 units higher.

Relative Solubility and Availability of Phosphorus in Superphosphates and in Mixtures

A greater proportion of the available P_2O_5 is present in water-soluble form in superphosphates than in mixed fertilizers. Similarly, a greater proportion of the total P_2O_5 is present in available form in superphosphates than in mixtures.

The solubility and availability characteristics of the P_2O_5 differed appreciably among the various superphosphates and mixtures as well as between the two classes of materials themselves. The

Table III. Total Phosphorus and Portion of Total Phosphorus in Water-Soluble and in Available Form, and of Available Phosphorus in Water-Soluble Form

Region and Item	No. of Samples	Total P_2O_5, %		Portion of Total P_2O_5, %				Portion of Available P_2O_5 in Water-Soluble Form, %	
				Water-Soluble Form		Available Form			
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
New England									
All superphosphates, 18–46%	12	18.7–46.7	22.7	72.6–92.2	83.3	96.1–99.6	98.0	75.0–92.9	85.0
Superphosphate, 18–20%	11	18.7–22.1	20.5	72.6–89.8	82.5	96.1–99.6	97.9	75.0–90.1	84.3
Superphosphate, 46%	1	...	46.7	...	92.2	...	99.2	...	92.9
All mixtures	16	8.2–16.9	11.3	15.7–82.3	41.5	94.1–99.1	96.6	16.2–83.6	42.8
P-K grades	2	14.5–16.9	15.7	77.4–82.3	79.8	98.3–98.4	98.4	78.7–83.6	81.2
N-P-K grades	14	8.2–15.5	10.7	15.7–54.7	36.0	94.1–99.1	96.4	16.2–56.3	37.4
Middle Atlantic									
All superphosphates, 18–20%	21	19.0–22.9	21.5	61.9–90.1	81.4	87.6–99.8	96.3	70.7–94.0	84.3
Superphosphate, 18–20%	21	19.0–22.9	21.5	61.9–90.1	81.4	87.6–99.8	96.3	70.7–94.0	84.3
All mixtures[a]	59	6.2–21.5	11.8	10.9–79.6	46.1	84.3–99.2	94.7	11.2–81.3	48.4
P-K grades	3	15.6–21.5	19.4	63.8–79.6	71.8	95.7–98.0	96.6	66.4–81.3	74.2
N-P-K grades	56	6.2–16.6	11.4	10.9–77.7	44.7	84.3–99.2	94.6	11.2–80.0	47.1
South Atlantic									
All superphosphates, 18–46%	12	17.8–48.0	22.9	60.2–90.0	81.2	94.9–99.8	97.6	61.9–92.6	83.1
Superphosphate, 18–20%	11	17.8–22.5	20.6	60.2–90.0	80.9	94.9–99.8	97.5	61.9–92.6	83.0
Superphosphate, 46%	1	...	48.0	...	84.8	...	99.5	...	85.2
All mixtures[a]	163	3.1–20.4	10.3	5.6–96.0	38.6	48.9–99.6	93.6	6.2–97.0	41.1
P-K grades	5	13.0–20.0	15.4	64.1–81.3	71.4	94.1–97.8	96.2	67.3–84.2	74.3
N-P-K grades	158	3.1–20.4	10.1	5.6–96.0	37.6	48.9–99.6	93.5	6.2–97.0	40.1
East North Central									
All superphosphates, 18–20%	12	18.5–22.2	21.1	34.2–88.1	77.0	95.1–99.8	97.0	35.6–88.3	79.2
Superphosphate, 18–20%	12	18.5–22.2	21.1	34.2–88.1	77.0	95.1–99.8	97.0	35.6–88.3	79.2
All mixtures[a]	111	6.7–23.4	14.2	10.4–81.3	48.6	86.0–99.9	95.4	11.0–83.1	50.8
P-K grades	8	10.3–23.4	18.6	39.5–81.3	65.8	94.7–99.0	96.9	40.9–83.1	67.9
N-P-K grades	103	6.7–21.6	13.9	10.4–79.7	47.3	86.0–99.9	95.3	11.0–81.3	49.4
West North Central									
All superphosphates, 18–48%	19	19.3–49.6	39.1	78.0–94.4	86.3	92.1–99.9	97.0	81.4–97.9	88.8
Superphosphate, 18–20%	6	19.3–22.2	21.0	80.2–94.4	86.5	92.1–99.9	96.6	81.4–97.9	89.5
Superphosphate, 42–48%	13	44.7–49.6	47.5	78.0–91.6	86.2	94.1–99.7	97.2	81.6–94.1	88.5
All mixtures	47	9.6–34.9	18.4	4.0–87.0	52.7	73.6–99.9	94.3	4.4–90.8	55.5
N-P grades	9	16.1–34.9	24.8	48.6–77.8	58.3	87.6–98.2	94.4	51.0–79.8	61.7
P-K grades	4	19.2–21.9	20.9	68.7–84.0	78.3	92.8–99.4	96.6	74.0–85.4	81.0
N-P-K grades	34	9.6–26.2	16.4	4.0–87.0	48.3	73.6–99.9	94.0	4.4–90.8	50.8
East South Central									
All superphosphates, 20–49%	10	20.2–51.1	26.8	74.2–93.4	83.5	95.9–99.6	98.0	77.4–93.8	85.1
Superphosphate, 20%	8	20.2–22.7	21.4	74.2–87.6	82.6	95.9–99.3	97.7	77.4–88.9	84.5
Superphosphate, 45–49%	2	45.7–51.1	48.4	80.8–93.4	87.1	99.3–99.6	99.4	81.4–93.8	87.6
All mixtures[a]	62	7.7–14.8	11.2	0.7–84.1	32.8	73.2–99.3	93.0	0.9–84.7	34.9
P-K grades	4	14.3–14.8	14.6	56.0–84.1	72.5	97.3–99.3	98.0	57.2–84.7	73.9
N-P-K grades	58	7.7–14.7	10.9	0.7–65.9	30.1	73.2–97.1	92.7	0.9–68.1	32.2
West South Central									
All superphosphates, 20–48%	4	21.9–51.4	42.1	81.7–92.2	86.9	95.3–99.3	96.7	85.8–92.9	89.9
Superphosphate, 20%	1	...	21.9	...	81.7	...	95.3	...	85.8
Superphosphate, 45–48%	3	47.3–51.4	48.8	86.3–92.2	88.7	95.6–99.3	97.1	90.3–92.9	91.3
All mixtures[b]	14	8.9–21.4	21.3	9.8–74.8	43.0	89.8–97.8	94.5	10.3–77.1	45.3
N-P grades	1	...	20.3	...	74.8	...	97.1	...	77.1
N-P-K grades	13	8.9–21.4	11.7	9.8–67.4	40.6	89.8–97.8	94.3	10.3–69.1	42.8
Mountain									
All superphosphates, 19–46%	12	20.4–48.6	38.0	61.3–87.2	78.4	70.9–99.5	93.3	70.1–90.5	84.1
Superphosphate, 19–20%	4	20.4–23.3	22.1	61.3–85.7	76.1	70.9–99.5	87.7	85.2–89.5	86.8
Superphosphate, 42–46%	8	43.0–48.6	46.0	66.6–87.2	79.5	91.1–98.8	96.1	70.1–90.5	82.8
All mixtures[b]	10	10.8–25.9	18.5	11.9–81.6	58.6	81.0–98.1	93.4	14.6–86.8	61.8
N-P grades	5	10.8–25.9	22.3	42.9–81.6	68.6	94.0–94.8	94.4	45.2–86.8	72.6
N-P-K grades	5	11.8–17.0	14.7	11.9–79.8	48.7	81.0–98.1	92.3	14.6–81.7	51.0
Pacific									
All superphosphates, 19%	1	...	20.4	...	83.7	...	97.9	...	85.5
Superphosphate, 19%	1	...	20.4	...	83.7	...	97.9	...	85.5
All mixtures[b]	4	7.7–10.4	8.9	76.2–93.1	85.1	96.7–99.4	98.5	78.7–93.6	86.3
N-P grades	2	7.1–10.2	8.7	84.0–93.1	88.5	...	99.4	84.5–93.6	89.1
N-P-K grades	2	7.7–10.4	9.1	76.2–87.1	81.6	96.7–98.5	97.6	78.7–88.4	83.6
United States									
All superphosphates, 18–49%	103	17.8–51.4	28.2	34.2–94.4	82.1	70.9–99.9	96.7	35.6–97.9	84.8
Superphosphate, 18–20%	75	17.8–23.3	21.1	34.2–94.4	81.1	70.9–99.9	96.5	35.6–97.9	83.9
Superphosphate, 42–49%	28	43.0–51.4	47.3	66.6–93.4	85.0	91.1–99.7	97.2	70.1–94.1	87.1
All mixtures	486	3.1–34.9	12.5	0.7–96.0	43.4	48.9–99.9	94.3	0.9–97.0	45.8
N-P grades	17	7.1–34.9	21.9	42.9–93.1	65.8	87.6–99.4	95.1	45.2–93.6	69.0
P-K grades	26	10.3–23.4	17.6	39.5–84.1	71.6	92.8–99.4	97.0	40.9–85.4	73.8
N-P-K grades	443	3.1–26.2	11.8	0.7–96.0	40.9	48.9–99.9	94.1	0.9–97.0	43.2

[a] No N-P grades collected.
[b] No P-K grades collected.

distribution of the superphosphates and mixtures in relation to degrees of solubility and availability of their P_2O_5 contents is shown in Table IV. Approximately 90% of the superphosphates in comparison to 55% of the mixtures contained 95% or more of the total P_2O_5 in available form. In the 1949–50 survey, these percentages were 78 and 38%, respectively. Similarly, the available portion of the P_2O_5 exceeded 90% in 97.1% of the superphosphates (97.8% in 1949–50), and in 91.7% of the mixtures (86.2% in 1949–50). Slightly more than 85% of the superphosphates (90% in 1949–50) but only 5.1% of the mixtures (7.4% in 1949–50) contained 80% or more of the available P_2O_5 in water-soluble form. Only one of the superphosphates (none in 1949–50) contained less than 50% of the available P_2O_5 in water-soluble form, whereas in 54.7% of the mixtures (49.0% in 1949–50) less than 50% of the available P_2O_5 was water-soluble. The percentage of mixtures containing less than 30% of the available P_2O_5 in water-soluble increased from 15.2 in 1949–50 to 25.4% in 1955–56, an increase of approximately 67%.

The lower values for water solubility and availability of the P_2O_5 exhibited in the 1949–50 survey (*5*) were attributed to formulation practices involving ammoniation, and incorporation of considerable quantities of liming materials to offset the acid-forming properties of other ingredients and as make-weight material. Changes in these formulation practices between 1949–50 and 1955–56 largely involved increased use of granulation and drying operations with the use of less limestone in relation to P_2O_5 content in the formulation of granular mixtures, continuous ammoniation, sulfuric and (or) phosphoric acid in the formula to permit greater use of nitrogen solutions, and nitric acid in the production of available phosphates. The increased use of phosphoric acid and the decreased use of limestone in certain formulations tend to favor increased water solubility and availability of the phosphorus content. As compared with 1949–50, the average fertilizer in 1955–56, however, exhibited slightly greater phosphorus availability but lower water solubility in relation to both total and available P_2O_5.

Adams and Scholl (*1*) reported that superphosphates supplied 97.6% of the P_2O_5 content of mixed fertilizers manufactured in 1954. Neglecting the effect of differences in the initial degrees of

Table IV. Distribution of Samples in Relation to Solubility and Availability of Phosphorus

Water-Soluble Portion of Available P_2O_5, Range, %	Available Portion of Total P_2O_5, Range, %							
	40.0–49.9	50.0–59.9	60.0–69.9	70.0–79.9	80.0–89.9	90.0–94.9	95.0–100.0	Total
	% of Total Samples in Indicated Ranges							
Superphosphates								
35.0–39.9	...	...	...	...	...	...	1.0	1.0
60.0–69.9	...	...	...	...	...	...	1.9	1.9
70.0–79.9	...	...	...	...	1.0	1.0	9.7	11.7
80.0–84.9	...	...	...	...	...	1.9	21.4	23.3
85.0–89.9	...	...	...	1.0	1.0	2.9	37.9	42.7
90.0–100.0	...	...	...	...	...	1.0	18.4	19.4
Total				1.0	1.9	6.8	90.3	100.0
Mixtures								
0–9.9	...	...	...	0.2	...	0.8	1.0	2.1
10.0–19.9	...	...	...	0.4	1.6	3.3	4.1	9.5
20.0–29.9	...	0.2	...	0.4	1.4	6.8	4.9	13.8
30.0–39.9	...	0.2	...	0.2	1.0	4.3	6.8	12.6
40.0–49.9	0.2	...	0.2	0.2	0.6	7.4	8.0	16.7
50.0–59.9	...	...	0.2	...	1.0	7.8	12.8	21.8
60.0–69.9	...	...	...	...	...	4.1	8.6	12.8
70.0–79.9	...	...	...	...	...	0.8	4.9	5.8
80.0–89.9	...	...	...	...	...	0.8	3.5	4.3
90.0–100.0	...	...	...	...	...	0.2	0.6	0.8
Total	0.2	0.4	0.4	1.4	5.8	36.4	55.3	100.0

Table V. Distribution of Total Phosphorus in Water-Soluble and in Available Form in Relation to 16 Principal Grades

Grade $N–P_2O_5–K_2O$	No. of Samples	No. of Manufacturers	Portion of Total P_2O_5, %				Portion of Available P_2O_5 in Water-Soluble Form, %	
			Water-Soluble Form		Available Form			
			Range	Mean	Range	Mean	Range	Mean
4-10-6	16	13	13.7–57.8	26.6	91.6–98.3	95.2	14.5–59.7	27.9
4-10-7	16	13	14.7–48.3	28.1	89.1–96.0	93.6	16.0–53.0	30.1
4-8-8	14	13	5.6–76.2	28.8	64.7–98.1	92.0	6.2–77.9	31.4
4-8-6	10	10	7.7–58.1	30.0	91.1–97.4	94.4	8.0–61.4	31.8
5-10-5	21	15	10.1–70.4	36.2	90.2–99.1	94.1	11.2–71.1	38.3
3-9-6	27	18	6.5–68.5	37.5	73.2–98.5	92.7	6.7–69.8	40.1
4–12–12	14	13	18.4–68.7	40.2	86.9–97.3	94.3	19.4–70.8	42.6
5–10–10	43	26	10.4–77.7	41.0	84.3–99.2	94.7	11.0–80.0	43.1
3-9-9	19	15	18.8–62.7	42.2	89.7–98.0	94.7	19.7–64.1	44.6
10-10-10	21	15	19.7–78.6	44.5	86.9–99.2	96.0	22.7–81.3	46.2
3-12-12	54	31	4.0–70.3	44.1	73.6–99.9	94.4	4.4–72.6	46.5
2-12-12	13	12	34.7–57.5	48.6	92.2–98.0	95.4	35.6–59.6	50.9
6-12-12	10	4	27.6–71.3	52.9	90.2–97.4	94.8	29.7–74.5	55.6
4-16-16	23	16	15.1–81.3	53.2	88.4–99.1	94.8	17.1–83.0	55.8
5-20-20	10	9	29.5–74.0	57.4	96.0–98.6	97.1	30.1–75.0	59.1
0-20-20	11	9	39.5–84.0	72.2	92.8–99.4	96.8	40.9–85.4	74.5
Total, 16 grades	322	113	4.0–84.0	42.1	64.7–99.9	94.5	4.4–85.4	44.3
70 other grades	164	83	0.7–96.0	46.1	48.9–99.9	93.9	0.9–97.0	48.7
Total	486	159	0.7–96.0	43.4	48.9–99.9	94.3	0.9–97.0	45.8

availability of superphosphates and the minor sources of phosphorus in mixed fertilizers, an average of 97.5% (96.1% in 1949–50) of the originally available P_2O_5 remained in available form after formulation of the mixtures, whereas only 52.9% (57.3% in 1949–50) of the water-soluble P_2O_5 was still water soluble.

Regional averages for the available portion of the total P_2O_5 remaining in available form ranged from 94.9% for the East South Central region to slightly more than 100% for the Mountain and Pacific areas. Similarly, regional averages for the water-soluble portion of the total P_2O_5 remaining in water-soluble form ranged from 39.3% for the East South Central region to more than 100% for the Pacific area. Regional averages for the water-soluble portion of the available P_2O_5 remaining in water-soluble form ranged from 41.0 to 100.9% and averaged 54.0% in comparison to 59.4% in 1949–50.

In general, higher averages for both water solubility and availability of the P_2O_5 contents of the mixtures were observed for those regions in which a greater proportion of the phosphate supply is available as concentrated superphosphate or in forms other than superphosphate. The lower average values were associated with regions in which ammoniation and formulation of physiologically neutral mixtures are more extensively practiced. Intermediate values were associated with the East North Central and West North Central regions.

Principal Mixed Fertilizer Grades

Data on the distribution of P_2O_5 between water-soluble and available forms are summarized in Table V. Although considerable variation exists for each grade in the distribution of P_2O_5 between water-soluble and available forms, listing these grades in the ascending order of the average percentages of total P_2O_5 in water-soluble form requires only the interchange of the 3–12–12 and 10–10–10 grades. Four of the 11 principal grades in the 1949–50 survey—2–12–6, 3–12–6, 4–12–4, and 6–8–4—were not classified among the 16 principal grades in the current survey.

The general tendency is for the water-soluble fraction of the total and of the available P_2O_5 to increase as the N–P_2O_5 ratio decreases, and in the case of the higher analysis mixtures to increase as the P_2O_5 grade of the mixture increases. These relationships reflect the economic pressure on manufacturers to derive a high proportion of the total nitrogen from relatively inexpensive ammoniating solutions. With solutions now available and particularly in connection with the use of supplemental acid as an aid in granulation processes all of the nitrogen for these grades may be supplied in solution form.

Grades in Relation to Nitrogen Content

In Table VI data are summarized in relation to the principal nitrogen grades of the mixtures. Of the 16 nitrogen grades included in the survey, the grades between 0 and 10% N represented 96.5% or 469 of the 486 samples. The total P_2O_5 in water-soluble and in available form in superphosphate decreases appreciably in the formulation of N–P–K mixtures. The water solubility of the phosphorus in the P–K mixtures also decreased appreciably, but to a much lesser degree than indicated by the 1949–50 data. Presumably in the case of the P–K mixtures the observed decrease resulted largely from incorporation of liming materials.

The 4, 5, and 6% N grades exhibit lower values for the portion of the total P_2O_5 in water-soluble and in available form and for the portion of the available P_2O_5 in water-soluble form than either higher or lower N grades. The 5% N grade, however, shows higher values for water-solubility and availability than the 4 and 6% grades. The data indicate that in general the P_2O_5 contents are subjected to higher rates of ammoniation in the formulation of 4, 5, and 6% N grades than in the formulation of the other grades.

Relative Solubility of Phosphorus in Commercial Solid Mixed Fertilizers, 1880–1956

The relative solubility and availability of the P_2O_5 content of phosphorus containing solid commercial mixed fertilizers as determined in the present

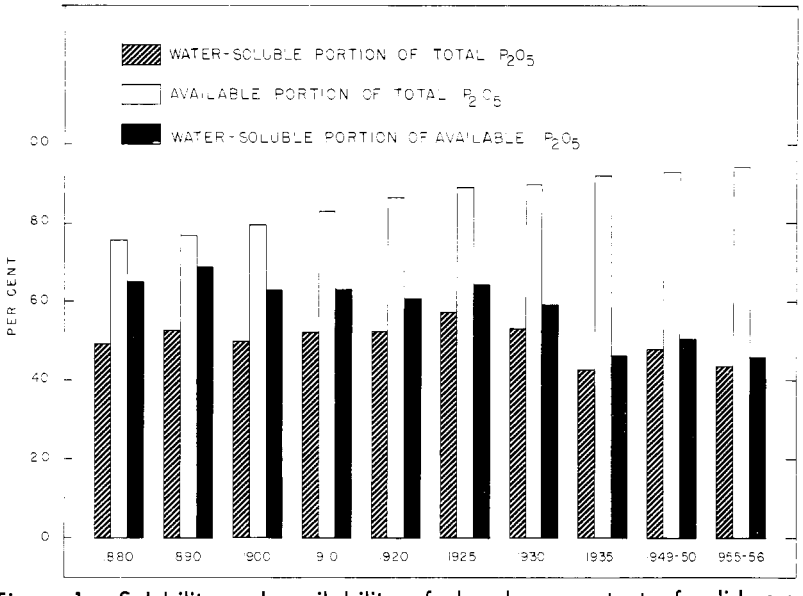

Figure 1. Solubility and availability of phosphorus content of solid commercial fertilizers for 1880–1956

Table VI. Distribution of Total Phosphorus in Water-Soluble and in Available Form in Relation to Nitrogen Grade

N Grade, %	No. of Samples	No. of Grades	No. of Manufacturers	Weighted Average Grade, N–P_2O_5–K_2O, %	Portion of Total P_2O_5, %				Portion of Available P_2O_5 in Water-Soluble Form, %	
					Water-Soluble Form		Available Form			
					Range	Mean	Range	Mean	Range	Mean
0	26	6	18	0–16.65–18.15	39.5–84.1	73.4	92.8–99.4	97.3	40.9–85.4	73.8
2	15	2	14	2–12.00–11.20	16.8–57.5	51.0	78.5–98.0	96.1	21.4–59.6	48.4
3	121	8	51	3–10.69–10.61	4.0–70.3	44.8	73.2–99.9	95.2	4.4–72.6	45.4
4	116	15	57	4–11.15–9.27	5.6–81.3	37.8	48.9–99.1	94.7	6.2–83.0	39.3
5	83	10	45	5–11.20–9.90	10.1–77.7	43.9	54.2–99.2	93.8	11.0–80.0	43.6
6	49	15	30	6–10.12–7.53	0.7–78.1	33.1	61.0–98.1	94.8	0.9–82.5	36.7
8	23	11	17	8–14.43–8.61	9.8–87.1	51.2	89.5–99.6	96.0	10.3–88.4	51.1
10	36	9	24	10–12.50–7.94	19.4–96.0	49.9	86.9–99.2	96.7	19.9–97.0	52.9
Subtotal	469	76	152	4.50–11.53–9.96	0.7–96.0	42.8	48.9–99.9	94.2	0.9–97.0	45.2
Eight other N grades	17	10	14	12.76–12.12–7.29	16.3–93.1	62.0	94.1–99.9	96.9	16.8–93.6	62.0
Total	486	86	159	4.59–11.55–9.87	0.7–96.0	44.5	48.9–99.9	95.3	0.9–97.0	45.8

survey is compared with similar information covering the period 1880–1950 (*5*) (Figure 1). Although the data for the years prior to 1949–50 probably are not as completely representative of the entire country as those obtained in the 1949–50 and 1955–56 surveys and may relate only to N–P–K mixtures, they indicate that the portion of the total P_2O_5 in water-soluble form increased from about 49% in 1880 to about 57% in 1925, and then decreased to 42.6% in 1935. The values were 46.9 and 43.4% in 1949–50 and 1955–56, respectively. The decreases between 1925 and 1950 followed development and utilization of ammoniating solutions, and those between 1949–50 and 1955–56 marked increased usage of such solutions in granulation and continuous ammoniation processes and the use of nitric acid in the production of available phosphates. Presumably the observed decrease between 1949–50 and 1955–56 was limited to some extent by introduction of sulfuric acid and (or) phosphoric acid into fertilizer formulas to aid granulation.

The available portion of the total P_2O_5 increased gradually from 75.5% in 1880 to 93.0% in 1949–50 and to 94.3% in 1955–56. This continued increase probably is due to the decrease in the portion of the P_2O_5 derived from organic materials, process improvements in the production of superphosphate, and the change in the official method for available phosphorus which in 1931 decreased the weight of the analytical sample from 2 grams to 1 gram and increased the period of digestion from 30 to 60 minutes (*3*). Between 1925 and 1935 the water-soluble portion of the available P_2O_5 decreased from approximately 64 to 46%, increased to 48.4% for N–P–K grades alone, and to 50.2% for all mixtures in 1949–50, and decreased to 43.2 and 45.8%, respectively, in 1955–56.

PART II. REACTION, PARTICLE SIZE, AND INERT MATTER CONTENTS

Analytical Methods

Moisture, acid-insoluble ash, and calcium carbonate equivalent of carbonate carbon contents, and the acid- or non-acid-forming qualities of the samples were determined by duplicate analyses (*2*). The vacuum-desiccation procedure was used in determination of the free-moisture content and the dried residues therefrom were used in the determination of the acid-insoluble ash.

The glass-electrode procedure was used in determining the acid-base balance required for calculation of the acid- or non-acid-forming quality. In most instances the fertilizer control officials supplied the nitrogen analysis needed in the calculation, but in a few cases where the nitrogen analyses were not available, the guaranteed nitrogen grade was used in the calculation. The uncertainty introduced by this substitution probably did not exceed 5 pounds of calcium carbonate equivalent per ton of mixture. The citrate-insoluble P_2O_5 values determined in connection with the phase of the survey concerned with the different forms of phosphorus were used in the calculation.

Two parts of cooled, boiled, distilled water were used per part of fertilizer to determine the pH of the sample at 25° to 30° C. by the glass electrode procedure.

The particle-size distribution of samples received in the unground condition was determined with the use of 4-, 6-, 20-, and 28-mesh Tyler standard screen scale sieves. The nested assembly of sieves was shaken in a Rotap machine for a period of 3 minutes, after which the weight of material retained on each sieve and collected in the pan was determined to the nearest 0.1 gram.

Screen Analyses. As indicated in Table VII, 83 of the 103 superphosphate and 393 of the 491 mixed fertilizer samples were received in unground condition and subjected to the screen analysis described above. The data are summarized in this table to indicate the distribution of the samples in relation to the +20-mesh and −28-mesh fractions.

As shown by both sections of the table, increases in the +20-mesh fractions are more or less regularly associated with corresponding decreases in the −28-mesh fractions with no distinct division between granular and nongranular materials. However, the samples may be conveniently classified into nongranular, semigranular, and granular materials by the broken lines in each section of the table. The nongranular classification then applies to products containing 0 to 40% +20-mesh material, semigranular to products containing

Table VII. Distribution of Superphosphates and Mixed Fertilizers in Relation to Screen Analyses

+20-Mesh Portion, Range, %	−28-Mesh Portion, Range, %										
	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	Total
	% of Total Samples										
83 Superphosphates											
0–10	1.2									1.2	2.4
11–20							1.2	9.6			10.8
21–30							25.3	3.6			28.9
31–40						7.2	4.8				12.0
41–50		1.2		1.2	6.0	2.4					10.8
51–60				7.2							7.2
61–70		1.2	6.0								7.2
71–80	2.4	4.8									7.2
81–90	1.2										1.2
91–100	12.0										12.0
Total	16.9	7.2	6.0	8.4	6.0	9.6	31.3	13.3	...	1.2	100.0
393 Mixed Fertilizers											
0–10								0.5			0.5
11–20							2.0	17.3	2.3		21.6
21–30	0.5			0.3		3.1	36.9	3.8			44.5
31–40					2.3	10.7	0.8				13.7
41–50				2.0	3.6						5.6
51–60			0.3	1.5							1.8
61–70		0.8	2.0								2.8
71–80	0.3	2.5									2.8
81–90	1.0	1.0									2.0
91–100	4.6										4.6
Total	6.4	4.3	2.3	3.8	5.9	13.7	39.7	21.6	2.3	...	100.0

survey is compared with similar information covering the period 1880–1950 (*5*) (Figure 1). Although the data for the years prior to 1949–50 probably are not as completely representative of the entire country as those obtained in the 1949–50 and 1955–56 surveys and may relate only to N–P–K mixtures, they indicate that the portion of the total P_2O_5 in water-soluble form increased from about 49% in 1880 to about 57% in 1925, and then decreased to 42.6% in 1935. The values were 46.9 and 43.4% in 1949–50 and 1955–56, respectively. The decreases between 1925 and 1950 followed development and utilization of ammoniating solutions, and those between 1949–50 and 1955–56 marked increased usage of such solutions in granulation and continuous ammoniation processes and the use of nitric acid in the production of available phosphates. Presumably the observed decrease between 1949–50 and 1955–56 was limited to some extent by introduction of sulfuric acid and (or) phosphoric acid into fertilizer formulas to aid granulation.

The available portion of the total P_2O_5 increased gradually from 75.5% in 1880 to 93.0% in 1949–50 and to 94.3% in 1955–56. This continued increase probably is due to the decrease in the portion of the P_2O_5 derived from organic materials, process improvements in the production of superphosphate, and the change in the official method for available phosphorus which in 1931 decreased the weight of the analytical sample from 2 grams to 1 gram and increased the period of digestion from 30 to 60 minutes (*3*). Between 1925 and 1935 the water-soluble portion of the available P_2O_5 decreased from approximately 64 to 46%, increased to 48.4% for N–P–K grades alone, and to 50.2% for all mixtures in 1949–50, and decreased to 43.2 and 45.8%, respectively, in 1955–56.

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The glass-electrode procedure was used in determining the acid-base balance required for calculation of the acid- or non-acid-forming quality. In most instances the fertilizer control officials supplied the nitrogen analysis needed in the calculation, but in a few cases where the nitrogen analyses were not available, the guaranteed nitrogen grade was used in the calculation. The uncertainty introduced by this substitution probably did not exceed 5 pounds of calcium carbonate equivalent per ton of mixture. The citrate-insoluble P_2O_5 values determined in connection with the phase of the survey concerned with the different forms of phosphorus were used in the calculation.

Two parts of cooled, boiled, distilled water were used per part of fertilizer to determine the pH of the sample at 25° to 30° C. by the glass electrode procedure.

The particle-size distribution of samples received in the unground condition was determined with the use of 4-, 6-, 20-, and 28-mesh Tyler standard screen scale sieves. The nested assembly of sieves was shaken in a Rotap machine for a period of 3 minutes, after which the weight of material retained on each sieve and collected in the pan was determined to the nearest 0.1 gram.

Screen Analyses. As indicated in Table VII, 83 of the 103 superphosphate and 393 of the 491 mixed fertilizer samples were received in unground condition and subjected to the screen analysis described above. The data are summarized in this table to indicate the distribution of the samples in relation to the +20-mesh and −28-mesh fractions.

As shown by both sections of the table, increases in the +20-mesh fractions are more or less regularly associated with corresponding decreases in the −28-mesh fractions with no distinct division between granular and nongranular materials. However, the samples may be conveniently classified into nongranular, semigranular, and granular materials by the broken lines in each section of the table. The nongranular classification then applies to products containing 0 to 40% +20-mesh material, semigranular to products containing

Table VII. Distribution of Superphosphates and Mixed Fertilizers in Relation to Screen Analyses

+20-Mesh Portion, Range, %	−28-Mesh Portion, Range, %										
	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100	Total
	% of Total Samples										
83 Superphosphates											
0–10	1.2									1.2	2.4
11–20							1.2	9.6			10.8
21–30							25.3	3.6			28.9
31–40						7.2	4.8				12.0
41–50		1.2		1.2	6.0	2.4					10.8
51–60				7.2							7.2
61–70		1.2	6.0								7.2
71–80	2.4	4.8									7.2
81–90	1.2										1.2
91–100	12.0										12.0
Total	16.9	7.2	6.0	8.4	6.0	9.6	31.3	13.3	...	1.2	100.0
393 Mixed Fertilizers											
0–10								0.5			0.5
11–20							2.0	17.3	2.3		21.6
21–30	0.5			0.3		3.1	36.9	3.8			44.5
31–40					2.3	10.7	0.8				13.7
41–50				2.0	3.6						5.6
51–60			0.3	1.5							1.8
61–70		0.8	2.0								2.8
71–80	0.3	2.5									2.8
81–90	1.0	1.0									2.0
91–100	4.6										4.6
Total	6.4	4.3	2.3	3.8	5.9	13.7	39.7	21.6	2.3	...	100.0

Table VIII. Summary of Screen Analyses of Superphosphates and Mixed Fertilizers

Item	Samples No.	Samples %[a]	Portion Retained or Passed by Indicated Sieves, % +4 Mesh Range	+4 Mesh Mean	−4, +6 Mesh Range	−4, +6 Mesh Mean	−6, +20 Mesh Range	−6, +20 Mesh Mean	−20, +28 Mesh Range	−20, +28 Mesh Mean	−28 Mesh Range	−28 Mesh Mean
Nongranular Materials, 0–40% +20 Mesh												
All superphosphates	44	53.0	0–5.1	0.4	0–9.0	2.6	7.0–34.5	22.5	2.3–27.1	7.4	55.9–77.2	67.1
Superphosphate, 18–20%	37	64.9	0–5.1	0.5	0–9.0	2.8	7.0–31.0	21.6	2.3–27.1	7.4	56.8–77.2	67.7
Superphosphate, 45–48%	7[b]	26.9	0–0.4	0.1	0–4.9	1.5	22.2–34.5	27.2	5.3–8.9	7.4	55.9–68.8	63.8
All mixtures	313[c]	79.6	0–4.8	0.2	0–6.7	1.9	1.1–37.6	22.3	3.0–26.7	9.1	45.1–86.3	66.5
Semigranular Materials, 41–60% +20 Mesh												
All superphosphates	15	18.1	0–1.9	0.3	0–7.4	2.7	34.1–55.8	46.4	5.9–34.6	10.9	19.1–51.6	39.7
Superphosphate, 19–20%	11	19.3	0–1.9	0.4	0–7.4	3.6	34.1–55.8	46.5	5.9–11.7	9.0	32.6–51.6	40.4
Superphosphate, 42–45%	4	15.4	...	0.0	...	0.0	42.5–48.5	46.1	7.1–34.6	16.2	19.1–45.7	37.7
All mixtures	29	7.4	0–1.3	0.1	0–8.8	2.8	35.5–55.9	44.7	6.4–19.3	12.0	30.8–50.3	40.4
Granular Materials, 61–100% +20 Mesh												
All superphosphates	23	27.7	0–9.9	0.5	0–65.7	6.6	19.1–100.0	78.2	0–14.7	4.8	0–27.2	9.9
Superphosphate, 20%	9	15.8	0–9.9	1.2	3.8–65.7	13.7	19.1–75.5	60.5	0.3–14.4	6.7	5.0–27.2	18.0
Superphosphate, 45–46%	14	53.8	...	0.0	0–21.4	2.0	63.7–100.0	89.6	0–14.7	3.6	0–25.4	4.8
All mixtures	48	12.2	0–23.0	0.9	0–23.6	6.4	23.6–97.3	74.5	0–17.5	6.9	0–27.5	11.3

[a] Based on numbers of samples: all superphosphates, 83; 18–20% superphosphates, 57; 42–48% superphosphates, 26; mixed fertilizers, 393.
[b] Not including one sample which analyzed 87.3% passing 20- and retained on 28-mesh sieves.
[c] Not including three samples two of which analyzed 69.9 to 71.1% passing 20- and retained on 28-mesh sieves and the third 70.4% passing the 20-mesh sieve with 37.5% passing the 28-mesh sieve.

Table X. Distribution of Superphosphates and Mixed Fertilizers in Relation to pH

pH Range	% of Total Samples: 103 superphosphates	491 mixed fertilizers
2.0–2.4	30.0	0.4
2.5–2.9	66.0	3.1
3.0–3.4	3.9	10.6
3.5–3.9	...	28.1
4.0–4.4	...	25.9
4.5–4.9	...	18.9
5.0–5.4	...	8.1
5.5–5.9	...	2.9
6.0–6.4	...	1.2
6.5–6.9	...	0.6
7.0–7.4	...	0.2
Total	99.9	100.0

Table XI. Distribution of Superphosphates and Mixed Fertilizers in Relation to Moisture Content

Moisture Content, Range, %	% of Total Samples: 103 superphosphates	491 mixed fertilizers
0–0.50	20.4	13.4
0.51–1.00	32.0	23.4
1.01–1.50	25.2	20.2
1.51–2.00	7.8	15.1
2.01–2.50	6.8	9.6
2.51–3.00	3.9	5.9
3.01–3.50	1.9	3.7
3.51–4.00	1.0	3.9
4.01–4.50	...	2.6
4.51–5.00	1.0	1.4
5.01–5.50	...	0.4
5.51–6.00	...	0.2
6.01–6.50	...	0.2
Total	100.0	100.0

41 to 60% +20-mesh material, and granular to products containing 61% or more +20-mesh material.

A summary of the screen analysis data grouped according to the above classification is given in Table VIII.

The proportion of mixed fertilizers marketed in granular form in 1955–56 (12.2%) compares well with the 8.8% of all mixed fertilizer reported consumed in granular form in the continental United States by Scholl, Wallace, and Fox (*8*). On the average typical nongranular products contained about one-fourth +20-mesh material and two-thirds −28-mesh. Semigranular products averaged about 50% of −20-mesh and 40% of −28-mesh, whereas granular products averaged 75 to 90% +20-mesh material.

Analytical Data. A summary of the analytical data in relation to the different classifications of superphosphates and mixtures is given in Table IX. This table also indicates for each region the average grade of the samples and the numbers of samples, manufacturers, and states involved.

In general, the superphosphates were more acid in reaction than the several groups of mixed fertilizers. The concentrated superphosphates were the most acid, with a median pH value of 2.3 in contrast to 2.6 for normal superphosphates, 3.0 for P–K, 3.9 for N–P, 4.1 for N–P–K, and 6.4 for N–K grades. The pH values for superphosphates ranged from 2.0 to 3.1, for N–P and P–K mixtures from 2.4 to 4.5, for N–K mixtures from 4.8 to 7.1, and for N–P–K mixtures from 2.8 to 6.9.

The moisture content of the superphosphates ranged from 0.2 to 4.7 and averaged 1.2% and that of the mixed fertilizers from 0.1 to 6.2% and averaged 1.6%. These values are believed, in general, to be somewhat lower than those prevailing at the time the samples were drawn owing to unavoidable changes during storage and sample preparation.

The distributions of the superphosphates and mixed fertilizers in relation to pH and to moisture content are shown in Tables X and XI, respectively.

Except for the N–P and P–K grades, the mixed fertilizers contained more acid-insoluble ash and except for the N–P grades more carbonate carbon than the superphosphates. In 1954, 97.6% of the P_2O_5 content of mixed fertilizers was supplied by superphosphates (*1*). On the average, therefore, and assuming that the P_2O_5 is derived entirely from superphosphates, a ton of mixed fertilizer in 1955–56 appears to have contained about 150 pounds of acid-insoluble ash in excess of that contributed by the superphosphate, and also about 95 pounds of calcium carbonate equivalent. These figures compare with 150 and 100 pounds, respectively, reported in the 1949–50 survey. Nearly one eighth of the average mixed fertilizer in both surveys, therefore, consisted of materials not primarily useful as sources of the major plant-nutrient elements—nitrogen, phosphorus, and potassium.

The N–P grades were second highest in average plant-nutrient content, 31.7%, contained the least acid-insoluble ash and carbonate carbon, and were highest in acid-forming quality, 923 pounds $CaCO_3$ equivalent per ton. The N–K grades were lowest in average plant-nutrient content, 23.6%, highest in acid-insoluble ash and carbonate carbon, and second highest in acid-forming quality, 491 pounds per ton. The P–K group was the highest in plant-nutrient

Table IX. Distributions of Superphosphates and Mixed Fertilizers in Relation to pH

Region Fertilizer Classification	No. of Samples	No. of States	No. of Mfrs.	Average Grade, %	pH Range	pH Median	Moisture, % Range	Moisture, % Mean	Acid-Insoluble Ash, Lb./Ton Range	Acid-Insoluble Ash, Lb./Ton Mean	$CaCo_3$ Equivalent, Lb./Ton: Carbonate Carbon Range	Carbonate Carbon Mean	Acid- or Non-Acid-Forming Quality[a] Range	Acid- or Non-Acid-Forming Quality[a] Mean
New England Superphos., 18–20%	11	3	9	0–19.50–0	2.4–3.1	2.6	0.4–4.7	1.7	53–214	102	0–33	5	4B–84	37
Superphos., 46%	1	1	1	0–46.0–0	...	2.3	...	1.0	...	41	...	1	...	35
All mixtures	16	3	13	5.81–10.50–11.75	2.9–5.2	4.6	0.5–4.4	2.1	23–477	173	0–333	6	141B–673	269
P–K grades	2	2	2	0–14.50–22.00	2.9–3.1	3.0	2.6–3.9	3.2	23–165	94	0–4	2	141B–26	58B
N–P–K	14	3	12	6.64–9.93–10.29	3.8–5.2	4.6	0.5–4.4	1.9	28–477	184	0–333	7	55 –673	316
Middle Atlantic Superphos., 18–20%	21	6	14	0–19.90–0	2.4–28	2.6	0.3–2.6	0.8	50–186	92	0–41	6	147B–59	18
All mixtures	59	5	26	5.61–10.81–9.14	2.8–5.5	3.9	0.3–3.7	0.9	33–540	206	2–415	97	212B–875	197
P–K grades	3	2	2	0–18.00–18.00	2.8–3.0	2.9	0.4–0.9	0.6	41–78	57	7–26	15	106B–24B	67B
N–P–K grades	56	5	26	5.91–10.43–8.66	3.3–5.5	3.9	0.3–3.7	1.0	33–540	214	2–415	102	212B–875	211
South Atlantic Superphos., 18–20%	11	4	9	0–19.45–0	2.5–2.9	2.6	0.4–3.3	1.6	57–208	105	1–10	4	42B–34	6
Superphos., 46%	1	1	1	0–46.00–0	...	2.3	...	2.4	...	29	...	1	...	82
All mixtures	168	5	64	4.63–9.18–8.45	2.9–7.1	4.3	0.2–6.2	2.1	3–1079	319	3–603	114	472B–1486	44
N–K grades	5	4	5	11.60–0–12.00	4.8–7.1	6.4	0.2–1.7	0.7	22–1079	263	6–360	173	144B–1486	491
P–K grades	5	3	5	0–14.80–15.60	2.9–3.4	3.2	0.7–2.7	2.0	58–150	99	3–74	19	129B–12B	73B
N–P–K grades	158	5	61	4.09–9.30–8.11	2.9–6.3	4.3	0.2–6.2	2.2	3–884	328	3–603	115	472B–958	34
East North Central Superphos., 20%	12	3	10	0–19.83–0	2.4–3.1	2.6	0.5–2.3	1.4	48–157	81	1–19	6	56B–59	14
All mixtures	111	5	31	4.28–13.22–14.16	3.1–5.6	3.8	0.2–4.0	1.2	21–445	106	0–335	48	481B–1229	208
P–K grades	8	3	6	0–17.50–21.25	2.6–3.5	3.1	0.4–1.7	1.1	21–80	50	0–221	39	481B–175	10B
N–P–K grades	103	5	31	4.61–12.88–13.61	3.1–5.6	3.8	0.2–4.0	1.2	21–445	111	1–335	49	142B–1229	231
West North Central Superphos., 18–20%	6	3	6	0–19.70–0	2.4–2.6	2.6	0.6–1.4	1.1	44–180	82	1–6	3	23B–72	26
Superphos., 42–48%	13	5	11	0–45.20–0	2.1–2.5	2.3	0.2–2.6	1.2	25–85	67	0–12	5	50B–102	38
All mixtures	47	5	28	7.04–16.95–9.36	2.4–5.9	3.9	0.1–5.2	1.2	2–574	81	0–607	39	407B–1824	458
N–P grades	9	4	9	10.00–23.30–0	3.4–4.2	4.0	0.7–5.2	1.8	23–53	37	3–9	5	335 –1072	693
P–K grades	4	3	4	0–20.00–14.00	2.4–3.0	2.7	0.3–1.2	0.6	41–455	150	2–9	5	195B–24B	121B
N–P–K grades	34	5	3	7.09–14.91–11.29	2.9–5.9	4.1	0.1–4.6	1.1	2–574	85	0–607	51	407B–1824	464
East South Central Superphos., 20%	8	3	7	0–20.00–0	2.3–2.6	2.5	0.3–3.3	1.2	50–100	73	2–6	3	4B–81	43
Superphos., 45–49%	2	1	2	0–47.00–0	2.0–2.4	2.2	0.4–1.2	0.8	66–84	75	2–12	7	32 –91	61
All mixtures	62	4	28	4.26–10.13–8.39	2.9–6.9	4.4	0.5–4.9	2.1	28–503	171	2–792	212	747B–515	8B
P–K grades	4	4	4	0–14.00–14.00	2.9–3.4	3.1	1.4–2.3	1.9	40–135	82	2–14	6	51B–7	21B
N–P–K grades	58	4	27	4.55–9.86–8.00	3.5–6.9	4.5	0.5–4.9	2.1	28–503	177	4–792	226	747B–515	7B
West South Central Superphos., 20%	1	1	1	0–20.00–0	...	2.4	...	0.5	...	62	...	4	...	14
Superphos., 45–48%	3	1	3	0–46.00–0	2.1–2.5	2.4	0.3–0.7	0.5	73–84	8	3–6	5	88B–24	43B
All mixtures	14	3	12	6.07–11.29–9.36	3.1–5.9	3.9	0.2–3.2	1.0	23–569	196	2–579	132	399B–930	250
N–P grades	1	1	1	10.00–20.00–0	...	3.5	...	0.6	...	45	...	5	...	793
N–P–K grades	13	3	11	5.77–10.62–10.08	3.1–5.9	4.0	0.2–3.2	1.1	23–569	208	2–579	142	399B–930	208
Mountain Superphos., 19–20%	4	2	3	0–19.50–0	2.5–2.6	2.6	0.8–2.4	1.4	87–148	109	1–10	5	14 –51	31
Superphos., 42–46%	8	3	4	0–44.90–0	2.2–2.6	2.4	0.5–2.7	1.2	43–81	65	1–14	4	30B–74	42
All mixtures	10	4	8	9.10–16.90–2.50	2.6–4.9	4.0	0.4–4.0	1.5	20–585	189	2–16	7	46 –1719	809
N–P grades	5	3	5	10.60–21.00–0	2.6–4.5	2.8	0.4–4.0	2.0	20–76	42	2–6	4	435 –1719	1037
N–P–K grades	5	3	4	7.60–12.80–5.00	2.8–4.9	4.2	0.8–3.0	1.4	37–585	336	3–16	9	46 –1134	581
Pacific Superphos., 19%	1	1	1	0–19.00–0	...	2.5	...	0.9	...	110	...	3	...	53
All mixtures	4	1	3	12.75–8.75–2.25	3.3–4.5	3.8	0.1–1.3	0.5	21–331	116	2–9	4	779 –1786	1291
N–P grades	2	1	2	16.50–8.50–0	3.7–3.9	3.8	...	0.1	21–30	26	2–3	3	1695 –1786	1740
N–P–K grades	2	1	2	9.00–9.00–4.50	3.3–4.5	3.9	0.7–1.3	1.0	82–331	207	3–9	6	779 –904	842
United States Superphos., 18–20%	75	26	41	0–19.70–0	2.3–3.1	2.6	0.3–4.7	1.2	44–214	92	0–41	5	147B–84	24
Superphos., 42–49%	28	12	17	0–45.40–0	2.0–2.6	2.3	0.2–2.7	1.1	25–85	66	0–14	5	89B–102	35
All mixtures	491	35	160	5.26–11.42–9.91	2.4–7.1	4.1	0.1–6.2	1.6	2–1079	203	0–792	98	747B–1824	171
N–P grades	17	8	14	11.00–20.70–0	2.6–4.5	3.9	0.1–5.2	1.4	20–76	38	2–9	4	335 –1786	923
N–K grades	5	4	5	11.60–0–12.00	4.8–7.1	6.4	0.2–1.7	0.7	22–1079	263	6–360	173	144B–1486	491
P–K grades	26	16	18	0–16.70–18.50	2.4–3.5	3.0	0.3–3.9	1.4	21–455	84	0–221	19	481B–175	78B
N–P–K grades	443	35	146	5.28–10.88–9.76	2.8–6.9	4.1	0.1–6.2	1.6	2–884	216	0–792	105	747B–1824	154

[a] B. Non-acid-forming.

content, 35.2%, next to the lowest in acid-insoluble ash and carbonate carbon, and the only group which was non-acid-forming, 78 pounds per ton.

The N–P–K grades were next to the lowest in plant-nutrient content, 25.92%, next to the highest in acid-insoluble ash and carbonate carbon, and the lowest acid-forming group, 154 pounds per ton.

The data on acid-insoluble ash, carbonate carbon, and acid- or non-acid-forming quality are summarized in Table XII in relation to the principal grades of mixed fertilizers and superphosphates. Wide variations in ash and carbonate carbon contents, and in net acid-base balance were noted for all grades listed. In comparison with the superphosphates the mixtures, with the exception of the 0-20-20, 4-16-16, 5-20-20, and 10-10-10 grades, contained considerably greater quantities either of acid-insoluble ash, carbonate carbon, or both.

Acid-Insoluble Ash and Calcium Carbonate Equivalent of Carbonate Carbon. Table XIII shows the distribution of the mixed fertilizer samples in relation to acid-insoluble ash content, sum of the ash content and $CaCO_3$ equivalent of the carbonate carbon and the interrelation of these quantities. More than 45% of the samples (45.4%, 223 samples) contained less than 100 pounds of acid-insoluble ash per ton, and about 57% of these (127 samples) exhibited sums of ash content and $CaCO_3$ equivalent of carbonate carbon of less than 100 pounds. Nearly 30% (28.6%, 64 samples) exhibited sums of 101 to 400 pounds and 14% (14.3%, 32 samples) sums between 401 and 700 pounds. More than three eighths of the samples (38.1%, 187 samples) contained 101 to 400 pounds of ash and of these more than one fourth (26.7%, 50 samples) contained 401 to 700 pounds of ash plus calcium carbonate equivalent of carbonate carbon. Approximately 16% of the samples (16.4%, 81 samples) contained more than 401 pounds of ash and of these at least 38 also contained appreciable amounts of carbonate carbon.

Carbonate Carbon and Acid-Forming Quality. Distribution of the samples in relation to the $CaCO_3$ equivalent of the carbonate carbon content, the $CaCO_3$ equivalent of the acid-forming quality or the acid-base balance, and the interrelation of these quantities is given in Table XIV. One hundred and fifty-eight (32.2%) of the samples

Table XII. Summary of Acid-Insoluble Ash and Calcium Carbonate Equivalent Contents, and Acid- or Non-Acid-Forming Quality of Superphosphates and Principal Grades of Mixed Fertilizers

Grade, %	No. of Samples	No. of States	No. of Mfrs.	Acid-Insoluble Ash, Lb./Ton		$CaCO_3$ Equivalent, Lb./Ton			
						Carbonate Carbon		Acid- or Non-Acid-Forming Quality[a]	
				Range	Mean	Range	Mean	Range	Mean
Superphosphates									
P_2O_5									
18 to 20	75	27	41	44–214	92	0–41	5	147B–84	24
42 to 49	28	12	17	25–85	66	0–14	5	89B–102	35
Total	103	30	54	25–214	85	0–41	5	147B–102	27
Mixed Fertilizers									
$N–P_2O_5–K_2O$									
3-12-12	54	12	31	31–416	159	1–607	80	407B–1824	102
5-10-10	43	14	26	34–526	219	2–390	88	212B–499	187
3-9-6	27	6	18	58–535	299	43–792	224	747B–153	127B
4-16-16	23	8	16	31–157	57	3–105	16	39 –396	201
5-10-5	21	13	15	35–596	277	2–579	185	399B–369	93
10-10-10	21	12	15	21–166	46	2–51	17	377 –1229	712
3-9-9	19	5	15	51–525	261	57–535	174	412B–40	105B
4-10-6	16	3	13	61–618	402	7–451	133	378B–235	13B
4-10-7	16	3	13	32–410	207	96–539	283	465B–26	148B
4-8-8	14	3	13	36–808	531	3–603	105	472B–260	27
4-12-12	14	4	13	56–280	156	4–188	55	87B–256	99
2-12-12	13	4	12	64–377	180	4–180	68	211B–95	31B
0-20-20	11	9	9	41–456	91	0–57	12	195B–175	84B
6-12-12	10	4	4	51–188	104	3–61	21	147 –515	345
4-8-6	10	2	10	606–844	678	5–212	63	178B–229	73
5-20-20	10	7	9	22–58	32	2–61	13	183 –424	270
16 grades	322	28	113	21–844	217	0–792	102	747B–1824	107
74 other grades	169	33	87	2–1079	177	0–533	89	481B–1786	295
Total	491	35	160	2–1079	203	0–792	98	747B–1824	171

[a] B. Non-acid-forming.

Table XIII. Distribution of Mixed Fertilizers in Relation to Content of Acid-Insoluble Ash, and Sum of Ash Content and Calcium Carbonate Equivalent of Carbonate Carbon

Acid-Insoluble Ash, Range, Lb./Ton	Acid-Insoluble Ash plus $CaCO_3$ Equivalent of Carbonate Carbon, Range, Lb./Ton										
	0–100	101–200	201–300	301–400	401–500	501–600	601–700	701–800	801–900	1001–1100	Total
	% of Total Samples										
0–100	25.9	6.3	5.1	1.6	3.7	2.2	0.6	...	...	...	45.4
101–200	...	5.7	4.3	2.4	1.6	0.2	0.2	...	0.4	...	14.9
201–300	...	...	5.7	3.3	1.4	0.8	...	...	...	...	11.2
301–400	...	...	...	4.1	4.7	2.6	0.6	...	...	...	12.0
401–500	...	...	...	...	2.4	3.5	0.6	...	...	...	6.5
501–600	...	...	...	...	...	3.1	2.0	0.4	...	...	5.5
601–700	...	...	...	...	...	...	0.6	0.8	...	...	1 4
701–800	...	...	...	...	...	...	...	1.4	0.4	...	1.8
801–900	...	...	...	...	...	...	...	...	1.0	...	1.0
1001–1100	...	...	...	...	...	...	...	...	...	0.2	0.2
Total	25.9	12.0	15.1	11.4	13.8	12.4	4.7	2.6	1.8	0.2	99.9

were non-acid-forming compared with 167 (34.0%) which contained more than 100 pounds of $CaCO_3$ equivalent of carbonate carbon per ton and 76 (15.5%) which contained in excess of 200 pounds of carbonate equivalent. Of the 167 samples containing more than 100 pounds of $CaCO_3$ equivalent of carbonate carbon per ton 52 (31.1%) were acid-forming and 115 (68.9%) non-acid-forming. Approximately 44% (44.4) or 218 samples were acid-forming in the range of 0 to 300 pounds of $CaCO_3$ equivalent per ton, compared with 144 (29.3%) which were non-acid-forming in the same range. Fourteen of the mixtures (2.8%) were non-acid-forming in excess of 300 pounds of $CaCO_3$ equivalent per ton and 23.4% (115 samples) were acid-forming in this range.

Phosphorus, Ash, and Carbonate Carbon Contents. Inasmuch as the superphosphates are the principal phosphorus sources used in formulating mixtures (*7*) and also the principal ingredients normally carrying an appreciable acid-insoluble ash content, a comparison of the ash content of mixtures and of superphosphates in relation to their phosphorus content provides an approximate measure of the inert material that was added. Such a comparison is presented in Table XV for mixtures collected in two or more grades for each of 17 different plant-nutrient ratios. Similar comparative data also are tabulated for $CaCO_3$ equivalent of carbonate carbon and acid- and non-acid-forming quality. Although these latter quantities are more closely related to nitrogen content than to the phosphorus in the mixtures, the values for grades having the same plant-nutrient ratio are in approximately the same proportion in either case. Inspection of the table indicates that the lower analysis mixtures contained from 1.5 to 14 times as much acid-insoluble ash as would normally be associated with their phosphorus content, assuming that the phosphorus is derived entirely from superphosphates. Seven of the principal grades—3-9-6, 3-9-9, 3-12-12, 4-8-8, 4-12-12, 5-10-5, and 5-10-10—contained 2.8 to 14 times as much ash as the equivalent superphosphate. The data indicate that it would be possible in many cases to formulate higher analysis grades of the same plant-nutrient ratio without the use of concentrated superphosphates, and that such a practice would appreciably reduce the necessity for the addition of make-weight material. In other cases this can be and is being done economically by partial replacement of normal superphosphate with triple superphosphate or other high analysis phosphates. The data also indicate that in general the acid-forming quality of the mixtures increases and the $CaCO_3$ equivalent of the carbonate carbon content decreases as formulation of an individual plant nutrient ratio proceeds to the higher analysis grades.

Economic Significance

As indicated in Table IX, the average mixed fertilizer marketed in 1955–56 contained 203 pounds of acid-insoluble ash (an increase of 3.6% over 1949–50) and 98 pounds of $CaCO_3$ equivalent of carbonate carbon per ton (a decrease of 10.1% compared to 1949–50). Similarly, the average normal superphosphate contained 92 pounds of ash and 5 pounds of carbonates per ton. The corresponding figures for concentrated superphosphate are 66 and 5 pounds, respectively. Scholl, Wallace, Fox, and Crammatte (*9*) reported that 14,529,159 tons of mixtures containing 1,880,409 tons of total P_2O_5 were marketed in continental United States in the year ending June 30, 1956. Based on the figure for acid-insoluble ash content, the mixtures marketed in 1955–56 contained 1,474,710 tons of insoluble ash, of which 404,290 tons would be associated with the normal superphosphate equivalent of the mixtures or 131,630 tons with the concentrated superphosphate equivalent. Inasmuch as both normal and concentrated superphosphates are used in the formulation of many mixed fertilizers, the acid-insoluble ash content of the mixtures in excess of that contributed by the superphosphate components lies between 1,070,420 and 1,343,080 tons. Assuming, however, that the usage of normal and concentrated superphosphates in mixed fertilizer formulation is approximately proportional to the available P_2O_5 contents of their productions in this period (1,526,115 and 754,694 tons of P_2O_5, respectively) (*11*), the estimate for acid-insoluble ash associated with the superphosphate content of the mixed fertilizers becomes 314,070 tons. Thus, a net of 1,160,640 tons (1,474,710 −314,070) of ash are estimated as being added in the make-weight and neutralizing materials used in preparing the mixtures.

Similarly, the marketed mixtures are estimated to contain 711,930 tons of $CaCO_3$ equivalent of carbonate carbon, of which 19,180 tons were associated with superphosphates. Thus, it is esti-

Table XIV. Distribution of Mixed Fertilizers in Relation to Content of Calcium Carbonate Equivalent of Carbonate Carbon and Acid- or Non-Acid-Forming Quality[a]

$CaCO_3$ Equivalent of Carbonate Carbon, Range, Lb./Ton		Acid- or Non-Acid-Forming Quality, Range, Lb. $CaCO_3$ Equivalent/Ton											Total	
		0–100	101–200	201–300	301–400	401–500	501–600	601–700	701–800	801–900	901–1000	1001–1900	Subtotal	Total
		% of Total Samples in Indicated Ranges												
0–100	A	10.6	13.6	11.6	7.1	4.1	1.0	1.2	1.8	1.6	1.4	3.1	57.2	66.0
	B	6.5	2.0	0.2									8.8	
101–200	A	3.3	1.6	0.6	1.0	0.2					0.2		6.9	18.5
	B	8.4	3.1	0.2									11.6	
201–300	A	2.0	0.6										2.6	7.3
	B	1.6	2.9			0.2							4.7	
301–400	A		0.2			0.2	0.2						0.6	2.9
	B	0.8	0.6	0.8									2.2	
401–500	A	0.2			0.2								0.4	3.1
	B		1.0	1.2	0.4								2.6	
501–600	A													1.4
	B				0.8	0.6							1.4	
601–800	A													0.8
	B					0.4	0.2		0.2				0.8	
Subtotal	A	16.1	16.1	12.2	8.4	4.5	1.2	1.2	1.8	1.6	1.6	3.1	67.8	100.0
	B	17.3	9.6	2.4	1.2	1.2	0.2		0.2				32.2	
Total		33.4	25.7	14.7	9.6	5.7	1.4	1.2	2.0	1.6	1.6	3.1	100.0	

[a] A, acid-forming; B, non-acid-forming.

Table XV. Comparison of Acid-Insoluble Ash and Calcium Carbonate Equivalent of Carbonate Carbon Contents and Acid- or Non-Acid-Forming Quality of Single and Multiple Strength Grades of Superphosphates and Mixed Fertilizers

Plant-Nutrient Ratio, $N-P_2O_5-K_2O$	Grade, %	No. of Samples	No. of States	No. of Mfrs.	Acid-Insoluble Ash, Lb./Unit Total P_2O_5		$CaCO_3$ Equivalent, Lb./Unit Total P_2O_5			
							Carbonate Carbon		Acid- or Non-Acid-Forming Quality[a]	
					Range	Mean	Range	Mean	Range	Mean
					Superphosphates					
0–1–0	18-20	75	26	41	2.0–11.7	4.3	0–2.0	0.2	6.8B–4.5	1.1
	42-49	28	12	17	0.5–1.9	1.4	0–0.3	0.1	1.9B–2.1	0.7
	Total	103	30	54	0.5–11.7	3.5	0–2.0	0.2	6.8B–4.5	1.0
					N–P Mixtures					
1–2–0	10-20-0	6	5	5	1.2–3.1	2.2	0.2–0.3	0.2	17.7 –54.2	35.3
	12-24-0	1	1	1	...	1.1	...	0.2	...	30.9
1–4–0	6-24-0	1	1	1	...	2.0	...	0.3	...	13.8
	8-32-0	3	3	3	0.7–1.2	0.9	0.1–0.3	0.2	13.4 –20.0	16.1
					P–K Mixtures					
0–1–1	0-14-14	9	8	8	2.7–11.4	6.3	0–1.0	0.4	4.6B–1.8	1.9B
	0-20-20	11	9	9	1.8–22.2	4.4	0–2.5	0.6	9.5B–7.5	4.2B
					N–P–K Mixtures					
1–1–1	5-5-5	1	1	1	...	45.4	...	4.5	...	2.1B
	6-6-6	3	1	3	16.5–24.7	20.7	1.7–2.3	1.9	28.1 –39.5	34.0
	7-7-7	1	1	1	...	6.4	...	38.9	...	18.8
	8-8-8	6	5	5	3.6–12.8	7.5	0.3–29.8	16.5	5.1B–76.0	42.6
	10-10-10	21	12	15	1.9–16.5	4.3	0.2–5.0	1.6	29.1 –108.1	64.9
	12-12-12	7	7	6	0.8–4.0	2.3	0.1–2.5	0.7	23.3 –125.1	71.7
	13-13-13	1	1	1	...	0.4	...	0.3	...	102.5
	14-14-14	1	1	1	...	0.2	...	0.0	...	98.9
1–2–1	5-10-5	21	13	15	3.1–55.8	25.4	0.2–55.6	17.1	38.3B–34.5	8.4
	6-12-6	1	1	1	...	11.4	...	0.4	...	30.4
	10-20-10	2	2	2	2.1–2.3	2.2	0.3–1.9	1.1	17.1 –34.0	25.6
1–2–2	4-8-8	14	3	13	4.4–97.1	61.6	0.3–76.2	12.6	59.6B–30.6	2.6
	5-10-10	43	14	26	3.3–53.7	20.2	0.2–35.3	8.0	19.2B–49.0	17.3
	6-12-12	10	4	5	4.1–14.3	8.1	0.2–4.9	1.7	12.0 –43.5	27.4
	8-16-16	4	4	4	1.4–2.4	1.8	0.4–10.6	3.4	21.4 –33.1	27.1
	10-20-20	1	1	1	...	0.2	...	0.2	...	47.0
1–2–3	4-8-12	1	1	1	...	42.2	...	21.1	...	4.6B
	5-10-15	3	2	3	4.3–8.7	7.1	0.4–14.8	9.5	23.8 –30.9	28.4
1–3–1	4-12-4	3	2	3	2.4–46.2	29.6	0.4–31.3	10.7	9.5B–25.5	11.2
	8-24-8	3	1	3	1.3–2.3	2.0	0.2–0.7	0.4	12.8 –20.1	16.8
1–3–2	3-9-6	27	5	9	6.4–56.2	29.7	4.2–90.4	23.0	83.5B–15.0	13.5B
	4-12-8	6	5	5	4.3–27.1	12.7	0.3–12.5	3.3	0.8B–14.9	9.7
1–3–3	3-9-9	19	5	16	5.0–54.9	26.5	5.7–53.9	17.8	41.5B–3.9	10.8B
	4-12-12	14	4	13	4.1–23.1	12.4	0.3–13.9	4.3	6.6B–20.3	7.8
1–4–2	3-12-6	8	3	7	6.3–41.4	19.2	0.2–19.8	9.9	13.2B–11.0	0.8
	5-20-10	1	1	1	...	3.4	...	0.1	...	16.2
	6-24-12	1	1	1	...	1.4	...	0.0	...	9.9
1–4–4	3-12-12	54	12	32	2.2–33.4	12.1	0.1–41.6	6.0	27.9B–140.8	7.7
	4-16-16	23	8	16	1.9–11.0	3.5	0.2–6.0	1.0	2.4 –22.6	12.0
	5-20-20	10	7	9	1.0–2.7	1.5	0.1–3.2	0.7	8.7 –20.0	13.1
1–6–3	2-12-6	2	1	2	19.8–24.0	21.9	0.5–0.6	0.5	3.6 –9.6	6.6
	3-18-9	2	2	2	3.6–4.6	4.1	0.4–5.3	2.9	0.2 –5.4	2.8
	4-24-12	1	1	1	...	2.1	...	0.2	...	3.5
2–2–1	8-8-4	1	1	1	...	42.8	...	0.4	...	100.9
	10-10-5	1	1	1	...	7.9	...	0.8	...	86.9
2–3–3	6-9-9	1	1	1	...	30.8	...	1.8	...	29.4
	8-12-12	1	1	1	...	2.8	...	0.0	...	30.2
2–3–4	6-9-12	2	1	1	9.6–18.8	14.2	...	8.6	36.1 –40.0	38.0
	8-12-16	1	1	1	...	2.2	...	0.7	...	48.6

[a] B. Non-acid-forming.

mated that 692,750 tons of $CaCO_3$ equivalent also were added during mixing operations. These estimates of 1,160,640 tons of acid-insoluble ash and 692,750 tons of $CaCO_3$ equivalent appear to be of the right order of magnitude.

In 1956, Stanfield (*10*) estimated that operating costs in solid mixed fertilizer plants ranged from $4.63 per ton of product for a plant with an annual sales volume of 40,000 tons to $17.20 for a 2500 ton plant. Scholl (*6*) reported 1223 mixing plants in the United States in the 1951–52 fertilizer season with a total rated annual production capacity of 27,597,700 tons. Assuming the same number of plants operative in 1955–56 the average annual production of each plant was 14,529,159/1223 or 11,880 tons. Based on Stanfield's estimates, the operating costs for this average plant

would be $9.16 per ton of product. With bags and bagging at $4.00 per ton and handling and transportation also at $4.00 per ton, the manufacturing and distribution cost of mixed fertilizer from the average-sized plant in 1955–56 appears to have been $17.16 in excess of the value of the primary plant nutrients. Using a figure of $17.00, the cost to the consumer of the acid-insoluble ash added to mixed fertilizers in 1955–56 amounted to $19,730,880. It is generally recognized that incorporation of liming material partially or completely to offset the acid-forming character of mixed fertilizers serves a useful purpose in some parts of the country, notably the South and Southeast. Assigning a value of $3.50 per ton of $CaCO_3$ equivalent for this purpose, the net manufacturing and distribution cost of the 692,750 tons of $CaCO_3$ equivalent of carbonate carbon added to the mixtures amounted to $9,352,125 (692,750 × $13.50). The total cost to the consumer, therefore, for the 1,853,390 tons of acid-insoluble ash and $CaCO_3$ equivalent of carbonate carbon in excess of any plant-nutrient value amounted to $29,083,005, or $2.00 per ton of mixed fertilizer (an increase of $0.17 per ton or 9.3% over 1949–50). The effect of omitting concentrated superphosphate from consideration as an ingredient of the mixtures would be to reduce the estimate for added acid-insoluble ash by 90,200 tons and the cost to the consumer from $2.00 to $1.90 per ton.

It appears that the unit cost of primary plant nutrients in mixed fertilizer can be substantially reduced, if the consumer limits his purchases to those grades which can be manufactured and distributed economically without excessive inclusion of either limestone or inert materials.

Acknowledgment

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PHOSPHORUS AVAILABILITY

Crop Response to Ammoniated Superphosphates and Dicalcium Phosphate, as Affected by Granule Size, Water Solubility, and Time of Reaction with Soil

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Crop response to phosphorus in a series of nonammoniated and ammoniated ordinary and concentrated superphosphates and dicalcium phosphate was determined in greenhouse experiments. Heavy ammoniation decreased the water solubility of phosphorus in ordinary superphosphate from 70 to 14% and in concentrated superphosphate from 89 to 57%, chiefly because of conversion to dicalcium and more basic phosphates. With band application, yields of dry matter and of phosphorus with the ammoniated superphosphates were closely related to the amount of water-soluble phosphorus applied; but other than for dicalcium phosphate, granule size was of little importance. With phosphates mixed throughout the soil, both water solubility and granule size of the phosphates greatly influenced yields on most soils. Response decreased with increasing time of reaction (3 and 6 months) of the superphosphates with soil prior to cropping. Decrease in response with time was much less with granular than with fine superphosphates. Liming acid Hartsells fine sandy loam had variable effects on crop response to phosphates.

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RECENT TECHNOLOGICAL ADVANCES in granulation techniques have given a marked stimulus to the production and use of granular fertilizers. The widespread availability of a variety of nitrogen solutions for use in ammoniating and granulating superphosphate-based fertilizers has also been important. Because of the generally lower cost of nitrogen in solutions than in solid materials, some fertilizer producers incorporate as much solution nitrogen as possible into various mixed fertilizers. Ammoniation is also a desirable step in most granulation processes.

Results from a series of greenhouse pot tests with phosphate fertilizers varying in granule size and water solubility of the phosphorus are presented. The results are discussed in terms of first-crop response, and should probably not be extrapolated to residual effects over a much longer period of time.

Experimental Procedure

Characteristics of Fertilizers. A series of seven nitrogen-phosphorus fertilizers was prepared in two granule sizes, −6 +14 and −35 mesh, using modifications of a continuous ammoniation-granulation process (*3*). Ingredients and chemical analyses are shown in Table I. Different contents of water-soluble phosphorus were obtained by ammoniating ordinary and concentrated superphosphates to varying degrees. Ammonia–ammonium nitrate solutions were used for ammoniating and granulating the superphosphates and for granulating anhydrous dicalcium phosphate.

Soils and Liming Treatments. Analyses of the soils used in the experiments are shown in Table II. For experiment 1, Hartsells fine sandy loam (pH 5.2) was limed to three levels with ground dolomite—to pH 5.5: 1 ton per 2,000,000 pounds of soil just prior to mixing fertilizers; to pH 6.8: 5 tons 1 month previously; to pH 7.5: 5 tons one month previously, plus 2.5 tons prior to mixing fertilizers and seeding. Fort Collins loam subsoil (pH 8.2) was also used.

For experiment 2, Groseclose silt loam (pH 5.4) and Nason silt loam (pH 5.3) were limed to pH 6.5 with 4 and 10 tons of limestone per acre, respectively. Iredell clay loam (pH 6.5), unlimed, was also used.

For experiment 3, Hartsells soil was limed to pH 6.5 4 months prior to seeding. Davidson clay loam (pH 5.8) was used without liming.

For experiment 4, the unlimed Hartsells soil (pH 5.5) and the same soil limed to pH 7.5 with 8 tons of dolomitic limestone 1 month prior to mixing fertilizers for the 6 months' reaction period, were used.

Amounts equal to 3000 grams of dry soil per No. 10 can were used for experiments 1, 3, and 4; and 1800 grams per smaller can in experiment 2.

Fertilizer Application and Cropping. For experiment 1, the phosphates were applied in a layer 1 inch below the surface at rates of 60 and 120 mg. of total phosphorus (P_2O_5) per pot (40 and 80 pounds per 2,000,000 pounds of soil) on November 28, 1956. Anderson wheat was planted November 30 and harvested as heads began to form February 6, 1957. After working up the surface soil only, red clover was planted February 18 without further addition of phosphates. This crop was harvested June 5 as blossoms began to form. Other nutrients were added in solution as follows, per pot: For wheat, nitrogen was equalized at 150 mg. and potassium at 100 mg. as ammonium nitrate and potassium chloride; and for red clover, 65 mg. of nitrogen as ammonium nitrate and 15 mg. of magnesium as magnesium sulfate, were added.

For experiment 2, a total of 180 mg. of nitrogen as ammonium nitrate, 224 mg. of potassium as potassium chloride, and 45 mg. of total phosphorus per pot (50 pounds of phosphate per 2,000,000 pounds of soil) from the various phosphates were mixed with the three soils on July 18, 1957. Sudangrass was planted on the same day and harvested on September 4.

For experiment 3, the phosphates were ground to −100 mesh and mixed with limed Hartsells fine sandy loam and unlimed Davidson clay loam at the rate of 60 mg. of total phosphorus per pot. Anderson wheat was planted November 30, 1957, and later thinned to 20 plants per pot. A total of 150 mg. of nitrogen and 100 mg. of potassium per pot as ammonium and potassium nitrates were added for this crop. Wheat was harvested February 11, 1958.

For experiment 4, the phosphates were mixed throughout the soil at rates of 60 and 120 mg. of total phosphorus per pot (92 and 184 pounds of phosphate per 2,000,000 pounds of soil) at three dates: 6 months prior, 3 months prior, and just prior to seeding. All of the soil was potted at one time, just before the 6 months' reaction period. Nitrogen was equalized at this time with ammonium nitrate at 180 mg. per pot, the maximum amount of nitrogen present with the phosphate fertilizers. Potassium chloride was also added to provide 100 mg. of potassium per pot. The soil was watered occasionally and allowed to

Table I. Characteristics of Fertilizers Used in Experiments

Fertilizer Ingredients[a]	No.	U. S. Mesh Size	Total N, %	Total P_2O_5, %	Available P_2O_5, %	Water-Soluble P_2O_5, % of Available
CSP + AN	1	−6 +14	14.0	26.0	98	89
		−35	15.5	24.7	98	86
OSP + AN	2	−6 +14	7.9	17.2	97	70
		−35	7.6	16.9	96	71
ACSP + AN	3	−6 +14	14.9	30.0	96	57
		−35	14.1	30.0	95	55
AOSP + AN	4	−6 +14	10.1	16.5	88	53
		−35	9.5	16.7	88	52
AOSP + AN	5	−6 +14	8.9	18.0	79	32
		−35	9.0	17.9	79	30
AOSP + AN	6	−6 +14	9.0	18.0	86	14
		−35	8.7	18.0	81	14
DCP + AN	7	−6 +14	13.6	26.3	100	3
		−35	15.7	22.9	100	3

[a] OSP and CSP, ordinary and concentrated superphosphates; AOSP and ACSP, ammoniated OSP and CSP; DCP, anhydrous dicalcium phosphate; AN, ammonium nitrate. Major phosphate phases identified with a petrographic microscope were: water-soluble P—monoammonium in 3, 4, 5, and 6; monocalcium in 1, 2, and 6; water-insoluble P—DCP in all the fertilizers; precipitated apatite in 4, 5, and 6.

Table II. Analyses of Soils Used in Experiments

Soil Type and Source	pH	P Soluble in 0.5N $NaHCO_3$, P.P.M.	Exchangeable Cations, Meq./100 G. Ca	K
Hartsells fine sandy loam, Ala.	5.1	2.1	1.6	0.24
Ft. Collins loam subsoil, Colo.	8.3	17.6	[a]	0.39
Davidson clay loam, Va.	5.8	6.6	5.3	0.16
Groseclose silt loam, Va.	5.4	6.0	2.8	0.18
Iredell clay loam, Va.	6.5	4.2	6.9	0.06
Nason silt loam, Va.	5.3	9.0	0.9	0.16

[a] Calcareous soil.

react over the full period. Columbia oats were planted February 27, 1958, and later thinned to 25 plants per pot. The crop was harvested April 4 as heads began to form.

Experiments 1, 3, and 4 were conducted at Wilson Dam, Ala., and experiment 2 at Blacksburg, Va. Each phosphate treatment was replicated three times in all experiments. All crops were dried at 80° C., weighed, ground, and analyzed for total phosphorus.

Results

Granular and −35 Mesh Fertilizers. Band Application. Yields of dry matter with various phosphates applied in a layer 1 inch below the surface of the soil (experiment 1) are shown in Table III. Marked response to phosphorus was obtained on Hartsells fine sandy loam with both wheat and red clover. Only wheat responded to phosphorus on the calcareous Fort Collins loam; so red clover yields are not reported. This soil contained much more soluble phosphorus than did the Hartsells.

There were no appreciable interactions in yields between the various fertilizers and either rate of application of phosphorus or liming treatments. Consequently, mean yields for the two rates of phosphorus and three liming levels are shown. Mean wheat yields for 60 and 120 mg. of phosphate were 5.5 and 7.2 grams per pot, respectively, on Hartsells soil and 6.2 and 7.0 grams on Fort Collins soil. Wheat yields were not increased by liming, as shown by the means of 6.8, 6.0, and 6.2 grams at pH 5.5, 6.8, and 7.5 on Hartsells and 6.6 grams on Fort Collins. Corresponding yields of red clover, however, were 6.1, 7.5, and 7.7 grams per pot for the liming treatments on Hartsells and 14.8 grams on Fort Collins soil.

Wheat yields on the Hartsells soil decreased markedly with decrease in content of water-soluble phosphorus as applied in the series of ammoniated superphosphates. Granule size had little effect on yields in this series of fertilizers, but much lower yields resulted with the granular dicalcium phosphate. Crop responses to fine dicalcium phosphate and to superphosphate containing about 50% of its phosphorus in water-soluble form were about equal.

A similar trend in yields of the second crop, red clover, was found on the Hartsells soil, but differences for water solubility of the phosphorus were less than for wheat. With this crop, fine dicalcium phosphate produced higher yields than any of the superphosphates, and granular dicalcium phosphate was comparatively more effective than for wheat.

The differences among wheat yields due to fertilizers applied on the Fort Collins soil were small and not closely related to water solubility. Yields were appreciably higher with concentrated superphosphate and dicalcium phosphate than with ordinary superphosphate.

Relative differences for uptake of phosphorus and yield of dry matter among the various fertilizers were similar. The relationship between uptake of phosphorus by wheat and the amount of water-soluble phosphorus applied is shown in Figure 1. The high linear correlation coefficients for the two crops and soils fertilized with the superphosphate materials, given in Table IV, again emphasize the marked effect of water-soluble phosphorus on uptake from the superphosphate fertilizers. Uptake from fine dicalcium phosphate was obviously not dependent on its content of water-soluble phosphorus.

Apparently very little of the phosphorus not readily soluble in water was utilized by the crops from the ammoniated superphosphates, while fine dicalcium phosphate supplied adequate

Table III. Yields of Wheat (First Crop) and Red Clover (Second Crop) as Affected by Granule Size and Water Solubility of Phosphorus. Band Application

Fertilizer and % Water-Soluble P	Mesh Size	Yield of Dry Matter, G. per Pot: Hartsells Fine Sandy Loam[a] Wheat	Hartsells Fine Sandy Loam[a] Red clover	Hartsells Fine Sandy Loam[a] Both crops	Ft. Collins loam, wheat[b]
CSP (89%)	−6 +14	8.04	7.44	15.48	6.70
	−35	7.93	7.46	15.39	6.99
OSP (70%)	−6 +14	7.34	7.39	14.73	6.06
	−35	7.41	7.54	14.95	6.19
ACSP (57%)	−6 +14	7.19	7.27	14.46	6.76
	−35	7.06	7.57	14.63	7.05
AOSP (53%)	−6 +14	7.00	6.85	13.85	6.37
	−35	6.51	7.03	13.54	6.23
AOSP (32%)	−6 +14	5.72	5.96	11.68	6.43
	−35	4.98	6.67	11.65	6.23
AOSP (14%)	−6 +14	4.49	5.68	10.17	6.50
	−35	4.51	6.65	11.16	6.58
DCP (3%)	−6 +14	3.86	6.05	9.91	6.67
	−35	6.86	8.26	15.12	7.22
No P	...	2.93	5.10	8.03	5.39
L.S.D., 1% level		0.51	0.62	...	0.29

[a] Mean yields for liming to pH 5.5, 6.8, and 7.5 and for 60 and 120 mg. P_2O_5 rates per pot (3000 grams soil).

[b] Red clover was also grown on the Ft. Collins soil, but showed no yield response to fertilizer P.

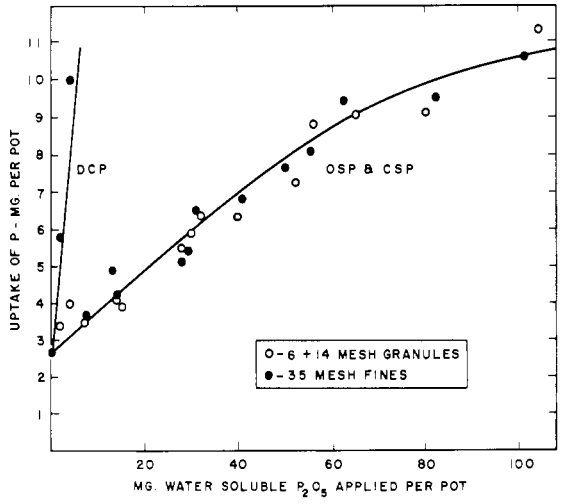

Figure 1. Uptake of phosphorus by wheat, as affected by band application of varying amounts of water-soluble phosphorus from −6, +14 and −35 mesh phosphates

Table IV. Correlation Coefficients[a] for the Relationship between Uptake of Phosphorus by Wheat and Red Clover and the Amount of Water-Soluble Phosphorus in Superphosphates Applied for Wheat

Soil and pH	Wheat	Red Clover
Hartsells, 5.5	0.950[a]	0.641[a]
Hartsells, 6.8	0.954[a]	0.913[a]
Hartsells, 7.5	0.940[a]	0.891[a]
Ft. Collins, 8.2	0.562[a]	N.S.[b]

[a] Significant at 1% level.

[b] Not significant.

phosphorus for good yields. Granular dicalcium phosphate dissolved too slowly to supply adequate phosphorus to the wheat crop, but later adequately supplied the clover crop.

Granular and −35 Mesh Fertilizers. Mixed Application. Yields of the first cutting of sudangrass for the fertilizers mixed thoroughly with three soils (experiment 2) are shown in Table V. Yields were rather low, but good response to phosphorus was obtained on all soils.

Yields were markedly lower on the Groseclose and Nason soils with −35 than with −6 +14 mesh superphosphates. This was true only for the nonammoniated superphosphates high in water-soluble phosphorus on the Iredell soil. The reverse was true for dicalcium phosphate on the first two soils and for ammoniated superphosphates and dicalcium phosphate on the Iredell soil. These results indicate the importance of soil characteristics, as yet poorly defined.

There was a pronounced increase in yield with increase in water-soluble phosphorus content of the superphosphates of both sizes on the Groseclose and Nason soils, but only for the −6 +14 mesh granules on the Iredell soil. Water solubility of the −35 mesh fertilizers had little effect on yields on this soil. Similar results were reported by Lawton, Apostolakis, Cook, and Hill (*4*).

Powdered Fertilizers. Mixed Application. Uptakes of phosphorus by wheat (first crop) and by oats (second crop) from powdered (−100 mesh) ammoniated superphosphates and dicalcium phosphate applied to two soils (experiment 3) are shown in Table VI. A marked response to applied phosphorus was obtained on both Hartsells fine sandy loam and Davidson clay loam. Uptake of phosphorus was least from ammoniated concentrated superphosphate (57% water-soluble) on both soils and highest from dicalcium phosphate on the Hartsells soil. Except for ammoniated concentrated superphosphate, there was no appreciable difference in uptake among fertilizers on the Davidson soil. Thus, the effects of water solubility on availability of phosphorus from the powdered fertilizers compared in this experiment were small, but still measurable. Generally, with powdered phosphates mixed with the soil, as pointed out by Lawton, Apostolakis, Cook, and Hill (*4*), the slow dissolution of phosphorus from small particles of low water solubility is just about balanced in terms of plant availability by fixation of phosphorus from highly soluble material.

Granular and −35 Mesh Fertilizers. Various Times of Mixed Application. Yields of dry matter by oats fertilized with the several phosphates mixed with Hartsells fine sandy loam (experiment 4) are shown in Table VII. Marked response to applied phosphorus was obtained, as indicated by the mean yields of 2.8, 6.4, and 8.5 grams per pot on the unlimed soil and 3.1, 6.7, and 8.5 grams per pot on the limed soil with none, 60, and 120 mg. of phosphorus applied per pot. These comparisons also indicate that liming increased mean yields only slightly.

Interactions of rate of application of phosphorus with liming, time of application prior to seeding, and granule size on plant uptake of phosphorus are shown in Figure 2. The widest range of yields, reflecting the greatest effect of fertilizer properties, occurred with granular materials applied just prior to

Table V. Yields of Sudangrass on Three Soils, as Affected by Granule Size and Water Solubility of Phosphorus. Mixed Application

Fertilizer and % Water-Soluble P	Mesh Size	Yield of Dry Matter, G. per Pot: Groseclose silt loam	Nason silt loam	Iredell silt loam
CSP (89%)	−6 +14	5.83	3.66	4.27
	−35	3.17	1.80	3.80
OSP (70%)	−6 +14	4.77	3.07	4.17
	−35	2.96	1.53	4.06
ACSP (57%)	−6 +14	4.37	2.50	3.73
	−35	1.47	1.00	3.80
AOSP (53%)	−6 +14	3.00	3.16	3.77
	−35	2.20	1.03	4.23
AOSP (32%)	−6 +14	2.87	1.60	3.57
	−35	1.67	0.77	4.83
AOSP (14%)	−6 +14	2.40	1.30	2.76
	−35	1.70	0.80	4.60
DCP (3%)	−6 +14	2.10	0.83	2.23
	−35	3.47	1.23	4.60
No P	...	1.30	0.77	0.83
L.S.D.				
5% level		1.40	0.77	0.99
1% level		1.90	1.04	1.34

Table VI. Uptake of Phosphorus[a] by Wheat (First Crop) and Oats (Second Crop) from −100 Mesh Fertilizers Varying in Water Solubility of the Phosphorus. Mixed Application

Fertilizer and % Water-Soluble P	Hartsells Fine Sandy Loam: Wheat	Oats	Total	Davidson Clay Loam: Wheat	Oats	Total
ACSP (57%)	11.95	6.87	18.82	9.87	5.62	15.49
AOSP (53%)	11.77	7.60	19.37	11.17	7.29	18.46
AOSP (32%)	11.63	8.70	20.33	11.33	7.34	18.67
AOSP (14%)	10.89	8.11	19.00	11.01	7.45	18.46
DCP (3%)	13.44	7.83	21.27	11.34	6.96	18.30
No P	2.31	3.57	5.88	7.35	4.51	11.86
L.S.D., 5% level	1.09	1.81	...	1.01	0.93	...

[a] Applied at rate of 60 mg. total P per pot (3000 grams soil).

Table VII. Yields of Oats, as Affected by Granule Size, Water Solubility of Phosphorus, and Time of Reaction with Hartsells Fine Sandy Loam. Mixed Application

Fertilizer and % Water-Soluble P	Mesh Size	Yields of Dry Matter, G. per Pot[a]: Applied Just Prior to Seeding: Unlimed	Limed[b]	Applied 3 Months Prior: Unlimed	Limed[b]	Applied 6 Months Prior: Unlimed	Limed[b]
CSP (89%)	−6 +14	11.13	11.49	9.44	10.19	8.87	9.55
	−35	9.51	11.20	5.75	9.03	4.86	7.58
OSP (70%)	−6 +14	11.17	10.80	9.01	9.77	8.96	9.14
	−35	9.77	10.03	5.94	8.44	5.14	7.25
ACSP (57%)	−6 +14	10.56	10.61	8.50	9.15	7.97	9.03
	−35	9.23	9.35	5.44	7.77	4.67	6.87
AOSP (53%)	−6 +14	11.01	9.90	9.06	8.91	7.81	7.89
	−35	8.79	8.23	5.24	6.44	5.06	5.77
AOSP (32%)	−6 +14	9.35	8.10	6.37	6.76	6.17	6.10
	−35	7.94	5.74	5.00	4.36	4.45	4.32
AOSP (14%)	−6 +14	7.47	5.54	5.86	4.72	5.30	4.09
	−35	7.65	4.34	4.91	3.93	4.44	3.54
DCP (3%)	−6 +14	5.82	3.98	5.40	3.89	5.61	4.13
	−35	9.72	6.51	6.60	7.33	5.74	7.31
No P	...	2.75	3.08	2.57	3.00	3.02	3.20
L.S.D.							
5% level		0.56	1.09	0.54	0.78	0.74	0.67
1% level		0.75	1.46	0.72	1.04	0.98	0.89

[a] Mean yields for 60 and 120 mg. phosphorus per pot (3000 grams soil).
[b] Eight tons of dolomitic limestone were applied 1 month prior to first date of application.

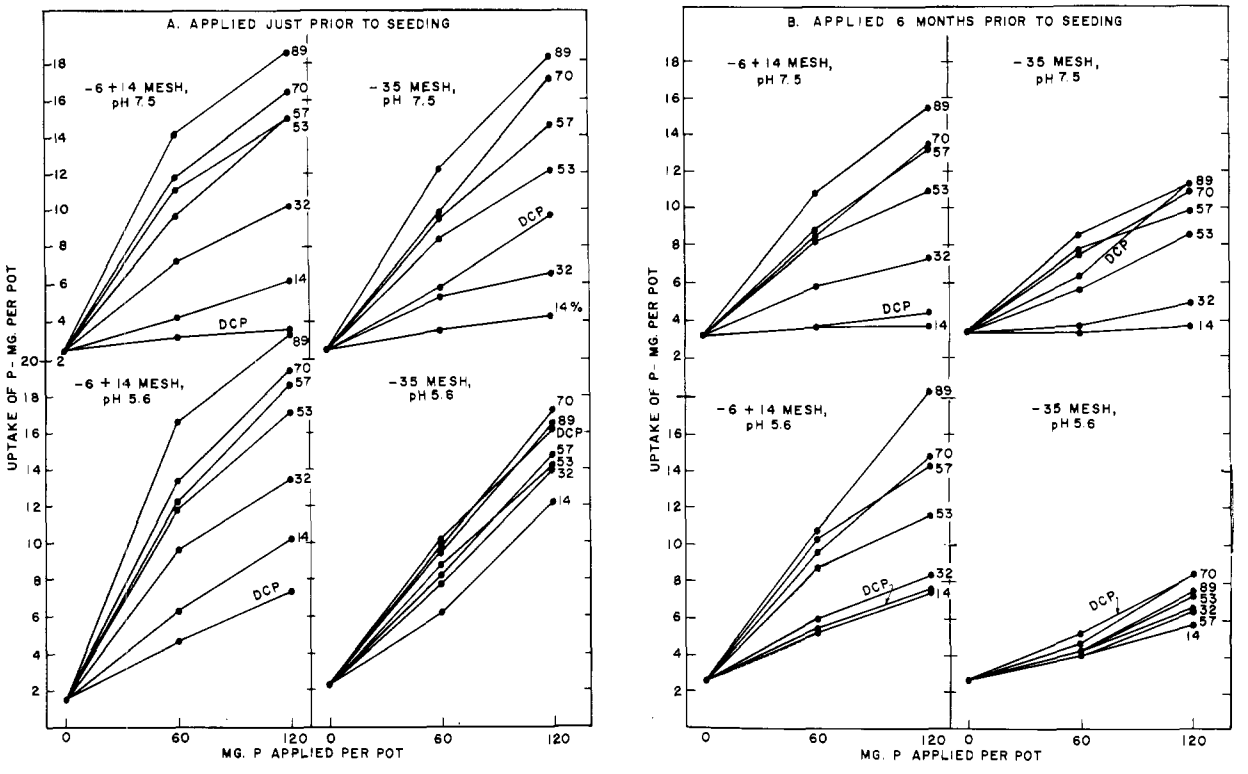

Figure 2. Uptake of phosphorus by oats in relation to rate of mixed application, as affected by granule size, liming, and time of reaction with soil prior to seeding

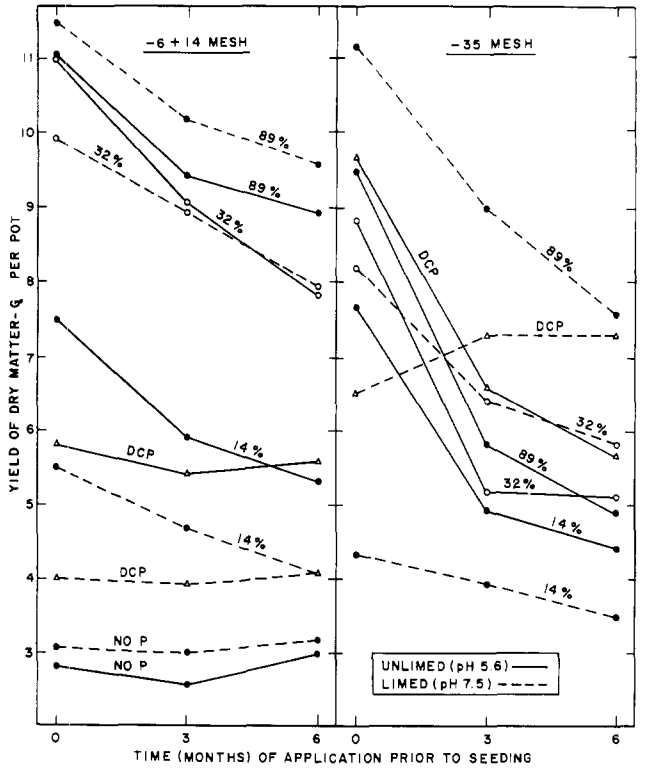

Figure 3. Yields of oats in relation to time of reaction with soil, as affected by water solubility of phosphorus, granule size, and liming

seeding. For 6 months' reaction with the soil, the narrowest range of yields occurred with −35 mesh fertilizers on the acid soil. The smaller yield differences with longer time of reaction with the soil apparently reflect the formation of reaction products similar in plant availability.

Effect of liming on crop response to individual fertilizers was marked, especially in relation to time of application prior to seeding the crop. With application just prior, liming tended to increase crop response to the phosphates of high water solubility, especially if finely divided, but decreased response to those of low water solubility. This was particularly marked with dicalcium phosphate and ammoniated ordinary superphosphate (14% water-soluble). With application of fertilizers 3 or 6 months prior to seeding, liming increased crop response to all the phosphates except ammoniated ordinary superphosphate (14 and 32% water-soluble) and to granular dicalcium phosphate, for which the reverse was true. Effects of liming on crop response to granular ammoniated ordinary superphosphate (53% water-soluble) were not appreciable. Apparently liming resulted in the formation of more soluble reaction products between water-soluble phosphates and soil. Except for fine dicalcium phosphate, liming reduced crop response to phosphates of low water solubility, with which rate of solution is the chief factor governing availability to plants.

The effect of time of reaction in relation to water solubility, granule size, and liming is illustrated in Figure 3. Yields with all of the superphosphate fertilizers decreased with time on both unlimed and limed soil. The extent of decrease was greater with fine than with granular materials and on unlimed than on limed soil. Decrease in yields with fine dicalcium phosphate on unlimed soil was similar to that for the super-

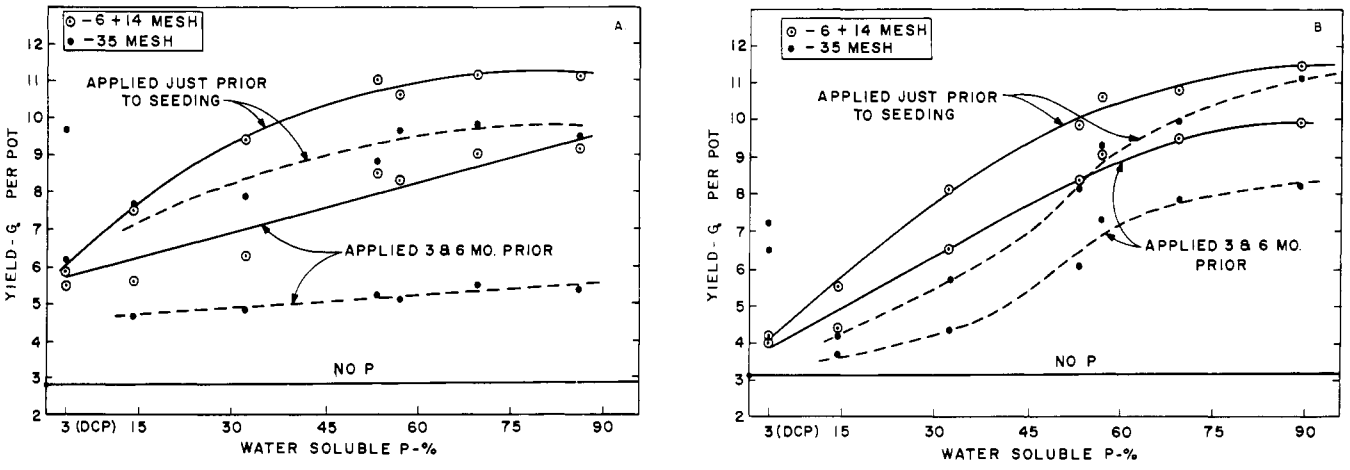

Figure 4. Yields of oats in relation to water solubility of phosphorus as affected by liming, granule size, and time of reaction

A. Oats, unlimed
B. Oats, limed

phosphates. Response to fine dicalcium phosphate increased somewhat with time on the limed soil, while response to granular material did not change appreciably with time on either unlimed or limed soil.

Effects of water solubility of phosphorus in the fertilizers on yields in relation to liming, granule size, and time of application prior to seeding are illustrated in Figure 4. Yields increased with increase in water solubility of the granular superphosphate fertilizers under all situations. The same was true for the −35 mesh superphosphates, except that after 3 and 6 months of reaction with the unlimed soil, availability of phosphorus to the crop was markedly reduced and water solubility no longer exerted an appreciable effect on yields. As in the other experiments, crop response to fine dicalcium phosphate was not related to its water solubility.

Discussion

Results from this series of experiments provide additional evidence that crop response to phosphorus in fertilizers is markedly influenced by a number of fertilizer and soil properties. Some fertilizer properties, such as water solubility of phosphorus, granule size, and composition, have been reasonably well defined, but the effects of soil properties, such as liming status, pH, and many others, are still rather poorly defined.

In experiment 4 (Figure 3) liming clearly influenced not only the initial phosphate behavior in soils, but also the course of reactions with time. These effects were particularly striking with −35 mesh materials, and differed with water solubility. Although crop response to the −35 mesh materials of highest water solubility decreased sharply with time on both limed and unlimed soil, liming lessened the rate of change to less soluble reaction products. The contrasting effects of liming on behavior of ammoniated ordinary superphosphate (14% water-soluble) and dicalcium phosphate strongly suggest that the characteristics of the insoluble residues differed initially and changed progressively with time. The increase in response to dicalcium phosphate with time in the limed soil probably results from release of phosphorus due to formation of octocalcium phosphate, as found by Lehr and Brown (*5*).

Differences in response by oats in favor of the granular superphosphates were greatest for application just prior to seeding and decreased with time. Response to granular was much less initially than to fine dicalcium phosphate and did not change appreciably with time. Because of relatively rapid migration of water-soluble phosphorus from fertilizer particles into, and reaction with the adjacent soil, plants obtain phosphorus largely from the reaction products rather than from the form initially present in the fertilizer. The effect of water-soluble fertilizer phosphorus, together with associated salts, is undoubtedly important both in increasing the concentration of phosphorus around the granules and in determining the volume of soil influenced by the applied phosphorus (*7*). These factors, together with differences in the rates of solution, probably account for higher availability from granular superphosphates than from granular dicalcium phosphate [Bouldin and Sample (*2*)].

Effectiveness of granular and fine superphosphates with further time and cropping, and the implications with respect to uptake of phosphorus throughout an entire cropping season of several months are subjects of continuing research.

Acknowledgment

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SOIL ORGANIC MATTER

Effect of Wheat Straw, Nitrogenous Fertilizers, and Carbon-to-Nitrogen Ratio on Organic Decomposition in a Subhumid Soil

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Further information on effects of nitrogen fertilizers and straw on organic decomposition and nitrogen transformation is desirable for sound field practice. Water-soluble and -insoluble fractions of straw, rates of straw, and forms of nitrogen had significant effects on carbon dioxide evolution. Ammonium hydroxide produced more rapid decomposition than ammonium or calcium nitrate. Apparent utilization of nitrate as an oxidant by denitrifiers decreased the nitrates and lowered carbon dioxide evolution. Level of available carbon and form of nitrogen influence nitrogen balance as well as rate and extent of decomposition. The carbon-to-nitrogen ratio often fails to predict the rate of microbial decomposition and effects on soil nitrogen. Definition of carbon levels should indicate availability as well as total amount. Straw additions prevented significant decreases in total nitrogen.

PLANT RESIDUES ADDED to soil undergo more or less rapid and extensive decomposition by microorganisms, with liberation of carbon as carbon dioxide. The rate and extent of this mineralization is governed by the nature of the organic matter and by environmental factors, especially moisture, temperature, and available nitrogen. Determination of the rate and amount of carbon dioxide evolution from a soil provides a measure of the rate and extent of the organic matter decomposition under the particular conditions imposed.

Wheat straw residues are extensively used in trashy fallow or stubble-mulch farming on the subhumid soils of Oregon and other western states as an erosion control measure. Nitrogen fertilizers are generally used to avoid deficiency of this element. Decomposition studies on such residues are important for indicating probable duration and effects on carbon-to-nitrogen relationships in the soil. As the nitrogen cycle and carbon cycle are biologically bound together and proceed simultaneously, organic matter decomposition is always accompanied by nitrogen transformations. The resulting changes in forms and availability of nitrogen lead to losses and gains, the balance being determined by carbon sources, carbon-to-nitrogen ratio, and conditions of environment. Although much attention is given to carbon-to-nitrogen ratio for its influence on decomposition and nitrogen availability, emphasis should be placed on the relative decomposability of the carbonaceous constituents of organic matter. Resistant material will have less pronounced and longer lasting effects. The significance of crop residues and nitrogen fertilizers in soil fertility may therefore be assessed more readily, if water-soluble as well as water-insoluble components are considered in relation to carbon and nitrogen changes resulting from additions of materials containing these elements.

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Materials and Methods

Soil. Walla Walla silt loam soil was selected for use in this study, because it is one of the most extensive and important wheat producing soils of Oregon (average annual rainfall is 15 to 18 inches). It is a Chestnut soil developed from fine-textured, wind-deposited material—loess. Accumulated humus from a native vegetation of bluebunch wheat grass (*Agropyron spicatus*) may be responsible for its dark color and exceptional fertility. Practically all of the soil is cultivated, and wheat is by far the most important crop. Summerfallow is commonly practiced.

Table I. Analysis of Walla Walla Silt Loam Soil

Analysis	Amount
pH	6.7
Nitrogen, p.p.m.	
Ammonium	10
Nitrite	10
Nitrate	4
Total	1,030
Total carbon, %	1.51
C:N ratio	14.66
Cation exchange capacity, meq./100 g.	18.75
Exchangeable cations, meq./100 g.	
Ca	9.95
Mg	3.12
Na	.52
K	1.49
Bicarbonate soluble phosphorus, p.p.m.	12.5
Water holding capacity, %	48.7
Mechanical analyses, %	
Sand	42
Silt	38
Clay	18
Microorganisms by plate count	
Molds, per gram	88,500
Mucors, %	12
Penicillium, %	49
Aspergillus, %	11
Demitaceae, %	24
Trichoderma, %	4
Bacteria, per gram	28,000,000
Streptomyces, %	21
Azotobacter, per gram	40,000

A bulk sample of the soil was collected in April 1956 from the Pendleton Branch Experiment Station. Random samples to a depth of 6 inches were composited from an alley along the end of a series of trashy fallow plots. The soil was brought to the laboratory, air-dried and passed through a 10-mesh sieve, and stored in covered 30-gallon galvanized iron cans for use as required. Moisture was determined each time soil was taken for an experiment, so that samples could be weighed out on the dry basis.

Chemical, physical, and microbial properties of the soil are shown in Table I. All data, except for pH, are given on the dry basis.

Exchange capacity and exchangeable cations were determined by the ammonium acetate method. Total phosphorus was determined by the bicarbonate method of Olson, Cole, Watanabe, and Dean (*17*).

For pH a soil-to-water ratio of 1 to 5 was used. Ten grams of soil, dry basis, and 50 ml. of distilled water were stirred in a 100-ml. beaker; after coarse particles had settled, readings were made with a Model N Beckman pH meter using a glass electrode.

Table II. Analysis of Straw

Moisture, %	5.75
Water holding capacity, %	573.0
Total carbon, %	45.23
Water-soluble carbon, %	3.03
Total nitrogen, %	0.37
Water-soluble nitrogen, %	0.00
C:N ratio	122.2
pH, 1/20 aqueous suspension	5.1

Table III. Mechanical Analyses of Milled Wheat Straw

Tyler Standard Screen, Meshs/Inch	Wiley Mill, 10-Mesh Screen, %	Weber Hammer Mill, No. 800 Screen,[a] %
+20	42.5	...
+40	37.4	...
+60	11.8	6.4
+80	4.1	18.4
+100	1.9	18.9
+150	1.0	16.7
+200	0.5	27.2
−200	0.8	12.1

[a] Approximately 60-mesh.
+ Indicates retained on screen.
− Indicates passing screen.

Table IV. Analysis of Extract and Extracted Wheat Straw

Analysis	Wheat Straw Extract, %	Extracted Wheat Straw, %
Moisture	...	2.9
Total solids	1.01	...
Total carbon	3.03	40.9
Total nitrogen	0.00	0.36
C:N ratio	...	113.3
pH	8.1	6.4[a]

[a] Of 1/20 water suspension.

Ammonium nitrogen was determined by the method of Shirkhande (*19*), which employs distillation of the sample with phosphate buffer at pH 7.4. By avoiding the use of strong alkali, liberation of ammonia from amino acids and other intermediate decomposition products of proteins was prevented.

Nitrites were determined by the diazotization method with sulfanilic acid, 1-naphthylamine hydrochloride, and sodium acetate buffer.

Harper's (*9*) phenoldisulfonic acid method for nitrates was modified by use of ammonium carbonate to remove the excess calcium hydroxide remaining in a 1-to-5 soil-water extract after clarification.

Total nitrogen was determined by the official AOAC Gunning method. For soil, this was modified to include nitrate.

Total carbon was determined by combustion, using the AOAC procedure.

Moisture was determined as loss in weight by drying samples at 105° C. for 24 hours.

Water holding capacity was calculated from the amount of water retained by samples in Gooch crucibles wetted from below by immersion and then allowed to drain to constant weight in a moisture-saturated atmosphere.

Mechanical analysis was made by the pipet method (*13*).

Numbers of microorganisms were estimated by plating procedures. Peptone glucose acid agar was used for molds, and sodium albuminate agar was employed for bacteria and streptomyces. *Azotobacter* counts were made after growth on the medium recommended by Martin (*14*).

Straw. A 10-pound sample of wheat straw, Elmar variety, 1956 crop, from Pendleton was obtained and ground in a Wiley mill to pass a 10-mesh screen. This material was used in a greenhouse experiment to be reported later. A subsample was further reduced in a hammer mill through an approximately 60-mesh screen for use in the laboratory. The prepared samples were stored in screw-capped amber bottles.

Chemical analysis of the straw is shown in Table II.

Mechanical analyses of the prepared straw samples are indicated in Table III.

A sample of milled straw weighing 100 grams was fractionated in a stack of Tyler standard screens on a Porter sand shaker for 10 minutes; the percentage of each separate component was then determined by weight. As particle size affects reactivity and rate of decomposition it is considered important to present the mechanical analysis. The hammer-milled sample was used for all the experiments. While nearly all the sample passed 60-mesh, a major proportion was much finer. Use of material of a uniform size would be desirable, but it is likely that different mechanical fractions differ in chemical constituents and that no one fraction would represent the composition of the whole.

Extracted Straw and Straw Extract. To 25 grams of the hammer-milled straw in a liter bottle were added 500 ml. of distilled water which was shaken for 10 minutes on a Miller paint shaker, then allowed to stand overnight. Next day, it was filtered through a Whatman No. 2 paper on a Büchner funnel and another 500 ml. of water were added to the residue. This was shaken for 10 minutes and then passed through the same filter. The extraction process was repeated three times, after which the filtrate was colorless. Following a final washing, washings and filtrates were combined, concentrated on a steam bath, made up to a volume of 1000 ml., and stored in a bottle in the refrigerator for use as required.

The residue of extracted wheat straw on the Büchner funnel was carefully removed, air-dried, and stored in a bottle. Analysis of the extract and extracted wheat straw gave the results shown in Table IV.

Nitrogen Sources. The following sources and rates of nitrogen were used: ammonium hydroxide, ammonium nitrate, and calcium nitrate at 0, 100, 200, and 400 p.p.m. of nitrogen. The sources except ammonium hydroxide were of C.P. grade; the ammonium hydroxide was U.S.P., 28% nitrogen. Carbon-to-nitrogen ratios of additions with straw were calculated to include the nitrogen content of the straw, but not of soil nitrogen.

Experimental

Decomposition Studies. Portions of soil weighing 200 grams, dry basis, were placed in pint milk bottles and were treated in factorial combination with three rates of straw with three sources and four rates of nitrogen. A randomized block design was used with two replications. The straw was used at rates of 0, 5, and 10 tons per acre—acre-furrow-slice, considered equivalent to 2,000,000 pounds dry soil—calculated on the dry basis. The 5-ton rate, corresponding to 0.5% of the soil by weight, is not much in excess of average field practice. Extracted straw and straw extract fractions were used in amounts equivalent to those supplied by whole wheat straw at 5 tons per acre. Additional treatments with extracted straw at a rate equivalent to total carbon in straw at 5 tons per acre were made also. The straw was mixed with the soil before transferring to bottles. Nitrogen sources and straw extract were added in solution, and the moisture in all treatments was made up to 50% of the water holding capacity of the soil. This was maintained by frequent addition of distilled water to restore loss of weight.

Temperature was maintained at 28 ± 1° C. The experiment was set up in the incubator and connected to a modified Potter and Snyder (*18*) respiration apparatus designed by Bollen (*1*). The carbon dioxide evolved was absorbed in tubes containing 1*N* sodium hydroxide. The tubes were replaced at the end of 21, 42, 84 hours, and 7, 14, and 28 days.

The absorbed carbon dioxide was determined by differential titration with a Beckman automatic titrator. The end points used were those recommended by Cooper (*6*): pH 8.5 for neutralization of excess sodium hydroxide and conversion of carbonate to bicarbonate; pH 5.0 to 4.6 for neutralization of bicarbonate, the particular end point varying according to the amount titrated. Sulfuric acid, 1*N*, in undetermined amount, was used to first bring the pH to about 8.5, after which 0.83*N* acid was used. Results are expressed as milligrams of carbon equivalent to carbon dioxide per 200 grams of soil on a cumulative basis.

Effect of Straw Fractions on Nitrogen Transformations. Eighty-gram portions of soil, dry basis, were used in pint milk bottles for each treatment. Treatments included straw at 0 and 5 tons per acre in factorial combination with the three sources of nitrogen at 0, 100, 200, and 400 p.p.m. nitrogen.

In each case the amount of soil was weighed and spread in a thin layer on a sheet of wrapping paper. The straw was added by sprinkling the weighed portion over the soil and then mixing well with a spatula, and rolling the mixture back and forth on the paper. It was transferred in four or five approximately equal portions to a pint milk bottle. Corresponding aliquots of distilled water, or solutions of the nitrogen sources when required, were added to each soil portion after it was placed in the bottle. Subsequent diffusion was relied upon to distribute the moisture and nitrogen treatments. This procedure was believed to give better distribution than mixing liquid additions with the soil before transfer to the bottle. It also avoided the risk of puddling. The bottles were capped, weighed, and marked before being placed in the incubator. Each cap was punctured with a 7-mm. hole to provide aeration. A table of random numbers was used in choosing nitrogen sources, in numbering treated bottles, and in selecting their position in the incubator.

Two replications were used. Because of the large number of treatments and analyses involved the replications were set up 7 days apart. The experimental design was a split-split plot with nitrogen sources as major treatments, dates as subtreatments, and nitrogen rates as subsubtreatments.

Soil from replicate bottles was analyzed at 0, 7, 14, and 28 days for pH, ammonium ion nitrogen, nitrite ion nitrogen, nitrate ion nitrogen, and total nitrogen.

Results and Discussion

Effects of Rates of Wheat Straw and Nitrogen Fertilizers on Decomposition. The untreated soil showed the least production of carbon dioxide (Figure 1), which indicated that only 2.5% of the native organic matter was completely decomposed in 28 days. Addition of each nitrogen source, regardless of the rate of application to the soil, only did not significantly increase carbon dioxide evolution at the end of 28 days (Figure 2). There were, however, slight increases during the first day or so; with ammonium hydroxide and ammonium nitrate these became apparent earlier than with calcium nitrate.

To discuss and interpret these carbon dioxide data it is necessary to make certain assumptions, particularly that any excess carbon dioxide produced in a soil treated with plant residues is derived wholly from the organic addition. However, it has been shown that the addition to soil of plant material containing

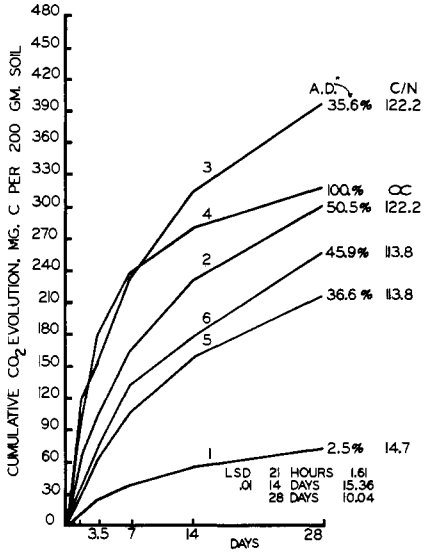

Figure 1. No nitrogen added

1. Soil only
2. Wheat straw 5 T/A
3. Wheat straw 10 T/A
4. Extract from 5 T/A wheat straw
5. Extracted straw from 5 T/A wheat straw
6. Extracted straw equivalent to total carbon in 5 T/A wheat straw

* A.D. = apparent decomposition

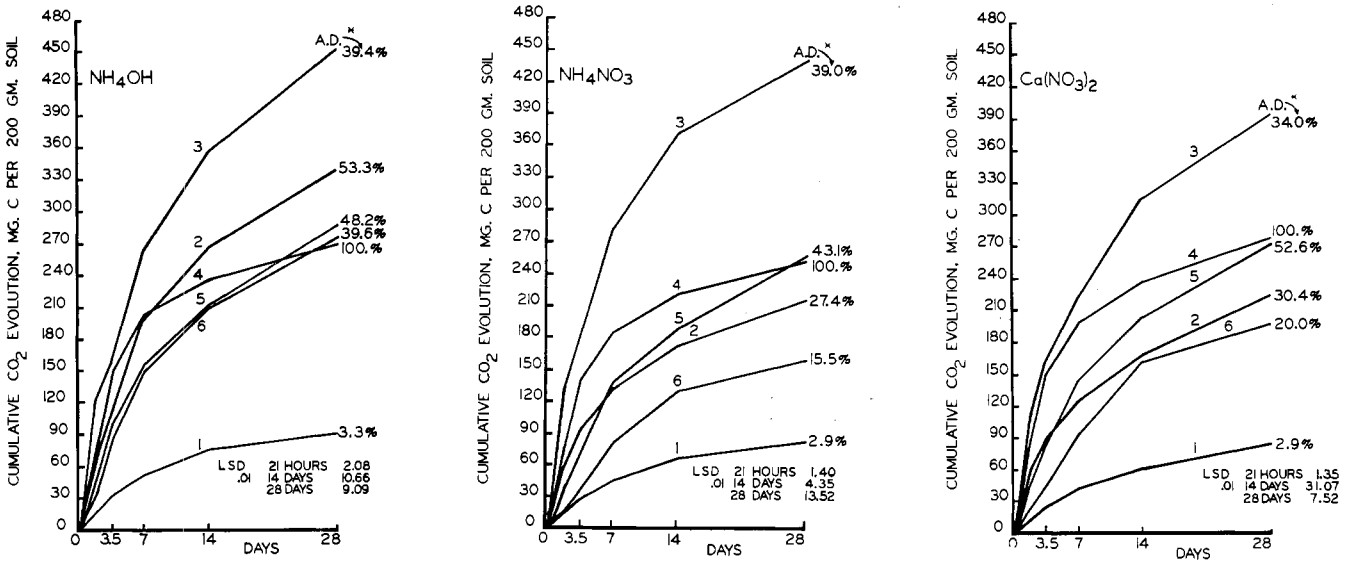

Figure 2. Nitrogen sources at 100 p.p.m. N

1. Soil + N, C:N = 13.4* 2. Wheat straw 5 T/A + N, C:N = 19.1 3. Wheat straw 10 T/A + N, C:N = 33.0 4. Extract from 5 T/A wheat straw + N, C:N = 1.5 5. Extracted straw from 5 T/A wheat straw + N, C:N = 17.3 6. Extracted straw equivalent to total carbon in 5 T/A wheat straw + N, C:N = 19.1

*C:N ratios refer to straw-fertilizer additions

labeled carbon increases the decomposition rate of the soil organic matter (*4*, *8*).

As these experiments were conducted without benefit of carbon-14 as a tracer, the increase in carbon dioxide production, when straw was added, was assumed to be due to decomposition of the straw itself. On this basis, added wheat straw was concluded to be extensively decomposed in 28 days. The extent of decomposition calculated from the carbon dioxide evolved and the total carbon added is referred to as apparent decomposition. Without added nitrogen, apparently one half of the total carbon in wheat straw at 5 T/A was converted to carbon dioxide, while about one third of the 10 T/A treatment was similarly decomposed. This is in accordance with the generally observed phenomenon that the greater the amount of mature plant material added to a soil, the longer time is required for a given percentage to decompose (*4*, *8*).

Results obtained with the different nitrogen sources at 100 p.p.m. of nitrogen are shown in Figure 2. Data for the two higher rates are not presented, as they gave essentially the same pattern.

Ammonium hydroxide increased the rate of decomposition of 5 T/A straw additions, the higher rates of nitrogen having increased effects. In part, this could have been due to effect on pH, although preferential assimilation could be involved. Ammonium and calcium nitrate at all rates depressed the decomposition, the lowest rate in each case being most depressive. This suggests that much of the nitrate served as an oxidant for denitrification, rather than as a direct nutrient for microbes effecting the decomposition. The ammonium and calcium nitrate, however, had closely similar effects; all the nitrate of the former and a similar amount, or one half the total, of the latter could have been involved in oxidizing activities.

With wheat straw at 10 T/A all sources of nitrogen slightly increased the total carbon dioxide evolved at 28 days. Ammonium hydroxide at each rate produced small and similar increases. Higher rates of ammonium nitrate were less effective than at 100 p.p.m. of nitrogen in increasing carbon dioxide production. Increasing rates of calcium nitrate increased apparent decomposition; at 400 p.p.m. of nitrogen, it had the greatest effect of any of the sources. The amount of straw in proportion to added nitrogen sources is a factor influencing the rate of decomposition. Larger amounts of straw could be expected to increase nitrogen fixation, to increase aeration, and to decrease denitrification accordingly. This is consistent with results of total nitrogen determinations (Table V), which showed a greater decrease with no straw. However, carbon dioxide production and nitrates showed a negative correlation. Other investigators have reported conflicting results on the existence of parallels between nitrate formation and carbon dioxide (*7*, *11*).

Decomposition of Water-Soluble and -Insoluble Fractions of Wheat Straw. The water-extract of wheat straw in an amount equivalent to that in whole wheat straw at 5 T/A was rapidly decomposed in all cases, even without added nitrogen (Figures 1 and 2). At 28 days, the carbon dioxide production was in excess of 100% apparent decomposition, indicating a simultaneously accelerated oxidation of the native soil organic matter. All sources and rates of added nitrogen significantly depressed the carbon dioxide evolution, which, however, was still more than enough to account for complete decomposition of the added water-soluble carbon. The larger the rate of nitrogen addition the less carbon dioxide was evolved. This suggests a sparing effect of nitrogen fertilizers on native organic nitrogen, microorganisms thus being relieved of the necessity for decomposing humus to satisfy nitrogen nutritional requirements.

Jansson, Hallam, and Bartholomew (*11*) and Jansson (*10*) reported the preferential utilization of ammonium nitrogen by bacteria in their studies on straw decomposition. This agrees with the results shown in Figure 2, which indicates that the ammonium nitrogen was generally more effective than the nitrate in promoting carbon dioxide production from added straw and straw fractions.

With the extracted wheat straw, containing no readily soluble carbon, addition of nitrogen increased the rate of decomposition as shown by carbon dioxide production. The increase in this case was least with ammonium hydroxide and greatest with calcium nitrate. Forms of nitrogen as well as resistance of organic matter thus seem to influence preferential utilization of these materials by soil microorganisms. Advantages of one form of nitrogen over another, as well as the function of each, are probably determined not only by the nature of the organic substance but also by the given set of environmental conditions.

Where extracted wheat straw was added at the higher rate, equivalent to total carbon supplied by whole straw at 5 T/A, the carbon dioxide evolution was less than from the whole straw at 5 T/A in all cases. With added nitrogen, less carbon dioxide was evolved from the higher application of the extracted straw, except with ammonium hydroxide at 400 p.p.m. nitrogen. Least carbon dioxide was produced with ammonium nitrate.

Effect of Straw Fractions on Nitrogen Transformations. Data showing changes in ammonium nitrogen, nitrification, denitrification, and total nitrogen are given in Table V. Essentially complete recoveries of added nitrogen were obtained at 0 days, within 1 hour after additions. The data for 7 and 14 days are omitted as the trends were in agreement with the results at 28 days. As changes in nitrate concentration can be due to more or less simultaneous denitrification and nitrification, and also to assimilation, the terms apparent nitrification and apparent denitrification are applied to the calculated percentage of these transformations as based on nitrate determination.

The data are in general consistent in showing that the added carbonaceous materials decreased nitrate accumulation. In the absence of straw, some nitrification occurred and ammonium hydroxide was appreciably nitrified, but nitrification of the ammonium ion of ammonium nitrate was overshadowed by nitrate disappearance in many cases. Nitrates disappeared rapidly and extensively from calcium nitrate. These results suggest that both denitrification and assimilation, even in absence of straw, are reasonable in view of the relatively wide carbon-to-nitrogen ratio of the soil. The amounts of ammonium ion nitrogen found in all cases were consistent with nitrate and total nitrogen changes.

Additions of wheat straw, straw extract, and extracted wheat straw gave differences significant at all times, at the 1% level between treatments. Replications showed no significant differences in either carbon dioxide evolution or nitrogen changes. Wheat straw extract had little effect on nitrate accumulation. As this extract provided only a relatively small amount of carbon, 310 p.p.m., it could induce only limited nitrogen assimilation; thus more nitrogen would be left for nitrification. Neither could this small amount of carbon be expected to retard nitrification. The generally accepted concept is that soluble carbonaceous material inhibits in pure cultures of nitrite- and nitrate-forming bacteria, but they can stand high concentrations of organic matter in soil, composts, and manure piles where the soluble carbonaceous material is higher. This tolerance is probably made possible by physical and chemical conditions of the soil and complex microbial associations.

Conflicting observations have been reported on the influence of water extract of straw on retardation of plant growth (*5*) and on nitrification in soil (*12*). Results reported in Table V agree with Jensen's (*12*) observations that the water soluble fraction of wheat straw did not interfere with nitrification.

With ammonium nitrate added to soil, with and without wheat straw and wheat straw fractions, there were decreases in nitrate during the first 7 days, followed by increases. This indicated a probable early assimilation of the nitrate fol-

Table V. Effect of Wheat Straw and Its Water-Soluble and Water-Insoluble Fractions on Nitrogen Changes in Walla Walla Silt Loam Soil with Different Sources and Rates of Nitrogen

Treatment		NH_4OH							NH_4NO_3							$Ca(NO_3)_2$						
		Days				Apparent nitrification,[a] %	Total Nitrogen, P.P.M.		Days				Apparent nitrification,[b] %	Total Nitrogen, P.P.M.		Days				Apparent denitrification,[c] %	Total Nitrogen, P.P.M.	
Straw, tons/acre	N, p.p.m.	NH_4^+ N, P.P.M.		NO_3^- N, P.P.M.					NH_4^+ N, P.P.M.		NO_3^- N, P.P.M.					NH_4^+ N, P.P.M.		NO_3^- N, P.P.M.				
		0	28	0	28		0	28	0	28	0	28		0	28	0	28	0	28		0	28
Whole																						
0	0	6	8	5	42	..	1044	1055	6	5	5	42	...	1050	1058	8	8	5	45	..	1050	1060
0	100	105	9	5	104	62	1138	1109	52	14	48	99	14	1146	1084	7	9	104	51	94	1153	1088
0	200	198	61	5	125	41	1238	1220	103	40	98	117	−25	1241	1178	8	9	204	102	72	1236	1168
0	400	394	118	5	174	33	1450	1367	201	39	197	184	−29	1446	1284	8	8	402	258	47	1436	1300
5[d]	0	7	2	5	3	..	1048	1118	8	3	5	2	...	1050	1130	5	4	4	2	..	1054	1121
5	100	104	6	5	76	34	1154	1169	51	7	45	33	−108	1154	1305	9	13	98	35	110	1169	1224
5	200	199	9	5	109	31	1260	1294	102	10	106	88	−54	1263	1360	10	12	204	84	81	1271	1337
5	400	391	19	5	146	26	1458	1479	198	39	183	164	−39	1452	1493	9	11	397	198	62	1463	1465
Extracted[e]																						
4.03	0	7	5	5	2	..	1048	1110	8	5	4	1	...	1046	1120	7	8	4	3	..	1049	1114
4.03	100	102	51	5	46	4	1146	1202	56	47	45	28	−128	1149	1208	34	41	98	46	99	1141	1221
4.03	200	201	75	5	59	8	1249	1300	103	75	97	72	30	1261	1316	23	33	195	75	85	1248	1321
4.03	400	390	221	5	99	14	1456	1500	200	93	194	152	55	1449	1490	19	25	391	212	58	1447	1494
Extracted = C in whole straw at 5 T/A[f]																						
5.35	0	5	5	5	1	..	1043	1209	5	7	5	1	...	1047	1205	6	8	5	1	..	1049	1207
5.35	100	104	40	5	42	0	1146	1345	55	51	48	15	−26	1151	1263	8	22	97	81	64	1153	1291
5.35	200	203	102	5	46	2	1249	1141	104	91	97	37	40	1250	1329	7	34	202	120	63	1254	1348
5.35	400	400	265	5	76	9	1442	1545	203	156	189	55	33	1453	1489	7	55	399	232	63	1458	1489
Extract[g]																						
0.94	0	8	25	5	56	..	1048	1152	8	25	5	56	...	1046	1160	10	14	5	55	..	1046	1163
0.94	100	106	42	5	97	55	1144	1220	57	44	49	101	118	1143	1213	17	33	105	29	116	1146	1200
0.94	200	201	135	5	113	36	1243	1315	104	75	99	144	102	1244	1314	19	46	204	66	90	1241	1230
0.94	400	403	246	5	157	28	1440	1416	201	144	199	224	91	1442	1424	29	70	402	99	87	1443	1420
$LSD_{0.01}$																						
(N × Straw)		8.60		0.48			36.07		11.52		0.45			31.01		7.09		0.27			31.51	

[a] Apparent nitrification, $\% = \frac{(\text{Increase in } NO_3^-\text{-N in treated soil}) - (NO_3^-\text{-N increase in soil only})}{NH_4^+\text{-N}} \times 100$

[b] Apparent nitrification, $\% = \frac{(NO_3^-\text{-N at 28 days}) - (NO_3^-\text{-N in soil only at 28 days}) - (NO_3^-\text{-N added})}{NH_4^+\text{-N added}} \times 100$

[c] Apparent denitrification, $\% = \frac{(NO_3^-\text{-N added}) - (NO_3^-\text{N at 28 days}) - (NO_3^-\text{-N in soil at 28 days})}{(NO_3^-\text{-N added})} \times 100$

[d] Equivalent to 2262 p.p.m. C.
[e] From wheat straw at 5 T/A; equivalent to 1952 p.p.m. C.
[f] Equivalent to C in whole straw at 5 T/A or 2262 p.p.m. C.
[g] From straw at 5 T/A; equivalent to 310 p.p.m. C.

lowed by nitrification of the residual ammonium ion nitrogen.

Generally progressive decreases in nitrate ion nitrogen occurred in all treatments with calcium nitrate. The decreases were greater with all straw additions, but losses of total nitrogen were less, showing that assimilation exceeded denitrification. The assimilation followed fairly well the order of availability of carbon in the different straw and extract treatments.

Total Nitrogen Changes. Increases in total nitrogen over soil only were obtained in most cases where straw was added, even in the presence of nitrogen additions as high as 200 p.p.m. It should be emphasized, however, that changes in total nitrogen amounting to less than 40 p.p.m. as determined by the Kjeldahl method are of doubtful significance even though the least significant difference, values may be less than this amount (LSD) (Table V). Thus from results shown in this table it appears that appreciable nitrogen fixation occurred consistently only with the extracted straw applied at 5.35 tons per acre without added nitrogen and also with each nitrogen source at 100 and 200 p.p.m. of nitrogen. No fixation resulted where nitrogen was added at 400 p.p.m. Large amounts of available nitrogen are known to inhibit assimilation of free nitrogen.

Gains in total nitrogen following straw additions have been reported previously by Vandecaveye and Villanueva (*20*) for Palouse soils, by Newton (*16*) for Western Canada's gray wooded, black earth, and brown prairie soils, and by Jensen (*12*) for a large number of Australian soils. Amounts of straw added to the soil in these several investigations ranged from 0.2 to 5%. Jensen (*12*) also found that hot-water extract of wheat straw increased nitrogen fixation. Much of this fixation could be due to *Clostridium* rather than to *Azotobacter* (*15*). Indirect evidence for nitrogen fixation in soils of semiarid regions is presented in reports by Bradley (*2*) and Bracken and Greaves (*3*). Certain dry wheatland soils in eastern Oregon and Utah were found to show no decline in total nitrogen after years of cropping.

Increases in total nitrogen averaging 112 p.p.m. were obtained with the cold-water extract without added nitrogen sources. As the extract was used at a rate equivalent to the amount in a 5-ton per acre application of whole straw, the actual amount of carbon added was relatively small. Nevertheless nitrogen increases with the extract were comparable to fixation with the whole straw. This points to the importance of an initial influence of the water-soluble carbonaceous constituents.

Extracted wheat straw, especially at the higher rate, gave greatest increases in total nitrogen. This as well as the greater nitrate assimilation indicated in Table V is not in accordance with the presumably more resistant nature of the

carbon. However, it should be pointed out that the actual amount of carbon, although water-insoluble, was much greater than that applied in the wheat straw extract. On the basis of unit amount of carbon, the amount of total nitrogen at the end of 28 days was generally greater for the soluble source of carbon, the only exceptions being at the higher rates of nitrogen added. Jensen (*12*) found that hot-water extracted straw did not favor any nitrogen fixation; it appears that cold-water extracted straw retains some available carbon sources for *Clostridium* if not for *Azotobacter*. Possibly hot and cold water have different effects on the physical structure and availability of the extracted straw.

The results of laboratory experiments, conducted under optimum conditions, are difficult to compare with results likely to occur in the field where distribution of straw, fertilizer, and moisture are irregular and variable. Nevertheless trends are indicated and inferences may be drawn. There is no doubt that straw additions influence nitrogen balance in the soil by promoting assimilation of available nitrogen, by affecting nitrification, by favoring denitrification, and by stimulating nitrogen fixation. Summation of these transformations determines the net loss or gain of available and total nitrogen.

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SOIL MAGNESIUM ANALYSIS

Elimination of Manganese Interference in the EDTA Titration of Exchangeable Soil Magnesium

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Agronomy Department, University of Illinois, Urbana, Ill.

Manganese titrates quantitatively and interferes in the determination of magnesium by EDTA [disodium (ethylenedinitrilo)tetraacetate] titration. This interference can be conveniently eliminated by addition of ferrocyanide to precipitate the manganese. Exchangeable soil magnesium can be titrated by EDTA with Eriochrome Black T (EBT) as the indicator in the presence of 1 meq. of exchangeable manganese per 100 grams of soil. If larger amounts of manganese are present, the precipitate of manganese ferrocyanide obscures the end point and must be removed by filtration before magnesium is titrated.

THE REAGENT EDTA [disodium(ethylenedinitrilo)tetraacetate] is widely used in the determination of calcium and magnesium. Cheng and Bray (*1*) adopted the procedures originated by Schwarzenbach, Biedermann, and Bangerter (*3*) to determine calcium directly and magnesium indirectly in plant materials and soils. To determine magnesium directly, calcium must first be removed. Tucker and Kurtz (*4*) obtained the most consistent results by tungstate precipitation of calcium.

Titrations with EDTA are subject to interference by foreign ions, such as aluminum, cobalt, copper, iron, manganese, and nickel, which are sometimes present in small but detectable amounts in soil and plant materials (*1*). Heavy metals such as cobalt, copper, nickel, and a small quantity of iron can be complexed with potassium cyanide (*1*). Pribil (*2*) screened aluminum from interference with triethanolamine, which also masked a small quantity of iron. When present in larger quantities, iron usually precipitates as the hydroxide at the pH specified for the titration.

Methods for eliminating manganese interference have not been convenient or satisfactory. This paper proposes a convenient procedure for such elimination in the titration of magnesium with EDTA. In neutral and alkaline solutions, manganous ferrocyanide is insoluble, but the calcium and magnesium salts of ferrocyanide are highly soluble and ionized in solution. Ferrocyanide precipitation was very effective in removing manganese interference from the titration of calcium and magnesium with EDTA. This modification gave very reproducible results when used in the analysis of soil for exchangeable magnesium.

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Reagents

STANDARD MAGNESIUM SOLUTION. Magnesium sulfate heptahydrate (5.068 grams) dissolved in 1 liter of distilled water (500 p.p.m. magnesium solution).

STANDARD CALCIUM SOLUTION. "Special" low magnesium reagent grade calcium carbonate (2.497 grams) was dissolved in a minimum quantity of 1 to 1 hydrochloric acid and diluted to 1 liter with distilled water (1000 p.p.m. calcium solution).

STANDARD MANGANESE SOLUTION. Manganese sulfate monohydrate (1.54 grams) was dissolved in 1 liter of distilled water (500 p.p.m. manganese solution).

STANDARD EDTA SOLUTION. A stock solution of 0.2% disodium dihydrogen (ethylenedinitrilo)tetraacetate has a convenient concentration for soil analysis. This solution was standardized against both the calcium and the magnesium standards.

EBT INDICATOR. Eriochrome Black T (0.2 gram) was dissolved in 50 ml. of methanol containing 2 grams of hydroxylamine hydrochloride. Fresh indicator should be prepared every 3 weeks to assure a sharp end point.

BUFFER SOLUTION (pH 10). Ammonium chloride (67.5 grams) was dissolved in 200 ml. distilled water, mixed with 570 ml. of concentrated ammonium hydroxide and diluted to 1 liter.

TUNGSTATE SOLUTION. Twenty grams of sodium tungstate were dissolved in 100 ml. of distilled water.

Interference-Removing Reagents. Potassium Cyanide Solution. Two grams of potassium cyanide were dissolved in 100 ml. distilled water.

Triethanolamine, pure reagent grade.

Potassium Ferrocyanide. Four grams of reagent grade potassium ferrocyanide trihydrate were dissolved in 100 ml. of distilled water.

Titration Procedures

Determination of Calcium Plus Magnesium. A suitable aliquot was diluted to about 100 ml. and adjusted to pH 10 by adding 15 ml. of ammonium hydroxide–ammonium chloride buffer. Ten drops of each of the interference-removing reagents and 10 drops of EBT indicator were added. Precipitation of manganese ferrocyanide was almost instantaneous when solutions were at 40° C. or warmer. When solutions were cold, titrations were delayed a few minutes to allow time for the precipitation to go to completion. Titrations were made from a microburet, with the standardized EDTA under fluorescent light, to the end point indicated by the color change of EBT from wine-red to pure blue.

Determination of Magnesium. A suitable aliquot was diluted to about 100 ml. and 20 ml. each of ammonium hydroxide–ammonium chloride buffer and tungstate solution were added. This mixture was warmed for 1 hour, allowed to cool, filtered through No. 42 Whatman filter paper, and washed with a solution containing 50 ml. of buffer per liter. Ten drops of each of the interference-removing reagents were added to the filtrate, which was then titrated in the manner described for calcium plus magnesium.

Table I. Effect of Ferrocyanide in Eliminating Manganese Interference

Present, Mg.			Mg + Ca Found as Ml.–EDTA Equivalent	
Mg	Ca	Mn	Without $Fe(CN)_6$	With $Fe(CN)_6$
0.50	0	0	4.06	4.06
0.50	0.50	0	6.40	6.41
0	0	0.50	1.74	0
0.50	0	0.25	4.92	4.08
0.50	0	0.50	5.80	4.07
0.50	0	2.50	12.72	Interfered
0.50	0.50	0.50	8.10	6.42
0.50	Removed	0.50	5.84	4.10

Table II. Precision of Determination of Exchangeable Calcium and Magnesium in Cisne Soil

Sample	Aliquot	Ca + Mg as Ml.–EDTA Equivalent	Magnesium		Calcium	
			EDTA, ml.	Mg.	EDTA, ml.	Mg.
I	1	8.18	3.61	0.445	4.57	0.976
	2	8.16	3.63	0.447	4.53	0.968
	3	8.16	3.60	0.444	4.56	0.974
	4	8.17	3.58	0.441	4.59	0.980
II	1	8.15	3.56	0.439	4.59	0.980
	2	8.12	3.54	0.436	4.58	0.978
	3	8.15	3.53	0.435	4.62	0.987
	4	8.15	3.54	0.436	4.61	0.985
III	1	8.18	3.60	0.444	4.58	0.978
	2	8.15	3.57	0.440	4.58	0.978
	3	8.17	3.60	0.444	4.57	0.976
	4	8.14	3.58	0.441	4.56	0.974
IV	1	8.19	3.62	0.446	4.57	0.976
	2	8.14	3.58	0.441	4.56	0.974
	3	8.19	3.61	0.445	4.58	0.978
	4	8.18	3.60	0.444	4.58	0.978

Results with Standard Solutions

Table I gives examples of data obtained by titrating solutions containing a constant amount of magnesium and different amounts of manganese with EDTA in both the presence and absence of ferrocyanide.

In the absence of ferrocyanide, manganous ions reduced the intensity of the metal-indicator complex so that more indicator was required for the detection of the end point. In other respects, manganese did not interfere with the end point, but titrated quantitatively along with the calcium and magnesium. Data in Table I show further that manganese, alone, in the absence of calcium and magnesium, was titrated quantitatively by EDTA with the EBT indicator and that manganese interference was not affected by the removal of calcium by tungstate precipitation.

In the presence of ferrocyanide, manganese did not influence the titration when present in amounts less than 0.5 mg. If larger amounts of manganese were present, the precipitated manganese ferrocyanide interfered with end point detection, and should be filtered out before the titration is made. If magnesium alone were determined in the presence of large amounts of manganese, the ferrocyanide could presumably be added with the tungstate, so that calcium and manganese would be removed simultaneously. Potassium ferrocyanide at the concentration of 4% has only a light yellowish color that does not affect the detection of the color change of the EBT indicator.

Results with Soil Extracts

Because consistent and reproducible results were obtained with standard solutions, the method was applied to soil analyses. Results obtained are illustrated in Table II with data from determinations on an acid Cisne soil (No. S 24065) from southern Illinois.

As the determinations of amounts of calcium and magnesium are of interest here, the exchangeable cations were removed from the soils by an equilibrium

Table III. Statistical Analysis of Precision of Analytical Methods

Analytical Method	Mean, Y	Variance, S^2	Standard Deviation, s	Coefficient of Variation, %
Ca + Mg	8.161	0.000308	0.01755	0.22
Mg direct	0.4416	0.00000445	0.00211	0.48
Ca indirect	0.9776	0.0000130	0.00360	0.37

extraction rather than by an exhaustive leaching. Four samples of 20 grams of soil were weighed, soaked in 100 ml. of 1*N* neutral ammonium acetate solution for 1 hour, agitated on a shaker for 30 minutes, and filtered through Whatman No. 42 filter paper. Aliquots of 10 ml. of this extract, each representing 2 grams of soil, were suitable for either direct determination of magnesium or the combined calcium and magnesium determination by the titration procedures previously described. The calcium equivalent of EDTA was obtained by subtracting the magnesium equivalent of EDTA from that of calcium plus magnesium.

The precipitated manganese ferrocyanide was slight and caused no difficulty in titrations, although Cisne soil is relatively high in easily soluble manganese. With the weight of soil sample and size of aliquots used in these experiments, interference from exchangeable manganese in amounts less than 1 meq. per 100 grams of soil is eliminated simply by the addition of ferrocyanide. If the amounts of manganese are higher, the precipitate should be removed by filtration to avoid difficulty in detection of the end point.

Data in Table II are from four aliquots of extracts of each of the four samples of soil and show excellent agreement within samples as well as among samples.

Statistical analyses of these data are shown in Table III. Coefficients of variation were 0.22, 0.48, and 0.37%, respectively, for the calcium plus magnesium titration, magnesium titration, and calcium determination by difference. Validity of these statistical statements is limited to the operator and conditions of these experiments, but good agreement is indicated.

Acknowledgment

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INSECTICIDE ASSAY

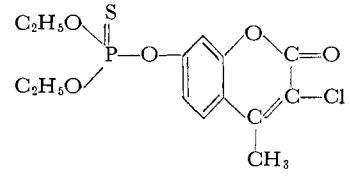
Assay of Co-Ral in Technical Material and Formulated Products

P. F. KANE, C. J. COHEN, W. R. BETKER, and D. MacDOUGALL

Research Department, Chemagro Corp., Kansas City, 20, Mo.

Two analytical methods for the determination of Co-Ral in technical material and formulated products are described. These involve alkaline hydrolysis and ultraviolet absorption spectrophotometry. The former method gives the more precise results. However, the latter allows for easy correction for 3-chloro-4-methylumbelliferone in the material and is less time-consuming than the former. Both methods have been used successfully for routine control purposes.

Co-Ral, formerly known as Bayer 21/199, is a commercial insecticide for the control of various insect pests of domestic animals. The compound is *O,O* - diethyl - *O* - 3 - chloro - 4 - methyl - 2-oxo-2*H*-1-benzopyran-7-yl phosphorothioate. The structural formula is



For the analysis of both technical material and formulations, a reproducible method of assay was required. The literature contains numerous references to analytical methods for coumarin derivatives, exemplified by the work of Ensminger (*2*). However, most of these methods have been designed for trace analysis and were not considered sufficiently accurate for assay purposes.

A preliminary examination of possible methods of analysis of the above compound indicated alkaline hydrolysis and ultraviolet absorption to be the most promising techniques for further study. Both of these approaches have been investigated and both have yielded satisfactory analytical procedures.

From a consideration of the structure of Co-Ral, hydrolysis was expected to form diethylphosphorothioic acid from saponification of the ester grouping and hydrochloric acid from the chlorine in the lactone ring. Ring opening in alkaline solution should form a carboxylic acid which should also be titratable. In addition to this, the above reactions would form two weakly acid phenolic groups. If ring closure to form a benzopyran ring occurs (*9*), only the remaining phenolic group would be titratable. Thus one might expect to obtain four or possibly five equivalents of acid on alkaline hydrolysis of Co-Ral.

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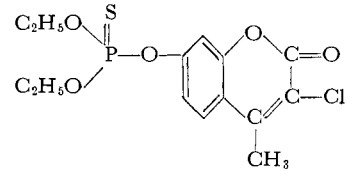

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Experimental

Hydrolysis of Co-Ral. The alkaline hydrolysis of Co-Ral was investigated in both water and isopropyl alcohol. Samples of recrystallized Co-Ral were hydrolyzed by refluxing for 2 hours in 1*N* aqueous and 1*N* isopropyl alcoholic

alkali. In both cases, the hydrolyzates were back-titrated with 0.5*N* hydrochloric acid using a Precision Scientific automatic titrator.

Figure 1 shows that the titration curves obtained are different, depending on whether the hydrolysis is carried out in water or in isopropyl alcohol. The aqueous titration curve has four inflections—at pH 11.0, 7.5, 5.5, and 2.6. These inflection points correspond respectively to 4.5, 3, 2.5, and 2 equivalents of acid per mole of Co-Ral. The alcoholic titration curve has only three inflections at pH 11.6, 7.5, and 3.0. These points correspond to 4, 3, and 2 equivalents of acid formed per mole of Co-Ral. The break which occurred at pH 5.5 following aqueous hydrolysis is not present in the titration curve for the alcoholic hydrolysis medium.

The appearance of the hydrolyzed solutions was markedly different, depending on whether water or isopropyl alcohol was used as solvent. Co-Ral is initially partially insoluble in the aqueous alkali, but after a few minutes of boiling, the solution becomes clear and remains so. After hydrolysis, the solution is a purple color and it undergoes a series of color changes during titration, turning gray-blue at pH 10.0, brown at pH 7.5, and orange-pink at pH 3.5. Below pH 4.0 the solution becomes turbid and a white crystalline precipitate forms on standing. Co-Ral is readily soluble in the alcoholic reagent, but insoluble material is formed during hydrolysis. This material dissolves readily in the water added before titration. At this point, the solution is dark green in color and highly fluorescent. During titration, this solution changes to brown at pH 11.5, and faintly pink at pH 7.0. Below pH 7.0 some turbidity is noted, but a definite crystalline precipitate does not form.

In both cases, the only inflection which is satisfactory for quantitative work is that which occurs at pH 7.5. In both the aqueous and alcoholic media, this corresponds to the formation of three moles of acid per mole of Co-Ral. It is assumed that these are diethyl phosphorothioic acid, hydrochloric acid, and a carboxylic acid formed by opening the lactone ring.

The effects of hydrolysis time and strength of alkali were investigated with both media using the end point at pH 7.5. The effect of hydrolysis time is shown in Figure 2. In both cases, no further hydrolysis occurs after 2 hours. The reaction is completed more rapidly in the alcoholic than in the aqueous medium.

The effect of dilution of the hydrolysis solution was tested, using both media. Increment amounts of water or isopropyl alcohol were added to the appropriate solutions before refluxing for 2 hours. In both cases, dilution to an alkali concentration of 0.5*N* decreased the amount of hydrolysis in 2 hours to 97 to 98% of the value obtained with 1.0*N* alkali. Further dilution of the alkali decreased the amount of hydrolysis markedly.

Consideration of the more definite stoichiometric relationship, more rapid reaction, and somewhat better end point indicated the choice of isopropyl alcohol over water as solvent for hydrolysis of this compound. Accordingly this was adopted for quantitative work.

Mechanism of Hydrolysis. According to Wawzonek (*10*) the initial action of alkali on coumarin structures is to open the lactone ring to form a salt of coumarinic acid. Acidification regenerates the original coumarin. That this happens with Co-Ral was demonstrated by shortening the hydrolysis time to a minimum. The solution was cooled as soon as the solid dissolved in 1*N* isopropyl alcoholic potassium hydroxide—about 2 minutes. Addition of water resulted in a pale yellow, slightly fluorescent solution. Titration of the hydrolyzate gave an inflection at pH 11.2. Precipitation of a white crystalline material began at pH 9. Each additional drop of acid was followed by a sharp drop in pH and then a slow return to pH 9 to 10. This continued until all of the alkali originally present was neutralized, indicating that no base had been consumed by the Co-Ral. The precipitate which formed on acidification was separated and identified by melting point and infrared absorption as Co-Ral.

This shows conclusively that the first action of alkali is the opening of the lactone ring (Reaction I).

On heating with alkali, three additional reactions can be visualized. The phosphate ester linkage would be split to form the potassium salt of diethylphosphorothionate. Reactions of this type have been described for many organic phosphate pesticides (*8*). The

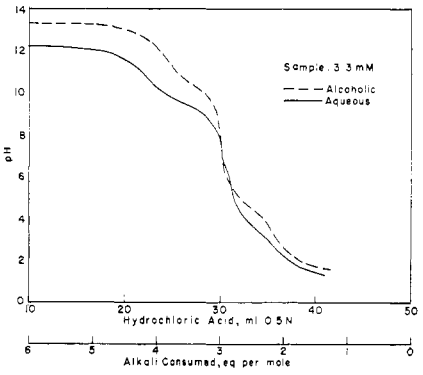


Figure 1. Titration of aqueous and alcoholic Co-Ral hydrolyzates with hydrochloric acid

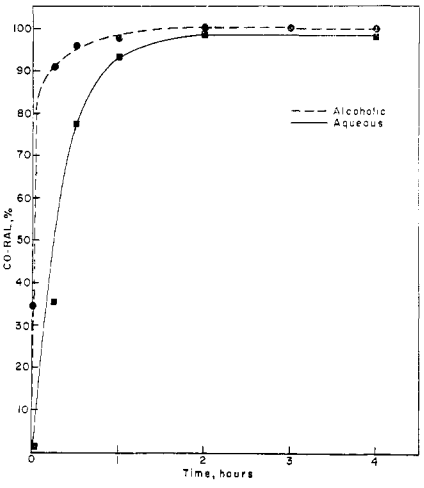
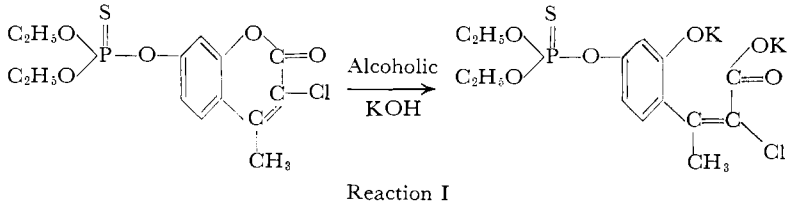
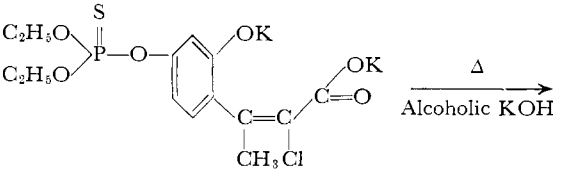


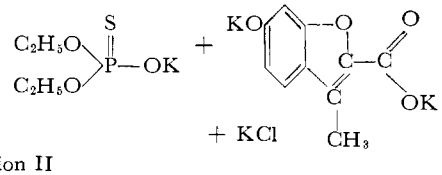
Figure 2. The effect of time on hydrolysis of Co-Ral

secondary phosphate esters are stable to alkaline hydrolysis (*7*) and, therefore, would not undergo further decomposition. The isomerization of the *cis*-coumarinic acid to a *trans*-coumaric acid has been described by Fries *et al.* (*3*, *4*). This would be expected to be followed by ring closure to form a benzofuran ring as originally described by Perkin (*9*).



Reaction I





Reaction II

The actual order in which the above reactions occur is not known, but the final products of hydrolysis are shown in Reaction II.

These reactions would explain completely the observations made on the hydrolysis of Co-Ral with alcoholic potassium hydroxide.

Ultraviolet Absorption. The principal interferences expected in an ultraviolet absorption method for Co-Ral would be chloromethylumbelliferone or Potasan (the unchlorinated compound corresponding to Co-Ral). The former compound could result either from incomplete reaction during manufacture or from hydrolysis as a result of decomposition of formulated products during storage. It was expected that the ultraviolet spectra of Co-Ral and chloromethylumbelliferone would overlap too closely to allow for the quantitative determination of one in the presence of the other. However, the free phenolic hydroxyl group in the latter compound offered the possibility of forming the phenolate in alkaline solution. Formation of phenolates from *m*-substituted phenols is known to cause a bathochromic shift (*6*). In view of this, the ultraviolet absorption spectra of Co-Ral and chloromethylumbelliferone were determined in both the presence and absence of sodium carbonate.

Solutions containing 10 γ per ml. of Co-Ral and 5 γ per ml. of chloromethylumbelliferone in absolute methanol were used in this experiment. The ultraviolet absorption spectra were determined in a Beckman DU spectrophotometer using a 1-cm. cell. The absorption spectra are shown in Figure 3. The results show a double peak for Co-Ral with maxima at 290 and 315 mμ. Chloromethylumbelliferone has an absorption maximum at 330 mμ. Direct estimation of one compound in the presence of the other would be possible by setting up simultaneous equations and measuring the absorbance at 290 and 330 mμ. As this would not be a very accurate procedure, the effect of sodium carbonate on the absorbance of both compounds was determined. In this experiment, solutions containing 10 γ per ml. of Co-Ral and 5 γ per ml. of chloromethylumbelliferone were prepared in 50% aqueous methanol. The solutions contained 0.5% of sodium carbonate. The spectrophotometric results are shown in Figure 3. Sodium carbonate causes a bathochromic shift in the absorbance of chloromethylumbelliferone from 330 to 380 mμ. Under the same conditions, Co-Ral is practically nonabsorbing at 380 mμ. The specific extinction values for Co-Ral and chloromethylumbelliferone in methanol at 290 mμ and for chloromethylumbelliferone in a solution of methanol, water, and sodium carbonate are shown in Table I.

The validity of correcting the Co-Ral absorbance at 290 mμ for that of chloromethylumbelliferone by measuring the latter at 380 mμ after adding sodium carbonate depends on the fact that Beer's law is obeyed in each of the three cases. This was tested and the results showed that there are no deviations from Beer's law in the concentration ranges required.

The stability of solutions of Co-Ral and chloromethylumbelliferone in methanol and of the latter compound in aqueous methanolic sodium carbonate were determined. Co-Ral and chloromethylumbelliferone did not change in absorbance at wave length 290 mμ during an 8-hour period. In 48 hours the Co-Ral solution decreased in absorbance by about 5%, while the chloromethylumbelliferone decreased by about 18%. The solution of chloromethylumbelliferone in sodium carbonate did not change appreciably in 15 minutes. Therefore, care must be taken to read the absorbance of these solutions immediately after adding the sodium carbonate. In 1.5 hours the sodium carbonate solution of chloromethylumbelliferone decreased in absorbance by about 8%.

In the determination of the Co-Ral content of technical material, dilute hydrochloric acid must be added before determining the absorbance in absolute methanol at 290 mμ. This is necessary because of the presence of some potassium carbonate in the technical material. The hydrochloric acid has no effect on the absorbance of Co-Ral.

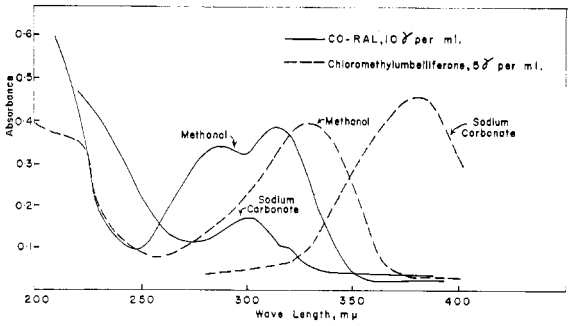


Figure 3. Ultraviolet absorption spectra of Co-Ral and chloromethylumbelliferone in absolute methanol and in 50% methanol containing sodium carbonate

Table I. Absorbance of Co-Ral and Chloromethylumbelliferone

Compound	Solvent	Wave Length, Mμ	Absorbance, $E^{1\%}_{1cm.}$
Co-Ral	Methanol	290	297
Chloromethylumbelliferone	Methanol	290	200
	Aqueous methanolic sodium carbonate	380	912

Analytical Procedures

Hydrolysis Method. REAGENTS. Only reagent grade chemicals should be used.

POTASSIUM HYDROXIDE, 1*N* solution in absolute isopropyl alcohol. Dissolve 66 grams of potassium hydroxide in 1 liter of isopropyl alcohol. Allow the insoluble material to settle. Use the supernatant liquid.

HYDROCHLORIC ACID, 0.5*N*, accurately standardized.

Procedure. Weigh about 1 gram of the Co-Ral sample to be analyzed into a 250-ml. Erlenmeyer flask. Add 25 ml. of 1*N* potassium hydroxide and reflux for 2 hours under a reflux condenser equipped with a soda-lime tube. Wash down the condenser with distilled water and transfer the contents of the flask to 250-ml. beaker. Adjust the final volume of solution to about 100 ml. with distilled water. Insert a calomel-glass electrode system and titrate to pH 7.5 with 0.5*N* hydrochloric acid. Carry out a blank determination. The titer difference between the sample and blank is converted to Co-Ral equivalents. One milliliter of 0.5*N* hydrochloric acid is equivalent to 60.47 mg. of Co-Ral.

The method can be applied to a 25% wettable powder by weighing a 4-gram sample into a Whatman extraction thimble and extracting with acetone for 1 hour in a Bailey-Walker extractor. After evaporation of the acetone, the procedure is employed as above.

Spectrophotometric Method. REAGENTS. Only reagent grade chemicals should be used.

Co-Ral, Standard Solution. Dissolve exactly 0.200 gram of Co-Ral (recrystallized, melting point 94° C.) in absolute methanol. Warm if necessary to dissolve. Dilute to 100 ml. with absolute methanol. This solution contains 2000 γ per ml. of Co-Ral. Make a further 1 to 20 dilution of the stock solution with absolute methanol. From this second solution, prepare fresh daily standards by diluting 10-, 15-, and 20-

ml. aliquots to 100 ml. with absolute methanol. The resulting solutions will contain, respectively, 10, 15, and 20 γ per ml. of Co-Ral.

Procedure. Weigh accurately approximately 0.2 gram of technical Co-Ral and place in a 250-ml. volumetric flask. Add 100 ml. of 1,4-dioxane and 5 ml. of 0.1N hydrochloric acid, stopper, and shake until the Co-Ral is dissolved. Dilute to volume with absolute methanol (Solution 1). Pipet a 2-ml. aliquot of the solution into a 100-ml. volumetric flask, and again dilute to volume with absolute methanol (Solution 2). Measure the absorbance in a 1-cm. silica cell at 290 mμ in a suitable spectrophotometer. Use a 0.8% v./v. solution of 1,4-dioxane in absolute methanol as a blank.

For the estimation of chloromethylumbelliferone, pipet 50 ml. of Solution 1 into a 100-ml. volumetric flask. Pipet in 50 ml. of 1% aqueous sodium carbonate solution. Cool rapidly to room temperature and dilute to volume with absolute methanol. Within 15 minutes of the sodium carbonate addition, read the absorbance in a 1-cm. cell at 380 mμ against a blank solution containing the same proportions of methanol, 1,4-dioxane, and aqueous sodium carbonate as are present in the sample solution. Calculate the chloromethylumbelliferone content by reference to the appropriate calibration curve. Multiply the chloromethylumbelliferone concentration by 0.67 and subtract from the Co-Ral value obtained at wave length 290 mμ.

Discussion

Reproducibility. The reproducibility of both methods is shown in Table II. The results show that the hydrolysis procedure is capable of somewhat greater precision than the spectrophotometric method. This is to be expected. However, the reproducibility of the spectrophotometric procedure is satisfactory for analytical control purposes. The precision of the spectrophometric method is reduced by running duplicate determinations. In general, the spectrophotometric method is preferred, because it permits a ready correction for chloromethylumbelliferone.

Table II. Reproducibility of Methods for Co-Ral Determination

Method	Material	Mean Value[a]	Standard Deviation
Hydrolysis	Recrystallized	100.73 (9)	0.15
Spectrophotometric	Technical	96.3 (4)	0.6
		94.9 (4)	0.5
		93.8 (4)	0.5
		92.3 (4)	0.6

[a] Number of values used to calculate each mean shown in parentheses.

The accuracy of the method for determination of chloromethylumbelliferone in Co-Ral was tested by preparing synthetic mixtures of the two compounds. The chloromethylumbelliferone results are shown in Table III. It is apparent that the method described will give very accurate values for chloromethylumbelliferone in the presence of Co-Ral.

Table III. Determination of Chloromethylumbelliferone Content of Co-Ral

Chloromethylumbelliferone, %	
Present	Found
0.99	0.99
0.99	0.99
2.00	1.98
2.01	2.00
3.02	3.01
3.01	2.99

The presence of more than small amounts of chloromethylumbelliferone can be detected by the hydrolysis method, although the actual concentration of interfering material cannot be determined accurately by this method. The presence of chloromethylumbelliferone will result in a change in the relative number of moles of acid obtained on titration to pH 7.5 and 3.0. In the case of pure Co-Ral, this ratio is 3 to 2 (Figure 1), while for chloromethylumbelliferone it is 1 to 1.

Acknowledgment

The authors thank Fred N. Larsen for technical assistance.

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FUNGICIDE ASSAY

Determination of Dyrene and Cyanuric Chloride in Technical Materials

P. F. KANE and K. GAIL GILLESPIE
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Dyrene (2,4-dichloro-6-*o*-chloroanilino-*s*-triazine), previously known as Kemate or B-622, is a new organic fungicide which has proved outstandingly effective for the control of fungus diseases on a number of crops. It is prepared by the reaction of cyanuric chloride with *o*-chloroaniline in the presence of sodium carbonate. This study was undertaken to develop a suitable assay method for this product in the presence of possible contaminants.

A residue method for Dyrene (*5*) is based on acid hydrolysis to form *o*-chloroaniline. The *o*-chloroaniline is then diazotized and coupled with *N*-1-naphthylethylenediamine to produce a colored complex. This method is time-consuming, as the hydrolysis requires 2 hours of reflux with hydrochloric acid. A second colorimetric procedure involving the Zincke reaction (*2*) has also been described. In general, volumetric methods are more accurate than colorimetric procedures in cases where the former can be employed. Therefore, it was decided to investigate the hydrolysis of the chlorine atoms of Dyrene, because chloride ion can be readily determined volumetrically. A second volumetric

ml. aliquots to 100 ml. with absolute methanol. The resulting solutions will contain, respectively, 10, 15, and 20 γ per ml. of Co-Ral.

Procedure. Weigh accurately approximately 0.2 gram of technical Co-Ral and place in a 250-ml. volumetric flask. Add 100 ml. of 1,4-dioxane and 5 ml. of 0.1N hydrochloric acid, stopper, and shake until the Co-Ral is dissolved. Dilute to volume with absolute methanol (Solution 1). Pipet a 2-ml. aliquot of the solution into a 100-ml. volumetric flask, and again dilute to volume with absolute methanol (Solution 2). Measure the absorbance in a 1-cm. silica cell at 290 mμ in a suitable spectrophotometer. Use a 0.8% v./v. solution of 1,4-dioxane in absolute methanol as a blank.

For the estimation of chloromethylumbelliferone, pipet 50 ml. of Solution 1 into a 100-ml. volumetric flask. Pipet in 50 ml. of 1% aqueous sodium carbonate solution. Cool rapidly to room temperature and dilute to volume with absolute methanol. Within 15 minutes of the sodium carbonate addition, read the absorbance in a 1-cm. cell at 380 mμ against a blank solution containing the same proportions of methanol, 1,4-dioxane, and aqueous sodium carbonate as are present in the sample solution. Calculate the chloromethylumbelliferone content by reference to the appropriate calibration curve. Multiply the chloromethylumbelliferone concentration by 0.67 and subtract from the Co-Ral value obtained at wave length 290 mμ.

Discussion

Reproducibility. The reproducibility of both methods is shown in Table II. The results show that the hydrolysis procedure is capable of somewhat greater precision than the spectrophotometric method. This is to be expected. However, the reproducibility of the spectrophotometric procedure is satisfactory for analytical control purposes. The precision of the spectrophometric method is reduced by running duplicate determinations. In general, the spectrophotometric method is preferred, because it permits a ready correction for chloromethylumbelliferone.

Table II. Reproducibility of Methods for Co-Ral Determination

Method	Material	Mean Value[a]	Standard Deviation
Hydrolysis	Recrystallized	100.73 (9)	0.15
Spectrophotometric	Technical	96.3 (4)	0.6
		94.9 (4)	0.5
		93.8 (4)	0.5
		92.3 (4)	0.6

[a] Number of values used to calculate each mean shown in parentheses.

The accuracy of the method for determination of chloromethylumbelliferone in Co-Ral was tested by preparing synthetic mixtures of the two compounds. The chloromethylumbelliferone results are shown in Table III. It is apparent that the method described will give very accurate values for chloromethylumbelliferone in the presence of Co-Ral.

Table III. Determination of Chloromethylumbelliferone Content of Co-Ral

Chloromethylumbelliferone, %	
Present	Found
0.99	0.99
0.99	0.99
2.00	1.98
2.01	2.00
3.02	3.01
3.01	2.99

The presence of more than small amounts of chloromethylumbelliferone can be detected by the hydrolysis method, although the actual concentration of interfering material cannot be determined accurately by this method. The presence of chloromethylumbelliferone will result in a change in the relative number of moles of acid obtained on titration to pH 7.5 and 3.0. In the case of pure Co-Ral, this ratio is 3 to 2 (Figure 1), while for chloromethylumbelliferone it is 1 to 1.

Acknowledgment

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FUNGICIDE ASSAY

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The hydrolysis of Dyrene and cyanuric chloride has been investigated in acid, neutral, and alkaline aqueous media. On treatment of Dyrene under reflux with 1*N* sodium hydroxide for 10 minutes, both of the chlorine atoms on the cyanuric chloride ring are released. Under the same conditions the three chlorine atoms in cyanuric chloride are liberated. On the other hand, refluxing with water completely liberates the chlorine from cyanuric chloride in 20 minutes, while under the same conditions no chlorine is liberated from Dyrene. These facts are utilized in an analytical method for Dyrene which contains cyanuric chloride. Inorganic chloride in the material is determined by solution in ice water and direct titration with silver nitrate.

possibility would have been to titrate the *o*-chloroaniline, formed on acid hydrolysis, with standard nitrite. However, the chloride determination has some advantages over the nitrite titration and therefore the former approach was investigated.

In the development of the chloride method for Dyrene, the principal interferences to be considered are inorganic chloride and cyanuric chloride, both of which are commonly present in the technical material as impurities. Considering the wide use of cyanuric chloride as a raw material, information on its reactions with aqueous reagents is surprisingly meager and, occasionally, contradictory. Fierz-David and Matter (*4*) state that cyanuric chloride is almost unaltered after heating for a short time with potassium hydroxide solution and with further heating is saponified completely to tripotassium cyanurate. In the same paper, however, they report that at 100° C., 10% sodium hydroxide gives only the dihydroxy compound. Comp and Heckle (*3*), on the other hand, found that these conditions yield cyanurate in 2 hours.

Experimental

Hydrolysis of Dyrene. The hydrolysis of Dyrene was investigated in aqueous media by refluxing 0.5 gram of recrystallized material for varying periods of time with 1*N* sodium hydroxide, water, and 1*N* hydrochloric acid. The curves obtained are shown in Figure 1. The figures for Dyrene hydrolyzed are based on the release of two equivalents of chloride per mole. In the case of the alkali hydrolyzates, the solution was acidified with nitric acid before titration with 0.1*N* silver nitrate, using a glass-silver electrode system to detect the end point. The aqueous and acid hydrolyzates were titrated without further treatment, allowance being made in the case of the hydrochloric acid hydrolyzate for the chloride added.

As can be seen, the alkaline hydrolysis is virtually complete within 10 minutes. With acid the reaction is much slower, although still appreciable. With water, there was no appreciable hydrolysis at least up to 30 minutes.

Hydrolysis of Cyanuric Chloride. The cyanuric chloride (Matheson Coleman & Bell, Cincinnati, Ohio), melting point 144 to 146 ° C., was hydrolyzed in 1*N* sodium hydroxide, 1*N* hydrochloric acid, and water by determining the chloride. In each case, a 0.3-gram sample of material was reacted with 50 ml. of reagent. The extent of hydrolysis in each medium was determined at 15 °, 25°, and 50° C., and reflux for varying periods of time. The results are shown in Figures 2, 3, and 4. The cyanuric chloride figures are based on 3 equivalents of chloride per mole.

The results show that cyanuric chloride is much more rapidly hydrolyzed in alkali than in water or acid. In the former medium, hydrolysis is virtually complete in 20 minutes even at 25° C. On the other hand, in acid or water, reflux temperature is required to obtain complete reaction in a reasonable length of time.

Comparison of Figures 1 and 4 shows that on refluxing with water, the hydrolysis of cyanuric chloride is essentially complete in 20 minutes. Under the same conditions, Dyrene is virtually unaffected. Thus, a possible method of determining cyanuric chloride in the presence of Dyrene presented itself.

As small amounts of inorganic chloride are liable to be present in technical Dyrene, a method for determination of cyanuric chloride in the presence of the former contaminant was sought. From Figure 4, it is shown that the rate of hydrolysis of cyanuric chloride decreases rapidly as the temperature is lowered.

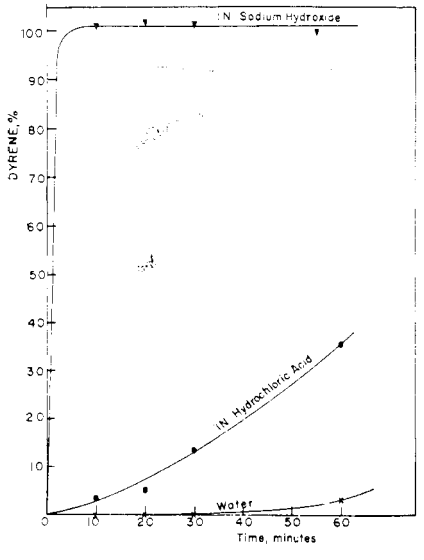

Figure 1. Hydrolysis of Dyrene at reflux

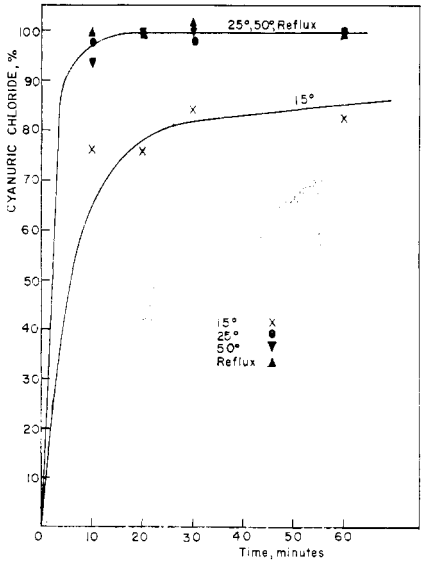

Figure 2. Hydrolysis of cyanuric chloride in 1*N* sodium hydroxide

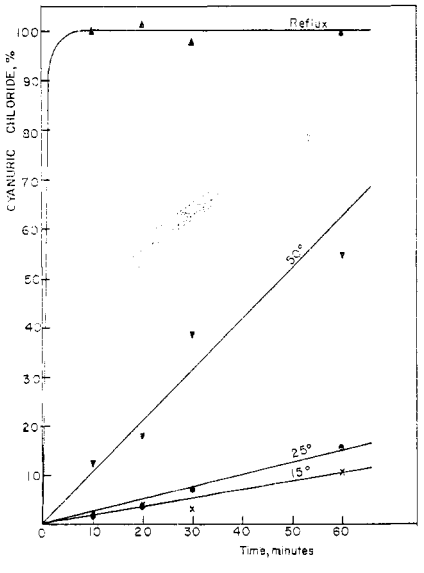

Figure 3. Hydrolysis of cyanuric chloride in 1*N* hydrochloric acid

The possibility that at 0° C. the hydrolysis of cyanuric chloride might be sufficiently slow to allow the direct titration of inorganic chloride was investigated. The results showed that a small amount of hydrolysis of cyanuric chloride occurs even at 0° C. Under these conditions, cyanuric chloride was 0.24% hydrolyzed in 10 minutes. However, a rapid titration of inorganic chloride in a solution held at 0° C. would give an almost correct result, if the cyanuric chloride content of the sample is not too great. Fierz-David and Matter (*4*) have reported that no hydrolysis of cyanuric chloride occurs in 12 hours at 0° C. The authors have been unable to confirm their results.

Application to Analysis of Technical Dyrene. Consideration of the various hydrolysis curves (Figures 1 to 4) indicated the following approach to the problem of analyzing technical Dyrene.

1. Determination of total hydrolyzable chloride by refluxing for 30 minutes with sodium hydroxide. This will determine chloride from Dyrene, cyanuric chloride, and sodium chloride.
2. Determination of water-hydrolyzable chlorine by refluxing with water. This will determine chloride from cyanuric chloride and sodium chloride.
3. Determination of sodium chloride alone by dissolving in ice cold water.

Determination of Total Hydrolyzable Chlorine. The method was first applied to recrystallized Dyrene by refluxing 0.5 gram for 30 minutes with 1*N* sodium hydroxide, acidifying with nitric acid, and titrating the liberated chloride potentiometrically with 0.1*N* silver nitrate. Dyrene is initially insoluble in the reagent, but rapidly passes into solution when hot. Eleven determinations on a recrystallized material gave a mean of 100.68% with a standard deviation of ±0.34%, a mean significantly higher than 100. This may possibly have been due to the formation of small amounts of cyanate. A further series of six determinations was carried out. The hydrolyzate was acidified under reflux for 30 minutes before titration. A mean of 99.19% was obtained with a standard deviation ±0.14%. Apparently, some volatile or acid-hydrolyzable material was removed as a result of the refluxing.

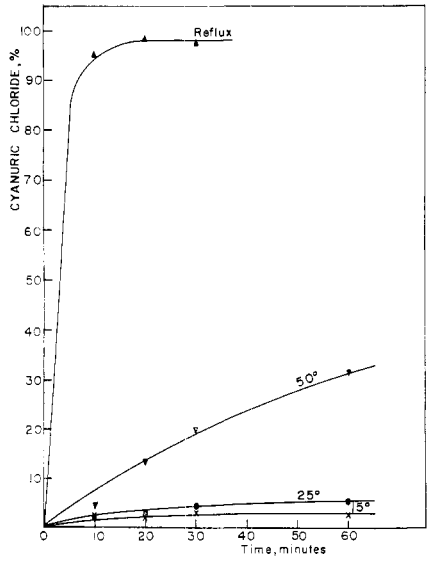

Figure 4. Hydrolysis of cyanuric chloride in water

Table I. Reproducibility of Various Chlorine Determinations on Mixtures of Dyrene, Cyanuric Chloride, and Sodium Chloride

Determination	Calcd., %	Found, % $\bar{x}$	s	n
Total hydrolyzable chlorine, as Dyrene	101.81	101.65	±0.11	6
Water hydrolyzable chlorine, as cyanuric chloride	3.42	3.45	±0.05	6
	3.46	3.48	±0.04	5
Ionizable chlorine, as sodium chloride	0.98	0.96	±0.07	5
	0.90	0.96	±0.04	5
	0.98	0.99	±0.01	5

Determination of Water-Hydrolyzable Chlorine. From the hydrolysis curves of Dyrene and cyanuric chloride it would appear that 20 minutes would be the optimum reflux time for the estimation of the latter compound in the presence of the former. However, under these conditions Dyrene is hydrolyzed to the extent of about 0.2%. Under the condition where a small amount of cyanuric chloride (less than 3%) is present in a large amount of Dyrene, the error due to Dyrene hydrolysis becomes appreciable. In an effort to compensate for this error, the hydrolysis time was reduced to 10 minutes. Under this condition the cyanuric chloride is only about 95% hydrolyzed, but the value will be compensated by a very slight hydrolysis of Dyrene.

Determination of Ionizable Chloride. The method proposed for this constituent was simply to stir the sample for 5 minutes with ice-cold water containing chipped ice and titrate potentiometrically with 0.1*N* silver nitrate at the same temperature. Because of the comparatively small cyanuric chloride content of technical Dyrene, interference from the hydrolysis of this compound would be expected to be negligible.

Analytical Procedure

Method. Weigh a 0.5-gram sample into a 300-ml. Erlenmeyer flask. Pipet 50 ml. of 1.0*N* sodium hydroxide into the flask, swirl, and attach a reflux condenser. Reflux for 30 minutes. Cool and rinse down the condenser with water. Add one drop of 1% phenolphthalein followed by sufficient dilute nitric acid—1 to 2—to discharge the color, then use 10 ml. in excess. Reflux for 30 minutes more. Cool, rinse down the condenser, and transfer the solution to a 400-ml. beaker. Insert a bright silver cathode and glass anode connected to a suitable potentiometer and titrate to the end point with 0.1*N* silver nitrate. One milliliter of 0.1*N* silver nitrate is equivalent to 3.546 mg. of chloride. Let the total hydrolyzable chlorine be A%.

Table II. Analysis of a Mixture of Dyrene, Cyanuric Chloride, and Sodium Chloride

Component	Present, %	Found, %
Dyrene	92.90	92.80
Cyanuric chloride	2.61	2.64
Sodium chloride	1.13	1.16

Weigh a 5-gram sample into a 300-ml. Erlenmeyer flask. Add 100 ml. of water, swirl, and reflux under a condenser for 10 minutes. Titrate with silver nitrate by the same method as above. Let the water-hydrolyzable chlorine be B%.

Weigh a 5-gram sample into a 400-ml. beaker. Add 100 ml. of ice-cold water and sufficient chipped ice to maintain the temperature during titration. Stir for 10 minutes and titrate with silver nitrate as before. Let the ionizable chloride be C%.

Calculation:

$$\text{Dyrene content} = (A - B) \times 3.886\%$$

$$\text{Cyanuric chloride content} = (B - C) \times 1.733\%$$

$$\text{Sodium chloride content} = C \times 1.648\%$$

Reproducibility and Accuracy. In order to test the reproducibility and accuracy of the analytical method developed, several synthetic mixtures of Dyrene, cyanuric chloride, and sodium chloride were prepared. These were analyzed for total hydrolyzable chlorine, water-hydrolyzable chlorine, and inorganic chloride. The results, as percentages, are shown in Table I. This experiment was designed to show the reproducibility of the different steps. These results cannot be used to calculate the actual Dyrene and cyanuric chloride contents of the samples, as the same mixtures were not used throughout. Table I shows that both accuracy and reproducibility are adequate for each of the three determinations. The results for inorganic chloride do not appear to be increased significantly due to slight hydrolysis of cyanuric chloride.

In reporting an analysis, the Dyrene content is calculated from the difference of two determinations, as is the cyanuric

Table III. Total Hydrolyzable Chlorine in Cyanuric Chloride

Conditions	$\bar{x}$	s	n
Direct titration	99.78	±0.32	5
30-minute reflux after acidification	100.08	±0.20	5

chloride content. As a final check on the procedures, a mixture of this Dyrene with cyanuric chloride and sodium chloride was analyzed for all components. The results are shown in Table II. Each of the determinations was carried out five or six times. It can be shown that the differences between the calculated and found figures for the mixture are not significant as based on the standard deviations of the previous table. If each determination were carried out in duplicate, a standard error for the Dyrene content would be ±0.13%, for cyanuric chloride ±0.05%, and for sodium chloride ±0.04%. Both accuracy and reproducibility are satisfactory for control purposes.

The method is subject to interference by pesticides such as DDT or BHC which are capable of undergoing alkaline dehydrohalogenation.

Application to the Analysis of Technical Cyanuric Chloride. Following on from the above, there seemed no reason why the relevant methods should not be applied to the assay of technical cyanuric chloride. The published method (*1*) for this determination involves the reaction of excess sodium methylate with cyanuric chloride, the excess reagent being hydrolyzed and determined acidimetrically in the usual way. The results with aqueous sodium hydroxide suggested a somewhat simpler procedure.

From the hydrolysis curves (Figure 2), one would expect hydrolysis at room temperature to be erratic. This was confirmed by hydrolyzing a series of 0.25-gram samples for 30 minutes in 1.N sodium hydroxide at about 22° C. The liberated chloride was determined by direct titration in acid solution. Five determinations gave a mean of 93.32% with a standard deviation of ±2.46. As hydrolysis at room temperature was obviously unsatisfactory, the reaction was reinvestigated by heating for 30 minutes at reflux. In this study the effect of boiling after acidifying the hydrolyzate was investigated for cyanuric chloride as it had been for Dyrene. The results obtained by the refluxing procedure are shown in Table III. Theoretical values are obtained whether the posthydrolysis boiling step is included or not and this step was omitted in applying the procedure to a mixture of cyanuric chloride and sodium chloride (2.47%). In this case a mean value of 100.0% (standard deviation ±0.19 for seven determinations) was obtained. The calculated value for the sample was 99.92%.

The method for free chloride as described for Dyrene was unsatisfactory for cyanuric chloride because of a small amount of hydrolysis of the latter compound in ice water. Accordingly, a procedure (*1*) involving solution of the sample in chloroform and shaking for 2 minutes with ice-cold water was employed. In this method the inorganic chloride is supposed to be extracted into the water without causing any hydrolysis of cyanuric chloride. When this method was applied to pure cyanuric chloride, five determinations gave a mean value of 0.09% as sodium chloride. This indicated that a slight hydrolysis of cyanuric chloride was occurring even under these conditions. That the value is real and not due to a slight contamination of the cyanuric chloride with inorganic chloride was shown by the fact that a plot for the hydrolysis of cyanuric chloride in water at 0° C. went through the origin. If there had been as much as 0.1% of inorganic chloride present, this would not have been true.

When the method was applied to a sample of cyanuric chloride containing 2% of added sodium chloride, the mean of six determinations gave a value 0.1% higher than the theoretical. However, for control purposes this was not considered serious.

The total hydrolyzable chlorine procedure is suggested as an alternative to the methylate procedure for the analysis of cyanuric chloride. It may be corrected satisfactorily for ionizable chloride by the method described above.

Acknowledgment

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ACARICIDE BIOASSAY

Two Organisms Suitable for Bioassaying Specific Acaricides

SUTHARM AREEKUL[1] and ROBERT F. HARWOOD

Department of Entomology, Washington State University, Pullman, Wash.

BIOASSAY with susceptible organisms is a well established method for determining residues of pesticides. However, the specific acaricides—pesticides specifically toxic to mites but virtually nontoxic to insects—cannot be successfully determined by bioassay with insects commonly used for this purpose.

Phytophagous mites, notably the two-spotted mite *Tetranychus telarius* (L.), have been used to screen candidate pesticides for acaricidal activity. Reports on this subject, found in the unpublished files of pesticide manufacturers, indicate a great deal of variability in response to any given level of toxicant. Several reasons may exist for such variability, but undoubtedly difference in host plants is a prominent factor. Thus variation in age and nutrition of plants probably affects the mites, and their almost continual dependence on plant food necessitates careful dipping or spraying practices, which are in turn modified by interactions between pesticide and plant surface. The instability of response to a given level of toxicant accounts for the present lack of bioassay methods for specific acaricides.

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Bioassay of specific acaricides was attempted because no suitable organisms for this type of analysis had been tested. The brine shrimp, *Artemia salina* Leach, and the stored products mite, *Tyrophagus putrescentiae* Schrank, showed promise for assaying this group of toxicants. The former was exposed in shell vials to toxicant in acetone introduced into salt solution, the latter to a dry film of acaricide in a chamber made from opposed microscope depression slides. Toxicity curves with solutions of highly purified toxicant were established for Aramite, Chlorobenzilate, Dimite, Kelthane, Mitox, ovex, and Sulphenone. A bioassay of Kelthane on pears and cherries, using extractives from these fruits to establish a standard curve, was in close agreement with the colorimetric method frequently employed.

ness of four organisms for bioassaying a number of pesticides showed that a stored product mite, *Tyrophagus putrescentiae* Schrank (Syn. *T. castellanii* Hirst, *T. noxius* Zakhvatkin, *T. bravni* Turk), and the brine shrimp, *Artemia salina* Leach, are suitably sensitive to specific acaricides in pure form. Methods of exposure and toxicity curves for seven of these compounds are reported here.

Organisms

The brine shrimp, *Artemia salina*, has been previously utilized to determine residues of insecticides. There are several advantages in its use (*6*). Eggs, which can be stored for long periods, are readily obtainable from tropical fish suppliers. They are easily hatched, and the nauplii are tolerant to a wide range of salt concentration. They tolerate small amounts of acetone, but minute amounts of insecticide are rapidly toxic to all age groups from 24-hour-old nauplii to adults. Sensitivity to methoxychlor, DDT, lindane, chlordan, toxaphene, endrin, Bayer 17147, parathion, and dieldrin has been reported (*6, 11*), but their usefulness in the assay of specific acaricides has not been determined.

A stored products mite, *Tyrophagus putrescentiae*, was tested for susceptibility to specific acaricides. This mite is world-wide in distribution, and has been reported to feed on many kinds of stored products, preferably flour and cheese. They have been reared on blocks of Cheddar cheese at 55° F., and acaricides have been evaluated for their control (*2*). This mite was demonstrated to be two to three times more productive in semolina than in wheat (*8*). It has a very high fecundity, and can seemingly be cultured indefinitely and easily under proper laboratory conditions. The minimal care required to maintain this mite aids in its desirability as an organism for bioassay.

Pesticides Tested

Dosage-mortality curves were established for 29 pesticides using these two test organisms. All test compounds were highly purified, or the technical chemical was used. The results related here are for preliminary tests with purified compounds against the two organisms used, and merely show their suitability as sensitive organisms provided relatively pure toxicant is employed. The following seven specific acaricides were tested:

Aramite [2-(*p-tert*-butylphenoxy)-1-methylethyl 2-chloroethyl sulfite]; Aramite standard A-7499; Naugatuck Chemical.

Chlorobenzilate (Geigy 338; ethyl-4,4′-dichlorobenzilate); pure chlorobenzilate; Geigy Agricultural Chemicals.

Dimite (DMC; 4,4′-dichloro-α-methylbenzhydrol); purified DMC, *p,p*-DMC, content 98.5%, OR-307; Sherwin-Williams Co.

Kelthane [FW-293; 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol]; purified Kelthane; Rohm & Haas Co.

Mitox (Chlorbenside; *p*-chlorobenzyl *p*-chlorophenyl sulfide); recrystallized Mitox, California Spray-Chemical Corp.

Ovex (Ovotran; *p*-chlorophenyl *p*-chlorobenzenesulfonate); highly purified ovex, Dow 360914; Dow Chemical Co.

Sulphenone (R-242; *p*-chlorophenyl phenylsulfone); technical Sulphenone; 4-chlorophenyl phenylsulfone 80%, 4,4-chlorophenyl phenylsulfone 5%, phenyl phenylsulfone 15%; Stauffer Chemical Co.

Rearing Methods

The salt solution previously recommended (*6*) was used for hatching brine shrimp eggs. Rather than endure the uncertainties of rearing the nauplii to adults, those hatched within 24 hours were used. The eggs were placed in the appropriate salt solution in one side of a crystallizing dish 6 inches in diameter, separated down the middle by a floating wooden divider. A light source illuminated the side lacking eggs and the photopositive nauplii swam to that side. In this manner nauplii were readily separated from unhatched eggs, and presumably only vigorous individuals uniform in their reaction to light were tested. Use of newly hatched nauplii precluded the variability in susceptibility of various growth stages previously noted (*11*).

The stored product mite was raised on wheat in 32-ounce wide-mouthed bottles. Only mechanically damaged grain is attacked (*8*); therefore the kernels were cracked in a grinding machine after a wash in hot water to ensure the elimination of other stored grain pests. The jars were filled almost to the top and about 3 grams of wheat germ and 1 gram of brewers yeast added. To promote fungal growth which is known to favor vigorous mite reproduction (*7*), 10 ml. of distilled water were deposited along the side of each filled jar to soak the bottom layer of the medium. Mites were introduced with grain from an old culture, and were found feeding at the bottom layer in considerable numbers within a week. Usually three or four jars were prepared simultaneously and placed on a metal pan surrounded by a barrier of motor oil. In 3 to 4 weeks tremendous numbers of mites developed, swarming on the filter paper covering each jar. A productive culture supplied sufficient mites for testing over 4 to 5 months. All cultures were maintained in a room at 75° F. and 40% relative humidity.

Method of Exposure

Brine Shrimp. Acaricides being tested were prepared in 1.25-ounce salve jars. The exposure medium consisted of salt mixture diluted with distilled water to one fifth of hatching concentration to which acaricide in acetone had been added. In practice this was accomplished by placing 8 ml. of distilled water and acaricide in each jar with 2 ml. of full-strength hatching medium containing approximately 50 shrimp. Solvents other than acetone in plant extracts of acaricide were evaporated in a fume hood and were replaced by acetone before the test exposure was made. The control (no acaricide) consisted of similarly diluted hatching solution, and diluted hatching solution plus acetone. Concentration of acetone varied within a test, but never exceeded 2% on a volume basis.

Brine shrimp and test solution were transferred to glass shell vials approximately 0.25 inch in diameter and 1.25

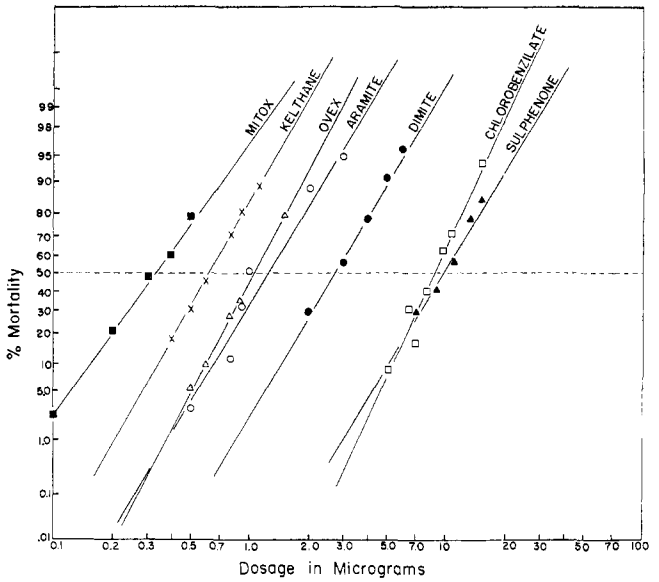

Figure 1. Log dosage–probit mortality regression lines of seven specific acaricides for brine shrimp, 24-hour exposure at 75° F.

Dosage expressed in micrograms per milliliter

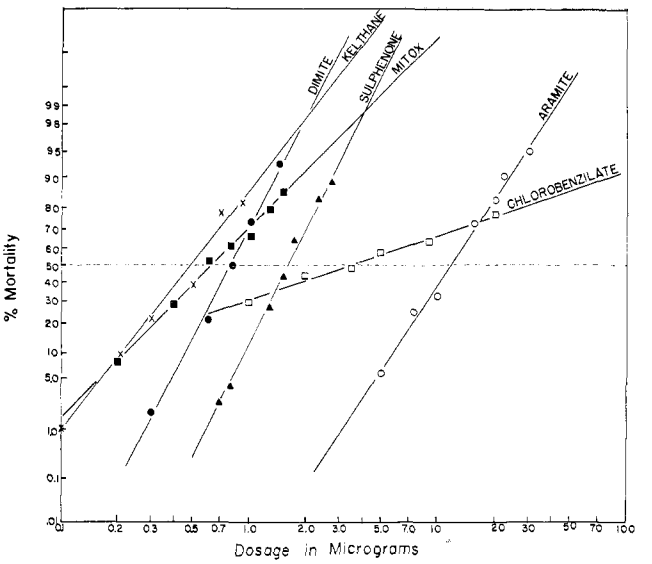

Figure 2. Log dosage–probit mortality regression lines of six specific acarides for the stored products mite, 24-hour exposure at 78° F.

Dosage expressed in micrograms per depression chamber

inches in height. Ten shrimp were placed in each vial, which was filled nearly to the top. Four vials were filled (1.2 ml.) with an identical concentration of salt solution and acaricide. The vials were then placed in a rack designed to permit easy observation of effects when placed before a dark background. Each concentration of toxicant was tested at least twice for a minimum of eight replications at any given level. All tests were conducted at 75° F. and 40% relative humidity.

Mortality, rather than time in relation to cessation of swimming activity (*6*), was the criterion used to determine levels of toxicant. Exposure was for 24 hours, during which time approximately 0.2 ml. of test solution evaporated. However, this was not considered particularly important, as in any case all test containers were subject to equal conditions.

In making a reading the exposure rack was placed in front of the light from two 15-watt fluorescent tubes and the shrimp were allowed approximately 1 minute to exhibit response to light before mortality was determined. Unaffected brine shrimp can cover the distance of the vial depth in less than 20 seconds, and move rapidly up and down the column of solution. In response to toxicants they sink to the bottom due to a loss of muscular coordination. They were counted as "dead" if they failed to travel at least 0.25 inch vertically during approximately 30 seconds of observation.

Stored Product Mite. Microscope slides possessing three shallow depressions were used as exposure chambers for the mites. The toxicant in solvent was placed in the slide depressions by calibrated micrometer syringe or small-volume pipet, a given amount being repeated in each depression for (a total of) three replications. Each depression had a capacity of approximately 0.1 ml. The solvents commonly employed for extraction of pesticides (acetone, benzene, carbon tetrachloride, chloroform, petroleum ether, Skellysolve B) were rapidly evaporated at room temperature, leaving a dry film of toxicant. For plant extracts it was sometimes necessary to evaporate the solvents more rapidly in a fume hood on a slightly warm surface because of wax and other plant residues retarding evaporation.

The mites were transferred to the treated depressions on pieces of dark blue or black paper formed with an insect point punch. In a vigorous culture they swarm over the filter paper covering the wide-mouthed rearing bottle. This was moistened with a few drops of water and the dry paper points were placed on top. In about 10 minutes the mites congregated on the surfaces of the dry points, which were transferred by forceps singly to treated slide depressions. The dark paper made the white mites plainly visible, so that points covered with 30 to 80 mites were readily chosen. The use of paper points for transfer minimized injuries caused by trying to handle the mites by other methods.

An enclosed exposure chamber was formed by opposing the depressions of each treated slide with those of an untreated one. A drop of 1% aqueous methylcellulose solution was placed at each end of the treated slide, causing a seal over all flat surfaces when the clean slide was placed on top. In addition to sealing off the exposure chambers, the methylcellulose solution appeared to maintain a desirable humidity during exposure. Mites that crawled out of the depressions before application of the untreated slide were crushed between the flat surfaces and not counted further in the test. Test chambers were laid horizontally in a light-excluding holding cabinet at 78° F. and 40% relative humidity. The mites were active and walked over both treated and untreated surfaces. Each level of acaricide was replicated at least six times.

Mortality counts were made following 24 hours of exposure. Each exposure chamber was readily observed when placed against a dark background in the field of a dissecting microscope at 15× magnification. The total number of living and dead mites was counted at this time. Living mites were activated by placing the exposure chambers for 15 seconds on a surface warmed to 95° F. Living individuals responded by active locomotion or movement of appendages. Dead individuals were motionless and often contorted and dried.

Results and Discussion

Percentage mortality was calculated by compensating for the mortality in untreated controls by Abbott's formula (*7*). For all tests the mortality of control brine shrimp was zero in diluted saline, less than 1% in saline solution with 2% acetone. Mortality of mites was less than 5% for all controls. Regression lines for specific acaricides against brine shrimp and mites were graphed on a log dosage-probit mortality scale (Figures 1 and 2). The lines were fitted statistically by means of the least square method (*3*), and the LD_{50} level was calculated (Table I). The mites were very insensitive to ovex under these experimental conditions, making a regression line impossible to plot. The LD_{50} level for DDT, parathion, and dieldrin, obtained under identical experimental conditions, is included in Table I.

Results indicate the toxicity of the acaricides to brine shrimp at the LD_{50}

evel to be: Mitox > Kelthane > ovex ≅ Aramite > Dimite > Chlorobenzilate > Sulphenone. A comparison at the LD_{50} level between the specific acaricides and three reference insecticides is as follows: Mitox > parathion > Kelthane > Aramite = DDT > Dimite > dieldrin > Chlorobenzilate. As the brine shrimp has been successfully employed by previous workers for the determination of insecticides, and as it is similarly sensitive to specific acaricides, exhibiting a satisfactorily steep slope for the dosage-mortality regression lines, it is obvious that this organism is suitable for the bioassay of residues of specific acaricides.

For the mites the toxicity of the acaricides at the LD_{50} was: Kelthane ≅ Mitox > Dimite > Sulphenone > Chlorobenzilate > Aramite. Ovex was virtually nontoxic to the postembryonic forms tested. In comparing specific acaricides to reference insecticides dieldrin > Kelthane > parathion > Chlorobenzilate > DDT = ovex.

From the standpoint of sensitivity at the LD_{50} level, and slope of the regression line of the seven specific acaricides tested, brine shrimp is more suitable for bioassaying Aramite and ovex. Under the same criteria *T. putrescentiae* is more suitable for the assay of Dimite and Sulphenone. They are roughly equivalent at the LD_{50} level for Kelthane and Mitox, but the brine shrimp possesses a steeper and therefore more satisfactory regression line for these two compounds. Although the brine shrimp is not as sensitive at the LD_{50} level as the mite to Chlorobenzilate, the steep pitch of the regression line suggests it to be a much more suitable organism for bioassay of this compound. In fact, the slope of the regression line for this compound against the mite suggests that it is unsuitable for determination of different levels. The response of brine shrimp to ovex is interesting, as the mite as well as two insects tested (*Drosophila melanogaster* and *Cryptolestes ferrugineus*) were virtually unaffected. This compound is used as an ovicide against mites, and it may be that brine shrimp are suitable for bioassay of other compounds that are primarily ovicidal in activity.

Assay of Plant Extractives. Tissue extractives, as might be expected, hindered the determination of specific acaricides by interfering with toxicant or by possessing naturally toxic substances. The strong-smelling extracts of turnips and onions, extracting 5 grams of tissue with 1 ml. of Skellysolve B, did not affect the mortality of mites in control exposure chambers. An extract of Kelthane from pears (6.45 grams of tissue per ml. of Skellysolve B) was toxic to brine shrimp, causing a calculated recovery of 163% based on the reference curve developed for acaricide and solvent only. Extract of cherries with Skellysolve B (15 grams per 1 ml.) did not significantly affect mortality of brine shrimp, yielding a 101% recovery. However, these same raw extracts appeared to mask the toxicity of Kelthane for mites, yielding only a 49.5% recovery for the pear extract, and an even smaller recovery for the cherry extract. Although standards prepared under identical conditions can be used to counterbalance such effects (*10*), the removal of plant-derived extractives as suggested (*4*, *5*) is desirable if they markedly modify toxicity.

The apparent agreement of chemical analyses of Kelthane with a limited number of bioassays indicates the validity of using brine shrimp and mites for determining residues of specific acaricides. Extracts of pears and cherries were obtained by tumbling them with Skellysolve B and anhydrous sodium sulfate for 1 hour. The filtered extract was determined colorimetrically (*9*), as 0.30 p.p.m. for pears and 2.3 p.p.m. for cherries. This same extract bioassayed with brine shrimp yielded determinations of 0.34 and 2.1 p.p.m., respectively, when compared to reference curves established with toxicant in appropriate plant extract. Bioassay with *T. putrescentiae* under similar conditions yielded determinations of 0.33 and 1.9 p.p.m., respectively. Apparently the differences in toxicity of pure solutions and plant extracts can be compensated for by establishing a toxicity curve with the plant extract in question.

Perhaps both organisms should be used in determining a residue sample. The results would serve as a cross check and might profitably be averaged to give a more accurate determination. For example, two samples of highly purified Chlorobenzilate and Kelthane were prepared by one investigator and determined by the other lacking previous knowledge of their concentration. For Chlorobenzilate a recovery of 96.2% (30.1 mg.) was obtained with brine shrimp, 116.7% (36.54 mg.) with mites. When averaged the recovery was 106.4%, actual amount being 31.30 mg. For Kelthane a recovery of 107.4% (7.57 mg.) was obtained with brine shrimp, 92.6% (6.53 mg.) with mites. When averaged the recovery was 100%, actual amount being 7.05 mg.

Table I. LD_{50} in Micrograms of Toxicant per Milliliter for Brine Shrimp, and per Exposure Chamber for Mites

	Organisms					
	Brine Shrimp			Mites		
Toxicants	LD_{50}	Dosage limits[a]	Slope	LD_{50}	Dosage limits[a]	Slope
Aramite	1.25	1.12–1.38	2.539	11.72	11.61–11.83	3.570
Chlorobenzilate	9.51	9.37–9.65	3.328	3.97	3.85–4.09	1.321
Dimite	2.56	2.45–2.67	3.207	0.71	0.60–0.82	4.422
Kelthane	0.75	0.63–0.87	3.302	0.42	0.34–0.60	2.538
Mitox	0.40	0.28–0.52	2.272	0.57	0.45–0.69	2.064
Ovex	1.16	1.04–1.28	3.534	100.0+	...	
Sulphenone	12.98	12.79–13.17	2.890	1.74	1.51–1.97	4.267
DDT	1.25	1.12–1.38	0.901	100.0+	...	
Dieldrin	5.45	5.29–5.61	0.589	0.041	0.029–0.053	2.969
Parathion	0.52	0.41–0.63	2.500	3.50	3.32–3.68	0.537

[a] Dosage limits were calculated by transforming the limits of % mortality at LD_{50} level to dosages, using the formula:

$$\text{Lower limit } X' = XLD_{50} - \frac{t05\ S\bar{y}.x}{b}$$

$$\text{Upper limit } X' = XLD_{50} + \frac{t05\ S\bar{y}.x}{b}$$

$$\text{where } S\bar{y}.x = \frac{S\bar{y}.x}{\sqrt{n}}$$

x = log dosage
y = probit mortality
$S\bar{y}.x$ = standard deviation
n = no. of observations
b = slope
$t05$ = value of t at 0.05 level

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INSECTICIDE METABOLISM

Metabolism of Malathion by Rat Tissue Preparations and Its Modification by EPN

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Homogenates of 11 rat tissues metabolized malathion at comparable rates and to similar metabolites. The main hydrolysis occurred at the carboxyester linkage. The hydrolysis at this linkage, the over-all hydrolysis, and the formation of malaoxon by various tissues in vitro were all inhibited by EPN in vivo. The synergism of EPN and malathion in vivo is therefore probably not attributable to an increased level of malaoxon in the body, but to a greater persistence of malathion and malaoxon in the tissues.

POTENTIATION" OR "SYNERGISM" as it is called in this paper, with organophosphate insecticides in mammals is of considerable interest, because of possible health hazards (*6*) and of the need for investigating the physiological background of the phenomenon (*3, 4, 10*).

Combinations of malathion and EPN deserve particular interest as after the initial report of Frawley *et al.* (*6*) it has frequently been confirmed that certain combinations of these two insecticides in the dog, rat, and mouse resulted in some remarkable synergistic effects (*5, 13*). It has been shown by Cook *et al.* (*3*) that malathion degradation by liver in vitro is inhibited by numerous organophosphates, and by Murphy and DuBois (*10*) that after EPN administration, the ability of the liver and serum to degrade malaoxon (the anticholinesterase which is a metabolite of malathion) is inhibited. Both groups, after studies on rats, concluded that their findings accounted for the observed synergistic effects.

The present study was initiated by the need for a broad approach to the metabolic background of the phenomenon, especially to locate the point of attack of EPN upon the metabolism of malathion. According to Cook and Yip (*4*), the principal liver metabolite of malathion in vitro is one of the half-esters—*O, O*-dimethyl *S*-(1-carboethoxy-2-carboxyethyl) phosphorodithioate. However, their Table I suggests that, in fact, more than one metabolite was produced; if this were so, it would reopen the problem of where EPN attacks.

This study examines the hypothesis that EPN inhibits degradation of malathion and/or malaoxon, and consequently raises the level of malaoxon in tissues. Such an effect in vivo would explain the potentiation of malathion by EPN.

Materials and Methods

Holtzman albino male rats, 160 to 180 grams, were used as sources of tissues which were pooled from at least two animals for every experiment. The animals were decapitated and the tissues immediately chilled after removal. The heparinized blood was separated into plasma and corpuscles (whole corpuscular fraction) by centrifugation.

The tissue preparations used were:

Natural form (plasma and corpuscles).

Homogenates, made with a Lourdes stainless steel homogenizer. Homogenates were used, because they were expected to yield primarily information on degradation. They were also the only type of preparation which could be used for all tissues.

Slices (thickness 0.2 mm.), cut with a Mickle tissue slicer, were used, because it was anticipated that they would yield information primarily on the balance between malathion oxidation and degradation (the procedure of slicing helps preserve cofactor-sensitive oxidative enzyme systems).

Acetone powders. One part of chilled tissue was homogenized in 20 parts of acetone at −50° C. and filtered, and the residue was rehomogenized twice in fresh, cold acetone portions. Acetone was removed by a Labline evaporator, and the powder was dried overnight under room conditions. Incubation suspensions were made up by homogenizing the acetone powders in isotonic solution. Acetone powders were used for comparison with the findings of Cook *et al.* (*3*) which were partially based upon this type of preparation.

Liver cell component fractions. Liver was homogenized in a Potter-Elvehjem type glass homogenizer and differentially centrifuged essentially according to Schneider (*14*); the preparation and incubation medium was isotonic sucrose solution (A) as specified below.

In experiments where only liver was used, not comparatively with other tissues, the liver was perfused before preparation with cold 0.9% sodium chloride solution in situ via the portal and hepatic veins in order to remove blood.

The incubation conditions were, unless stated otherwise: fresh tissue preparation (or the equivalent of fresh whole tissue as acetone powder or live cell component fractions) incubated at a concentration of 5% in isotonic solution A (0.018*M* calcium chloride in 0.25*M* sucrose) containing $6.8 \times 10^{-4}M$ P^{32} malathion for 30 minutes at 37.5° C. while shaken at 120 cycles per minute.

In experiments especially indicated below, a buffered isotonic solution, referred to as B, was used as incubation medium: 0.15*M* sodium chloride, 0.154*M*

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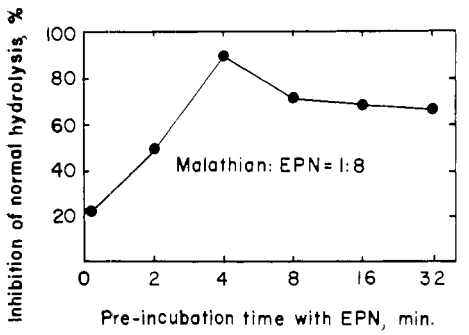

Figure 1. Effect of preincubation with EPN upon malathion hydrolysis

Malathion, 200 γ added after preincubation with 1600 γ EPN in isotonic solution A containing 5% liver homogenate

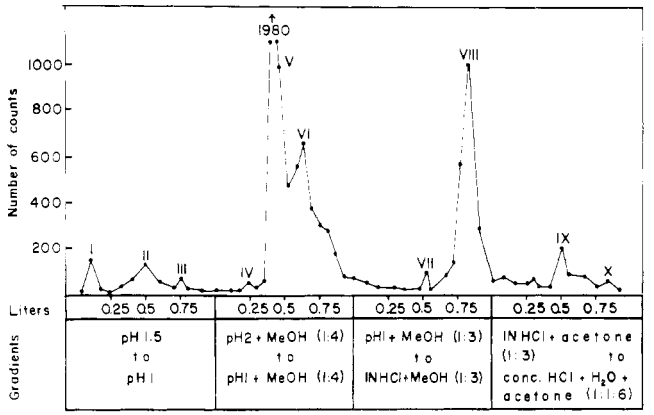

Figure 2. Ion exchange separation of water-soluble metabolites of P^{32} malathion

Liver acetone powder of untreated rats was incubated 30 minutes in isotonic solution A. Gradients indicated were applied in all ion exchange columns throughout the study

Peak No.	Metabolite
I	Phosphoric acid
II	Monomethyl phosphate
III	Dimethyl phosphate
IV	Unidentified
V	O,O-dimethyl S(1-carboethoxy-2-carboxy)ethyl phosphorodithioate or malathion monoacid
VI	O,O-dimethyl S(1,2-dicarboxy)ethyl phosphorodithioate or malathion diacid
VII	Unidentified
VIII	O,O-dimethyl phosphorothioate
IX	Unidentified
X	O,O-dimethyl phosphorodithioate

potassium chloride, 0.11*M* calcium chloride, 0.154*M* potassium acid phosphate, 0.154*M* magnesium sulfate heptahydrate, 0.1*M* phosphate buffer (pH 7.4), 2% Triton X-100, 0.36*M* glucose.

EPN (E.I. du Pont de Nemours and Co., Wilmington, Del.) was recrystallized *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate.

Synthesis of P^{32} Malathion. Phosphoric acid containing P^{32} (100 mc.) dissolved in hydrochloric acid was evaporated to dryness with dry air in a round-bottomed reaction flask, 0.25 gram of phosphorus pentasulfide was added, a condenser attached, and the flask was gassed with dry carbon dioxide. The contents were heated gently in the beginning, then vigorously for 10 additional minutes. Yellow crystals of phosphorus pentasulfide containing P^{32} formed on cooling (*2*). Five milliliters of toluene and a stirring bar were added to the reaction flask, the condenser was replaced, and 0.2 ml. of anhydrous methanol dissolved in 2 ml. of toluene was added dropwise. The contents were refluxed between 80° and 90° C. until all $P_2{}^{32}S_5$ had reacted.

The temperature was reduced to 50° to 60° C. and 0.425 ml. of diethyl maleate in 3 ml. of toluene were added. The mixture was refluxed for 4 hours at 85° C. with stirring. The residue was concentrated by evaporation, then taken up in chloroform and washed with an equal amount of 10% sodium carbonate. The compound was purified by chromatography on a Celite column with iso-octane–methanol (*1*) as eluent. The radioactive material was obtained in 70% yield and with an activity of 15,000 counts per minute per microgram.

Extraction Procedure. The protein of the incubation suspensions was precipitated and the pH was adjusted to 2.5 by saturated trichloroacetic acid solution. The suspension was partitioned against 30 ml. of chloroform, 3 grams of Celite were added, the suspension was filtered through a Büchner funnel, and the precipitate was washed alternately with 50 ml. each of water and chloroform. The precipitate was washed on another filtering flask with 20 ml. each of methanol and acetone, the washings were evaporated to dryness and taken up in the water and chloroform fraction, which was then centrifuged, and the water layer was removed. The chloroform was repartitioned against fresh water at pH 9 and centrifuged, and the water fractions were combined. An average of 5% of the total counts were not extractable from the protein-Celite filter residue.

The efficiency of the partitioning procedure was checked: (1) by repeated repartitions with fresh portions of water and chloroform, respectively: less than 2% of the total counts were found in the second partitioning step; (2) by partitioning a liver homogenate incubation suspension which had been inactivated (precipitated) prior to incubation: 0.4% of total counts were in the average found in water, and 99.8% in the malathion fraction—i.e., extracted by chloroform, and eluted by benzene from the aluminum oxide column (see below).

Column Chromatography. Water-Soluble Metabolites. The method described by Plapp and Casida (*12*) was employed with some slight modifications: An ion exchange resin (Dowex No. 1-X 8, 100 to 200 mesh, medium porosity, washed and stored in hydrochloric acid at pH 3) in a chromatography tube (21 mm. in diameter, 250 mm. long) was used with 3-pound pressure. The elution gradients are shown in Figure 1. Seven-milliliter samples were collected and counted with a Tracerlab 64 scaler or a Nuclear Chicago automatic unit.

The various water-soluble metabolites were tentatively identified by cochromatography with known compounds, whose position was determined by phosphorus assay. The possibility that one of the "unidentified" metabolites was malaoxon was examined, but it was found that this compound was not eluted off the column by the gradients shown in Figure 2 which were applied throughout the study.

Chloroform-Soluble Materials. Anionotropic aluminum oxide, activity grade 1, was used in column tubes 10 mm. in diameter and 170 mm. long. Two methods of adsorbent preconditioning have been employed: Aluminum oxide was thoroughly mixed with 2% (v./w.) water, slurried in hexane, and filled into the tube. The sample of chloroform-solubles, evaporated to dryness, and taken up in hexane, was added to the column. Two hundred milliliters each of hexane, benzene (eluting malathion), and chloroform (eluting malaoxon) were then run through successively one after another. As the aluminum oxide after such preconditioning retained between 20 and 55% of the radioactive material, another method based on the data of Malina *et al.* (*8*) was developed which resulted in 2 to 10% adsorption. Aluminum oxide was thoroughly mixed with 15% water, slurried in hexane, and filled into the tube. Unlabeled malathion (0.2 gram) in 1 ml. of hexane was added and 50 ml. of hexane were run through.

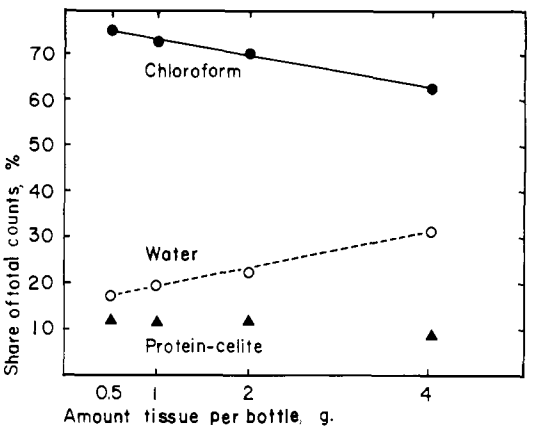

Figure 3. Hydrolytic activity as function of tissue concentration

Indicated amounts of liver homogenate per 20 ml. of suspension incubated for 30 minutes in isotonic solution A at 37.5° C.

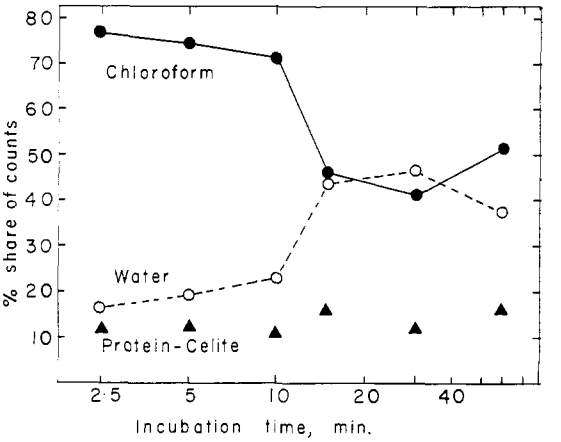

Figure 4. Hydrolytic activity as function of incubation time

Liver homogenates incubated as 5% of incubation suspension A for indicated times at 37.5° C.

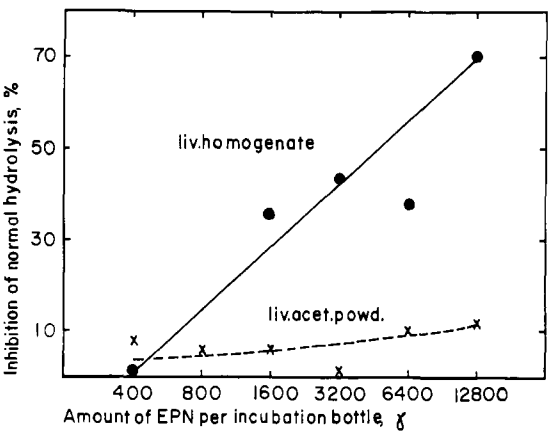

Figure 5. Influence of concentration of EPN upon degree of malathion hydrolysis in simultaneous incubation

Malathion, 400 γ per 20 ml. of liver homogenate suspension, 30 minutes of incubation.

Subsequently, the labeled sample taken up in hexane was added to the column and eluted with 50 ml. of hexane, 150 ml. of benzene, and 200 ml. of chloroform.

The ratio of malathion to malaoxon eluted off by both techniques was found to be equal.

The properties of the eluted malathion and malaoxon were identical with those reported by Gunther and Blinn (*7*), March *et al.* (*9*), and O'Brien (*11*) as checked by cholinesterase assays and infrared spectra.

Results

Favorable conditions for tissue concentration and incubation times were first established. Figure 3 shows that the extent of hydrolysis varied linearly with homogenate concentration: 2 grams of fresh weight tissue (or equivalents, see above) at 5% final concentration were used throughout subsequent experiments. Figure 4 shows that after 15 minutes of incubation, the hydrolysis has already leveled out at about 50% under the conditions stated. To ensure that despite varying conditions the incubation time was sufficiently long, and as respiratory experiments on slices showed regular oxygen consumption up to 50 minutes, 30 minutes were chosen as the standard incubation time.

Homogenates of various tissues were assayed. Table I shows the malathion hydrolyzing and oxidizing activities. The number and amounts of the water-soluble metabolites for some of them are listed in Table II, parts 1 to 3. All preparations listed formed water and chloroform-soluble metabolites of the same nature and to comparable extents. It was most surprising to find that homogenates exerted substantial oxidative properties. Contrary to what was anticipated from previous reports (*10*), liver was not one of the most hydrolytically and oxidatively active preparations. However, for comparison's sake with other reports, liver preparations were used as standard throughout the study. In confirmation of a previous study (*11*), the microsomes were the most effective fraction of liver in oxidizing malathion.

Effect of LD_{50} of EPN. For investigating the influence of EPN in vivo upon malathion metabolism in vitro, rats were injected with 40 mg. per kg. of EPN intraperitoneally and sacrificed after 5 hours of exposure (when symptoms were at their peak). This high dose was chosen to maximize the biochemical effects. As Table I shows, both hydrolysis and oxidation were inhibited to various degrees in all the preparations assayed. In every case, there was a net reduction in malaoxon production, rather than the anticipated increase.

The influence of various concentrations of EPN in the incubation medium upon malathion hydrolysis by liver homogenates and acetone powders was studied. Figure 5 shows that the relationships between concentration of EPN and inhibition of malathion hydrolysis were proportional, but with different factors for the two types of preparation. For one combination of EPN with malathion in vitro, the number and amounts of water-soluble metabolites are listed in part 4 of Table II. The formation of the monocarboxylic acid was severely inhibited, and phosphatase products, particularly dimethyl phosphate, increased correspondingly.

The influence of EPN in vitro and in vivo upon malathion metabolism in vitro was expected to vary with time of exposure, because of formation of metabolites of EPN whose effectiveness would possibly not be identical with that of EPN itself. Figure 5 shows that in vitro up to 4 minutes of preincubation with EPN before addition of malathion

resulted in increased inhibition of malathion hydrolysis, whereas longer preincubation times were less effective, probably as a result of the formation of less active EPN metabolites.

The results of the corresponding in vivo experiments are shown in Table III as far as the extent of hydrolysis and malaoxon formation are concerned, whereas Table II, part 5, shows the alterations in the nature and amounts of the water-soluble metabolites of malathion. As more pronounced effects with respect to the malaoxon levels were found with slices (instead of homogenates) incubated for 45 minutes, (instead of 30 minutes) in the buffered isotonic solution B (instead of A) these were selected for further study. The most significant metabolic shift caused by EPN was that from the normal of 35% carboxylic acid derivatives to 13% at 5 hours of exposure time to EPN and corresponding alterations in other metabolic pathways, indicating that it was mainly carboxyesterase which was inhibited by EPN. Formation of the monocarboxylic acid derivative, in particular, was severely inhibited.

There is good synchronization of the maximum peak of symptoms, minimal malathion hydrolyzing and oxidizing activity (Table III), and minimum in carboxylic acid derivatives (Table II, part 5). A peculiar stimulation of hydrolysis activity was repeatedly found at 125 hours, perhaps reflecting stimulated synthesis of esterases.

Effect of Low Dose of EPN. The unexpected finding that EPN actually depressed the level of malaoxon in all tissue preparations studied gave rise to the suspicion that such an effect was due to the use of a relatively high dose of EPN. Rats were therefore treated with $^1/_{10}$ of the LD_{50} of EPN—i.e., 4 mg. per kg.—and a comparative experiment was set up, the results of which are presented in Table IV. There was a depression of the extent of hydrolysis, the percentage of chloroform-solubles as malaoxon, and the absolute level of malaoxon. These effects were usually less after 4 mg. per kg. than after 40 mg. per kg. of EPN.

As these results obtained with liver preparations were somewhat unexpected, analogous assays were conducted with homogenates of nonhepatic tissues, under the same conditions as those under

Table I. Effect of LD_{50} of EPN in Vivo upon Subsequent Hydrolysis and Oxidation by Rat Tissue Homogenates Compared with Those of Untreated Rats

Preparation	Hydrolysis: Normal, % of total counts as water-solubles	Hydrolysis: EPN treated, % inhibition of normal, 5 hr. after 40 mg./kg. EPN	Malaoxon: Normal, % of total counts as malaoxon	Malaoxon: EPN treated, % inhibition of normal, 5 hr. after 40 mg./kg.EPN
Liver acetone powder	77	21	4	64
Kidney homogenate	66	85	4	50
Heart homogenate	61	23	...	...
Blood plasma	60	52	9	83
Ileum homogenate	59	0	...	...
Spleen homogenate	58	38	...	...
Lung homogenate	57	86	...	...
Testis homogenate	56	41	10	20
Muscles (diaphragm) homogenate	55	60	...	...
Blood corpuscles	53	62	...	...
Liver homogenate	52	14	7	86
Brain homogenate	41	0	8	83
Liver				
Nuclear fraction	71	...	2	...
Mitrochondrial fraction	69	...	4	...
Supernatant	52	...	1	...
Microsomal fraction	45	...	11	...

Preparations incubated in isotonic solution A for 30 minutes at 37.5° C.

Table II. Malathion Hydrolysis Products Formed by Rat Tissue Preparations in Vitro

Part	Preparation		Phosphoric Acid Derivatives[a] I	II	III	VIII	X	Total[b]	Carboxylic Acid Derivatives[a] V	VI	Total[b]	Unidentified Metabolites[a] IV	VII	IX	Total[b]
1	Liver homogenate		<1	1	0	0	3	4	30	53	83	<1	<1	11	11
	Lung homogenate		<1	<1	1	9	<1	10	33	55	88	0	1	<1	1
	Kidney homogenate		<1	<1	0	9	<1	9	39	50	89	0	1	<1	1
	Blood plasma		<1	<1	0	7	<1	7	50	42	92	0	0	0	0
	Brain homogenate		1	1	3	18	1	24	18	52	70	1	1	4	6
2	Liver slices		2	0	0	15	20	37	19	41	60	1	0	2	3
3	Liver acetone powder		2	3	0	<1	<1	5	32	38	70	0	19	5	24
4	Liver homogenate + 200 γ malathion + 2000 γ EPN		1	1	1	24	1	28	6	54	60	3	1	8	12
5	Liver homogenates from rats after 40 mg./kg. EPN intraperitoneally sacrificed after indicated times	Untreated	<1	1	0	0	3	4	30	53	83	<1	<1	11	11
		0.2 hr.	1	3	2	41	11	58	5	30	35	5	1	1	7
		1	2	10	6	28	3	49	2	13	15	2	7	27	36
		5	4	3	7	29	2	45	1	12	13	1	10	31	42
		25	<1	2	9	30	8	49	3	11	14	4	8	24	36
		125	2	<1	2	2	3	9	4	36	40	1	27	23	51

Results are as fraction of total water-solubles in per cent.

[a] Roman numerals indicate metabolites listed in Figure 5.

[b] Numbers for total amounts within a group are approximations, as metabolite levels below 1% were not determined precisely.

Table III. Dependency of Malathion Hydrolysis and Oxidation by Liver Slices in Vitro upon Time of Exposure to EPN in Vivo

	Normal	0.2 Hr.	1 Hr.	5 Hr.	25 Hr.	125 Hr.
Water-soluble metabolites	65.7	61.0	51.0	31.2	42.8	92.0
Chloroform-soluble metabolites	31.2	36.0	42.3	60.4	47.8	5.2
Retained in protein-Celite	3.1	3.0	6.7	8.4	9.4	2.8
Malathion	17.4	19.8	38.5	60.2	45.4	4.1
Malaoxon	13.8	16.2	3.8	0.3	2.4	1.1

40 mg./kg. EPN injected intraperitoneally. Liver slices were incubated with malathion in the buffered isotonic solution B for 45 minutes. Results are per cent of total counts.

which liver had been tested—45 minutes of incubation in buffered isotonic solution B. Table V gives information on the extent of hydrolysis and malaoxon levels normally and after EPN at $^1/_{10}$ LD_{50}; as with liver, hydrolytic and oxidative activity were both depressed in these tissue preparations. The depression of both the oxidation and hydrolysis was particularly marked in brain.

Discussion

The study has shown that a variety of preparations of hepatic and nonhepatic tissues are capable of malathion hydrolysis and oxidation in vitro. Although there are quantitative rather than qualitative differences between the same type of preparation of various tissues and between different types of preparation of the same origin, there is a surprisingly narrow range of deviation. Ten water-soluble degradation products have been found, of which the monoacid and diacid derivatives are the most abundant in all tissues examined. The fact that so many tissue preparations were capable of breaking down malathion to comparable extents may account for the rapidity of malathion detoxification in vivo (9).

All tissue preparations from rats treated with the LD_{50} of EPN showed significant depression of malathion hydrolyzing and oxidizing activity, the maxima of which coincided with the maximum of intensity of externally visible symptoms. Treatment with EPN at one tenth of the LD_{50} affected the hydrolyzing and oxidizing activities to a significant but smaller extent and did not result in externally visible symptoms. There are significant differences in the extents of hydrolysis, malaoxon levels, and water-soluble metabolites depending merely on the type of preparation or treatment; whereas with liver slices the phosphoric acid derivatives amount to about 35% of the total radioactivity, they constitute only about 5% in homogenates and acetone powders (Table II, parts 1, 2, and 3). The values for the carboxylic acid derivatives range between 60 and 80% and for unidentified metabolites from 3 to about 25%. A more conspicuous difference is represented in Figure 4, which shows that EPN has a very small effect upon metabolism by liver acetone powders as compared to homogenates. These extracts are probably unable to oxidize EPN to a potent inhibitor. Alternatively, acetone extraction may inhibit or remove a system which degrades malathion and which is sensitive to EPN.

The metabolic shift in water-soluble metabolites induced by EPN in vitro and in vivo (from abudant formation of carboxylic acids to more phosphoric acid derivatives, parts 4 and 5, Table II) indicates that carboxyesterase(s) is/are primarily inhibited by EPN.

The malaoxon levels determined are resultants of two oppositely directed processes: the oxidative formation from malathion and the hydrolytic decomposition to water-soluble products. In the case of homogenates and acetone powders, the oxidizing system was probably working under suboptimal conditions, because various cofactors are known to be of importance in such systems (*11*). But with liver slices a clear reduction of malaoxon formation was also noted, and an inhibition by EPN of the oxidation of malathion must be inferred. The explanation for this could be a competition of both malathion and EPN for the same oxidizing system(s).

The tissues of rats treated in vivo with EPN thus show a reduced capacity to hydrolyze and oxidize malathion in vitro. This is true for the LD_{50} or $^1/_{10}$ LD_{50} of EPN, for various exposure times, for the 11 tissues studied and for different types of preparation. There is, therefore, no evidence from these in vitro studies to support the hypothesis that synergism between EPN and malathion observed in vivo is due to a generally increased level of malaoxon resulting from inhibition of malathion and malaoxon hydrolysis. The data are compatible with an alternative hypothesis—namely, that EPN by virtue of its inhibition of hydrolysis and oxidation causes a greater persistence of malathion in the body tissues. The synergistic effect itself, however, must be due to an increased level or persistence of malaoxon at the target site; presumably the persistence of malathion in the various body tissues maintains the malaoxon level at the target for a much greater time, although that level may be somewhat lower than in the absence of EPN. Vandekar and Heath (*15*) were able to produce enhanced symptoms in rats and a much more irreversibly inhibited brain cholinesterase by artificially maintaining persistent body levels of another dimethyl phosphorothionate (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothionate). This procedure may be analogous to the effect of EPN.

Table IV. Effects of Two Dosages of EPN in Vivo at Two Exposure Times upon Malathion Hydrolysis and Oxidation by Liver Slices and Homogenates in Vitro

	Hydrolysis Products		Malaoxon	
	Liver slices	Liver homogenates	Liver slices	Liver homogenates
Normal	65.6 ± 1.3	84.4 ± 3.3	14.1 ± 1.1	13.3 ± 0.9
4 mg./kg. EPN				
1-hr. exposure	90.8 ± 0.1	86.2 ± 2.1	1.5 ± 0.1	1.9 ± 0.02
5-hr. exposure	88.5 ± 2.8	81.4 ± 8.4	2.6 ± 0.3	4.8 ± 1.3
40 mg./kg. EPN				
1-hr. exposure	55.6 ± 7.5	60.4 ± 12.9	6.6 ± 1.4	9.1 ± 1.4
5-hr. exposure	33.2 ± 2.5	29.0 ± 7.1	6.4 ± 2.1	0.82 ± 0.01

All preparations incubated for 45 minutes in buffered isotonic solution B. Results are per cent of total counts, ± standard error. At least 4 animals used for each determination.

Acknowledgment

The authors express their gratitude to H. R. Krueger for synthesizing labeled malathion, and to Claire Asselin for technical assistance. The malathion metabolites for cochromatography were kindly provided by Shirley DuBreuil and R. W. Young, American Cyanamid Co., Stamford, Conn.

Table V. Effect of 1/10 LD_{50} of EPN in Vivo upon Subsequent Hydrolysis and Oxidation of Malathion in Vitro by Rat Tissue Homogenates

	Hydrolysis			Malaoxon		
	% of Total Counts as Water-Solubles			% of Total Counts as Malaoxon		
	Normal	4 Mg./Kg. EPN		Normal	4 Mg./Kg. EPN	
		Exposure Time			Exposure Time	
		1 hr.	5 hr.		1 hr.	5 hr.
Plasma	90.2 ± 5.3	50.6 ± 2.3	85.6 ± 3.1	4.4 ± 0.1	3.9 ± 0.3	3.0 ± 0.2
Kidney homogenate	85.2 ± 1.2	69.0 ± 11.1	76.4 ± 3.7	6.1 ± 0.9	3.2 ± 0.3	5.8 ± 0.3
Brain homogenate	85.0 ± 7.3	14.2 ± 1.2	76.5 ± 6.4	4.7 ± 1.0	0.8 ± 0.0	0.9 ± 0.1
Liver homogenate	84.4 ± 3.3	86.2 ± 2.1	81.4 ± 8.4	6.1 ± 1.3	3.2 ± 0.2	6.5 ± 0.2
Testis homogenate	83.8 ± 2.8	72.5 ± 4.6	86.3 ± 2.3	8.3 ± 1.3	7.9 ± 0.7	7.1 ± 1.1

Tissue homogenates were incubated in buffered isotonic solution B for 45 minutes. Results are per cent of total counts.

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INSECTICIDE METABOLISM

Prechromatographic Purification of Insecticides from Insect Tissue Extracts

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Paper chromatograms of acetone extracts of insecticides and their metabolites from insect tissues often are smeared and unrecognizable. When a crude extract is spotted on filter paper and eluted with acetonitrile, the solvent selectively extracts insecticides, metabolites, and 20 to 30% of the insect lipide material, but not the interfering lipides. The purified extracts give clear chromatograms, on which insecticides and metabolites can be identified. This rapid and simple purification is very useful in studies of insecticide metabolism.

PAPER CHROMATOGRAPHY is used in the study of insecticide metabolism in plants, insects, and higher animals. In the course of studies on the metabolism of DDT, Systox, Thimet, and Sevin in houseflies, mosquitoes, German roaches, and milkweed bugs, it became evident that acetone extracts of insect tissues contain large amounts of lipoid material which causes smearing of paper chromatograms. This results in poorly defined spots, incomplete separation of spots, and shifting of R_f values. Therefore a method had to be devised for removing interfering materials from the extract before running a paper chromatogram. The acetonitrile method described in this paper has been successful with all the insecticides and insects so far studied in this laboratory, but only a few typical examples are presented here.

Procedure

Insects amounting to several hundred milligrams wet weight are homogenized in 5 ml. of acetone. A pinch of anhydrous sodium sulfate is added to remove water present in the homogenate, which is then centrifuged at 2000 r.p.m. for 20 minutes. The supernatant liquid is drawn off and the residue is re-extracted once or twice with 5 ml. of acetone. The combined volume (about 15 ml.) of acetone extract is evaporated in a stream of nitrogen gas to a final volume of 30 to 50 μl. and is transferred by self-filling capillary tubes onto the center of a strip of Whatman No. 4 chromatographic filter paper, 2.5 inches long and 1 inch wide, but at one end leading to a tapering point. Disposable glass capillaries are more convenient than micropipets, give faster transfer, and require no cleaning. One microliter of a 0.5% solution of *N,N'*-dimethyl-*p*-1-naphthylazoaniline (NDN) in benzene is spotted over the extract spot. In tests using carbon-14–DDT, carbon-14–Systox, and Thimet (detected by the sensitive color reaction with 2,6-dibromoquinonechloroimide), in extracts of houseflies and German roaches, it was found that when the NDN was completely extracted from the spot, no insecticide was detectable in the spot, but all the insecticide moved with the dye. The dye is a useful visual indicator of complete extraction. Both dye and insecticides move out of the spot gradually as very diffuse bands, by a slow elution process, probably analogous to a Soxhlet extraction.

Extraction is effected either by an upward-washing or a downward-washing method. The former is more convenient, but requires that the entire sample be spotted on one chromatographic strip. The latter allows the use of several aliquots of the sample on several strips if desired.

Upward Washing. The broad end of the spotted strip is dipped into a 50-ml. beaker containing 5 to 10 ml. of acetonitrile. The ascent of this solvent by capillarity carries insecticide, reference dye (NDN), and some lipoid material to the pointed tip of the strip. The bulk of the lipoid material stays in the original spot. The strip is held upright by clamping between two glass microscope slides (3 × 1 inch). Solvent is transferred from the tip by contact with a paper chromatographic strip to form a spot. This is dried and more solvent transferred to it repeatedly until transfer is complete—as indicated by complete removal of the dye from the tip (*5*).

Downward Washing. The spotted strip is hung in a closed tube in a cup containing acetonitrile, with the tip pointing down and touching a glass rod standing in a receiving cup (Figure 1). After 0.5 hour the solvent is collected from the receiving cup, concentrated to a small volume, and spotted on a paper chromatographic strip.

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INSECTICIDE METABOLISM

Prechromatographic Purification of Insecticides from Insect Tissue Extracts

J. J. MENN[1], M. E. ELDEFRAWI, and H. T. GORDON

Department of Entomology and Parasitology, University of California, Berkeley, Calif.

Paper chromatograms of acetone extracts of insecticides and their metabolites from insect tissues often are smeared and unrecognizable. When a crude extract is spotted on filter paper and eluted with acetonitrile, the solvent selectively extracts insecticides, metabolites, and 20 to 30% of the insect lipide material, but not the interfering lipides. The purified extracts give clear chromatograms, on which insecticides and metabolites can be identified. This rapid and simple purification is very useful in studies of insecticide metabolism.

PAPER CHROMATOGRAPHY is used in the study of insecticide metabolism in plants, insects, and higher animals. In the course of studies on the metabolism of DDT, Systox, Thimet, and Sevin in houseflies, mosquitoes, German roaches, and milkweed bugs, it became evident that acetone extracts of insect tissues contain large amounts of lipoid material which causes smearing of paper chromatograms. This results in poorly defined spots, incomplete separation of spots, and shifting of R_f values. Therefore a method had to be devised for removing interfering materials from the extract before running a paper chromatogram. The acetonitrile method described in this paper has been successful with all the insecticides and insects so far studied in this laboratory, but only a few typical examples are presented here.

Procedure

Insects amounting to several hundred milligrams wet weight are homogenized in 5 ml. of acetone. A pinch of anhydrous sodium sulfate is added to remove water present in the homogenate, which is then centrifuged at 2000 r.p.m. for 20 minutes. The supernatant liquid is drawn off and the residue is re-extracted once or twice with 5 ml. of acetone. The combined volume (about 15 ml.) of acetone extract is evaporated in a stream of nitrogen gas to a final volume of 30 to 50 μl. and is transferred by self-filling capillary tubes onto the center of a strip of Whatman No. 4 chromatographic filter paper, 2.5 inches long and 1 inch wide, but at one end leading to a tapering point. Disposable glass capillaries are more convenient than micropipets, give faster transfer, and require no cleaning. One microliter of a 0.5% solution of *N,N'*-dimethyl-*p*-1-naphthylazoaniline (NDN) in benzene is spotted over the extract spot. In tests using carbon-14–DDT, carbon-14–Systox, and Thimet (detected by the sensitive color reaction with 2,6-dibromoquinonechloroimide), in extracts of houseflies and German roaches, it was found that when the NDN was completely extracted from the spot, no insecticide was detectable in the spot, but all the insecticide moved with the dye. The dye is a useful visual indicator of complete extraction. Both dye and insecticides move out of the spot gradually as very diffuse bands, by a slow elution process, probably analogous to a Soxhlet extraction.

Extraction is effected either by an upward-washing or a downward-washing method. The former is more convenient, but requires that the entire sample be spotted on one chromatographic strip. The latter allows the use of several aliquots of the sample on several strips if desired.

Upward Washing. The broad end of the spotted strip is dipped into a 50-ml. beaker containing 5 to 10 ml. of acetonitrile. The ascent of this solvent by capillarity carries insecticide, reference dye (NDN), and some lipoid material to the pointed tip of the strip. The bulk of the lipoid material stays in the original spot. The strip is held upright by clamping between two glass microscope slides (3 × 1 inch). Solvent is transferred from the tip by contact with a paper chromatographic strip to form a spot. This is dried and more solvent transferred to it repeatedly until transfer is complete—as indicated by complete removal of the dye from the tip (*5*).

Downward Washing. The spotted strip is hung in a closed tube in a cup containing acetonitrile, with the tip pointing down and touching a glass rod standing in a receiving cup (Figure 1). After 0.5 hour the solvent is collected from the receiving cup, concentrated to a small volume, and spotted on a paper chromatographic strip.

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Other solvents (chloroform, cyclohexane, and iso-octane) were also tried. They either fail to extract the insecticides completely, or also extract most of the interfering lipoid material. Acetonitrile has been previously used (*1*, *4*) as the eluting solvent to separate insecticides from plant lipoid extractives by an alumina-wax or alumina-polyethylene column without loss of toxicant.

Results and Discussion

This technique has given apparently quantitative extraction of microgram quantities of DDT, DDE, Systox, Isosystox, Thimet, and Sevin. Precise recovery data cannot be presented, because precise quantitative analysis of quantities of the order of 1 γ is difficult. Comparison of the areas under peaks recorded by a scanner for radioactive spots on chromatograms indicates that at least 90% of the insecticide is extracted by acetonitrile, and no residual insecticide (radioactivity) can be detected. With some very polar insecticide metabolites such as the sulfoxide of the oxygen analog of Systox, or naphthoquinones formed by oxidation of 1-naphthol, recoveries seem to be less than quantitative, especially when quantities of less than 1 γ are involved. However, the chromatography and identification of very polar compounds are difficult, and no definite conclusions are possible.

The success of the method rests on the fact that acetonitrile extracts the insecticides, but not the bulk of the tissue extractives. Table I shows data on the amount of tissue extractives before and after purification of extracts from three insect species. The number of insects is that used in experiments on insecticide metabolism. The fresh weight is a few hundred milligrams, and the extracted fat weighs 2 to 4 mg. Acetonitrile extracts from 20 to 30% of this fatty material together with the insecticides, but the acetonitrile-soluble material does not cause much interference on paper chromatograms.

Figure 2 shows an example of acetonitrile purification taken from work on the metabolism of organic phosphates. The reference curve on the left shows the separation of a mixture of carbon-14–labeled Systox, Isosystox, and two possible metabolites, the sulfoxide of Systox and its oxygen analog sulfoxide. The radiogram at the upper right is of the same mixture added to an extract from five male German roaches before chromatography. The radiogram at the lower right shows the effect of acetonitrile purification of the section of the upper strip between dotted lines. The large smeared spot is now clearly resolved into three peaks. The R_f values of these peaks are lower than those of the reference radiogram at the left. This is an effect of the heavy load (about 0.6 mg.) of acetonitrile-soluble roach lipoid not separated from the insecticides by the purification procedure. This effect can be compensated for by adding a set of four dyes to the original spot. According to the unpublished procedure of Gordon (*2*, *3*), the position of the insecticide spots relative to that of the reference dyes—shown as dotted circles in Figure 2—is constant, even when R_f values show large variations.

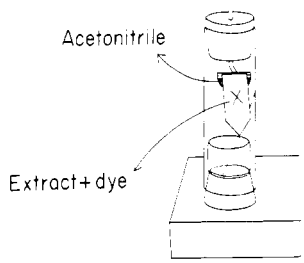

Figure 1. Method of downward-washing purification by acetonitrile extraction on filter paper strip

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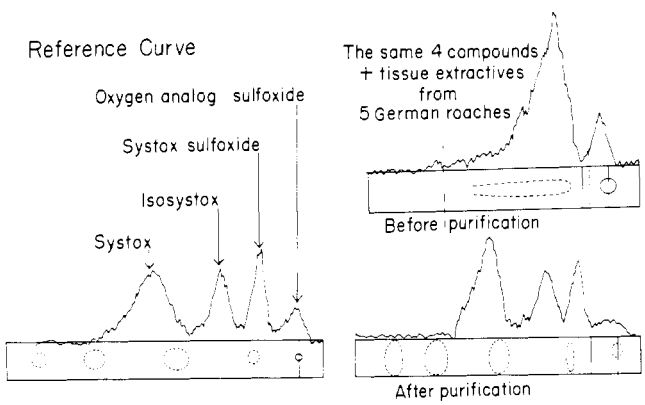

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100 mosquito larvae	2.8	0.6	21
	2.2	0.8	27
25 houseflies	3.8	1.0	26
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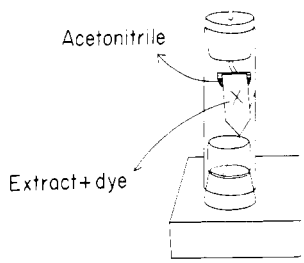

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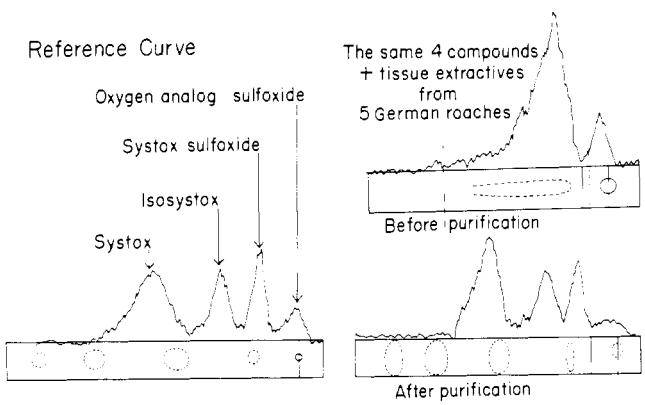

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Effects of Parathion and Malathion Separately and Jointly upon Rat Esterases in Vivo

F. W. SEUME[1], J. E. CASIDA, and R. D. O'BRIEN[2]

Department of Entomology, University of Wisconsin, Madison 6, Wis.

Effects of parathion and malathion in acute and subacute doses upon several rat esterases in vivo were studied. The effects of the two compounds were qualitatively similar in depression and recovery of the esterases. Combinations of malathion and parathion each in subacute dosage were antagonistic with respect to externally visible symptoms. After acute doses the effects were at least additive on the basis of both symptoms and esterase inhibition. Two procedures of tissue homogenization were compared to evaluate the significance of previous reports that the standard homogenization technique of tissues from poisoned animals gave inhibition values which were too high. Inhibition of acetylcholine hydrolysis was found to be consistent with the two techniques, whereas a slight difference occurred with o-nitrophenylacetate under the experimental conditions described.

RECENTLY it has been shown that certain combinations of organophosphate insecticides when administered to mammals are more toxic than anticipated from the "theoretical sum" of their individual effects (*7, 9, 10, 14*). This might result if one compound affected the rate of detoxification of the other, or if the specificity in blocking the physiologically important sites involved varied with the compounds. As the organophosphates display their most potent effects as antiesterase agents, a broad approach of studying esterase inhibition patterns in vivo was used in the initial survey reported here. The short-term processes of esterase depression and recovery in rats treated with organophosphates have been examined. Previous investigations have dealt mainly with long-term processes, where esterase resynthesis as well as recovery is involved (*8*).

In mammals the toxic effects of most organophosphates are primarily attributable to inhibition of cholinesterases and subsequent processes. However, studies with insects (*12*) have shown that other esterases may be severely inhibited by organophosphates, and it has been suggested that it may be the inhibition of these other esterases which causes death (*4*). Comparable studies with mammals have not hitherto been carried out in vivo. This paper deals with effects upon cholinesterases (ChE) as assayed with acetylcholine (ACh), and other esterases hydrolyzing triacetin (TA), *o*-nitrophenyl acetate (NPA), and diethyl maleate (DEM). Each substrate may be hydrolyzed by several esterases in a particular tissue homogenate (*1, 2*), but it was feasible to assay only total tissue preparations, and this limitation must be considered in interpreting the results.

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[2] Present address, Pesticide Research Laboratories, University Sub Post Office, London, Ontario, Canada.

Materials and Methods

Albino male rats (Holtzman Farms, Madison, Wis.), 180 to 200 grams, were lightly anesthetized with ether and injected intraperitoneally with the organophosphates. The solutions for injection were prepared by dissolving the insecticides in minimal acetone and making up to volume with corn oil (U.S.P.), so that 1 µl. of solution per gram of rat was administered. All combinations were injected simultaneously from the same syringe.

The experimental rats were decapitated and the blood was collected in 0.1 ml. of 2% sodium heparin (2000 U.S.P. units per ml.) per ml. of blood. The blood was centrifuged for 20 minutes at 10,800 × G and at 2° C. After the plasma had been removed, the corpuscles were washed once with buffer (0.15*M* sodium chloride, 0.04*M* magnesium chloride, 0.025*M* sodium bicarbonate) and centrifuged for 10 minutes at 6775 × G and at 2° C. The other tissues were chilled immediately after removing them from the body, weighed, and homogenized in glass homogenizer tubes. Two types of homogenization and dilution media were used: For the "unprotective" homogenization technique bicarbonate buffer and for the "protective" technique the appropriate substrate solution in its final concentration were employed.

ACh was dissolved directly in bicarbonate buffer to yield a 1*M* stock solution. NPA and DEM were dissolved in 95% ethyl alcohol to make 1*M* stock solutions. The 0.1*M* solutions were made by mixing 1 ml. of the stock solution with 0.3 ml. of Triton X-100 (Rohm and Haas Co.) and making up to 10 ml. with bicarbonate buffer. The 1*M* stock solutions of TA were made in the same way, the 0.1*M* solutions like those of NPA and DEM, but without emulsifier. Dilutions were made fresh for every experiment. The substrates yielded true solutions or finely dispersed, stable emulsions with the buffer tissue suspensions; the formulation media alone gave no carbon dioxide evolution with tissue homogenates.

The esterase assays were carried out by the Warburg manometric technique at 37.5° C. The tissue preparation (0.2 ml.) was in the side arm; the substrate in buffer (1.8 ml.) was in the main compartment. During preparation the vessels were kept in an ice-water mixture; then the flasks were attached to manometers, gassed (5% carbon dioxide in nitrogen) and equilibrated, and the tissue preparation was tipped in. Starting 5 minutes later—"zero time"—seven readings were taken at 5-minute intervals.

Parathion (99% *O,O*-diethyl *O-p*-nitrophenyl phosphorothionate) and malathion [96% *O,O*-dimethyl *S*(1,2-bis ethoxycarbonyl ethyl) phosphorodithioate] were donated by the American Cyanamid Co.

The results reported are average values from three or more determinations, the individual replicates varying only slightly from the mean during the entire experimental period.

Table I. Esterase Activity in Various Tissue Preparations from Untreated Rats Homogenized by the Unprotected Technique

Tissue	Tissue Wet Weight/ Flask, Mg.	Substrate	Substrate Concn., M	CO_2/30 Min., μl.	Enzyme Activity, μl. CO_2/Mg. Tissue
Brain	50	ACh	0.05	132	2.64
	50	NPA	0.03	343	6.86
	50	TA	0.02	172	3.44
Corpuscles	200	ACh	0.05	58	0.29
	50	NPA	0.03	242	4.85
	50	TA	0.02	175	3.5
Kidney	1	NPA	0.01	215	215
	2	TA	0.02	61	30
	50	DEM	0.02	193	3.85
Liver	1	NPA	0.01	215	215
	2	TA	0.02	99	49
	10	DEM	0.02	83	8.3
Plasma	200	ACh	0.05	51	0.25
	50	NPA	0.03	400	8
	200	TA	0.02	174	0.87
	200	DEM	0.02	279	1.39

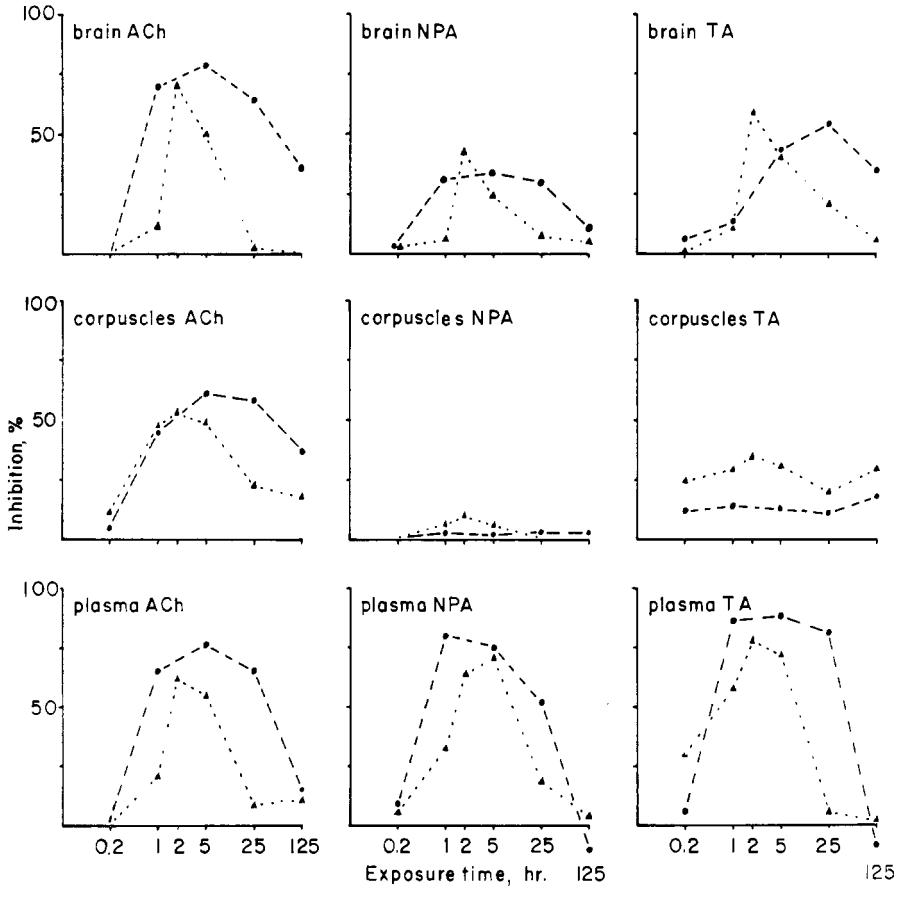

Figure 1. Effect of parathion and malathion, given singly at ALD/2 to rats, upon esterases

Table II. Per Cent Inhibition of Normal Hydrolytic Activity at Maximum and after 125 Hours' Exposure

(Unprotective homogenization)

Tissue	Parathion ALD/2	Parathion ALD/4	Malathion ALD/2	Malathion ALD/4
Maximal Inhibition				
Brain				
ACh	80	60	70	37
NPA	33	28	43	18
TA	55	59	59	18
Corpuscles				
ACh	62	64	53	56
Plasma				
ACh	76	79	61	33
NPA	80	68	71	48
TA	88	85	79	67
Inhibition 125 Hours after Treatment				
Brain				
ACh	35	34	0	7
NPA	11	14	5	3
TA	35	32	5	0
Corpuscles				
ACh	36	25	18	14
Plasma				
ACh	16	48	11	11
NPA	−8	0	5	0
TA	−10	6	1	15

Results and Discussion

Without Inhibitors. The carbon dioxide evolution was proportional to homogenate concentration under the experimental conditions (Table I). Whereas usually the activity-time relationship was linear—i.e., the carbon dioxide liberation per 5-minute reading interval was constant from the first to the sixth interval—this was not the case with NPA: In all reaction systems containing NPA, the carbon dioxide evolution rate dropped such that 25% of the total gas per 30 minutes was evolved in the first 5-minute reading interval and only about 10% in the sixth interval. This effect was observed in systems prepared by the method of unprotective and protective homogenization, from treated and untreated rats. It was not due to the formulation constituents (ethyl alcohol and Triton X-100) nor to inadequate substrate. Possible explanations might be that some kind of product poisoning occurred or that the pH decreased abnormally during assay (*6*).

Single Inhibitors. The acute lethal dose—i.e., the minimum dose which invariably was lethal to all of the 10 rats used in the ultimate determination—was established. The depression recovery curves of a number of esterases after parathion and malathion treatment were established using half of this acute lethal dose (ALD/2) which produced severe symptoms, but allowed the animals to survive. The ALD/2 for parathion was 5 mg. per kg., for malathion 500 mg. per kg. The results (Figure 1) show that the degree of tissue sensitivity to treatment with either compound was of the same order; however, considerable quantitative differences occurred.

The curves for depression and recovery of esterases after treatment with parathion and malathion are resultants of: the distribution pattern of each toxicant—i.e., their activation products—in the body; reaction with the esterases; degradation of the toxicants; and reversal of the enzyme blockage. Synthesis of new esterases is probably negligible in the time periods involved in this study (*8*). With malathion,

metabolism and excretion occur very rapidly and at least seven metabolites are formed in the mouse and hen (*13*). Degradation of malaoxon [*O,O*-dimethyl-*S*-(1,2-bis ethoxy carbonyl) phosphorothiolate] by mammals takes place not only in the liver and kidney, but in various other organs (*14, 15*). The degradation of malathion in the rat has been attributed to the hydrolytic activity of an enzyme system in liver which has been provisionally termed "malathionase" (*7*). The rate of inhibition of different esterases in vitro is known to vary greatly with the organophosphate used as an inhibitor (*16*).

Bergmann (*5*) concluded that many esterases have esteratic sites very similar to those of cholinesterase; as these are the sites attacked by organophosphates and as the curves in Figure 1 are qualitatively comparable, it appears likely that similar recovery processes are involved.

Malathion and parathion were next administered individually to rats at their ALD/4 dosages. Neither induced any visible symptoms, but both caused esterase inhibition (Table II). The esterase inhibition with parathion was similar at the ALD/2 and the ALD/4 dosages, whereas with malathion the lower dosage gave generally less esterase inhibition. The times for maximal esterase inhibition were approximately the same for the ALD/2 and ALD/4 dosages.

Combined Inhibitors. Administration of malathion and parathion simultaneously at their individual ALD/4 dosages produced no symptoms nor any significant inhibition of esterase activity. This effect is interpreted as antagonism, because at this level the compounds individually affected the esterases (see above and Table II).

Application of both compounds each at ALD/3 resulted in symptoms which were more severe than from either compound at the ALD/2, and led to death within an hour after injection (Table III).

If the responses were simply additive, one would expect that the effects of administering the compounds jointly each at ALD/3.5 should be somewhat greater than those produced by either at ALD/2. Table III shows that the effects were at least additive based upon the severity and persistence of symptoms. Therefore, this combination was selected for studying effects upon esterases (Figure 2). A rough comparison (an exact comparison cannot be made because of the differing dose levels) of Figures 1 and 2 shows that the effects of the combined dosages are intermediate between either compound alone at ALD/2. Once more, there is a striking discrepancy between the effects as judged by visible symptoms and by esterase inhibition, indicating that the degree of esterase inhibition does not necessarily parallel the severity of symptoms.

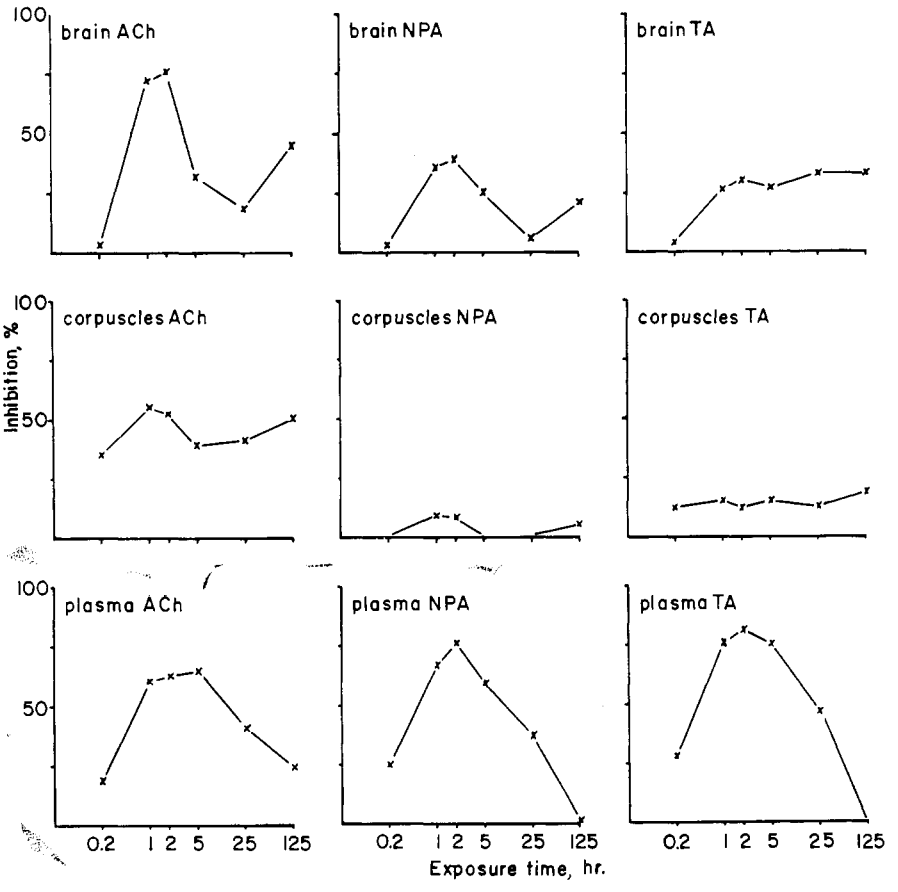

Figure 2. Effect of simultaneous administration of parathion and malathion each at ALD/3.5 upon rat esterases

Table III. Symptoms Following Treatment with Parathion and Malathion[a]

(Beginning and end of intoxication stages in hours after injection intraperitoneally, 4 rats per dosage)

Conditions	Malathion, ALD/2		Parathion, ALD/2		Malathion + Parathion, Each ALD/3.5		Malathion + Parathion, Each ALD/3	
	Start	End	Start	End	Start	End	Start	End
Hypersalivation	0.4	1.8	0.5	2.8	0.2	23	0.1	0.9
Facial muscular twitches	1	2	0.8	3	0.4	19	0.2	0.9
Muscular twitches of whole body	1.2	3.8	1	7.5	0.5	20	0.2	0.9
Apathy (including diarrhea, rough hair)	1.5	4	2	7	0.5	54	0.2	0.9
Death	...		...		...		0.9	

[a] No symptoms with malathion plus parathion each at ALD/4.

Artifacts in Preparing Tissue Homogenates. In poisoned animals certain amounts of organophosphates (and/or their active antiesterase metabolites) may be present at the time of sacrificing, which come into contact with the enzyme only during homogenization and subsequent stages of preparation (*3, 4, 11*). In order to prevent such an artifact, van Asperen (*4*) homogenized the tissues not in buffer (as it is usually done), but in the appropriate substrate solution, thus protecting the enzyme from further reaction with excess of inhibitor. This technique is termed the protective method, whereas the method of homogenization in buffer is called the unprotective method.

Tissues from untreated rats prepared by protective or unprotective homogenization yielded the esterase activities shown in Figure 3. The activity values differed considerably with the two methods. In the case of ACh, it is well known that inhibition occurs at high substrate concentrations. The protective technique was therefore examined in more detail. Experiments were carried out with additional substrate added 5 minutes before zero time or to the homogenization-reaction medium. The design and results of this experiment are given in Table IV. The values obtained with different concentrations of ACh and brain gave the characteristic concentration-activity relationship with

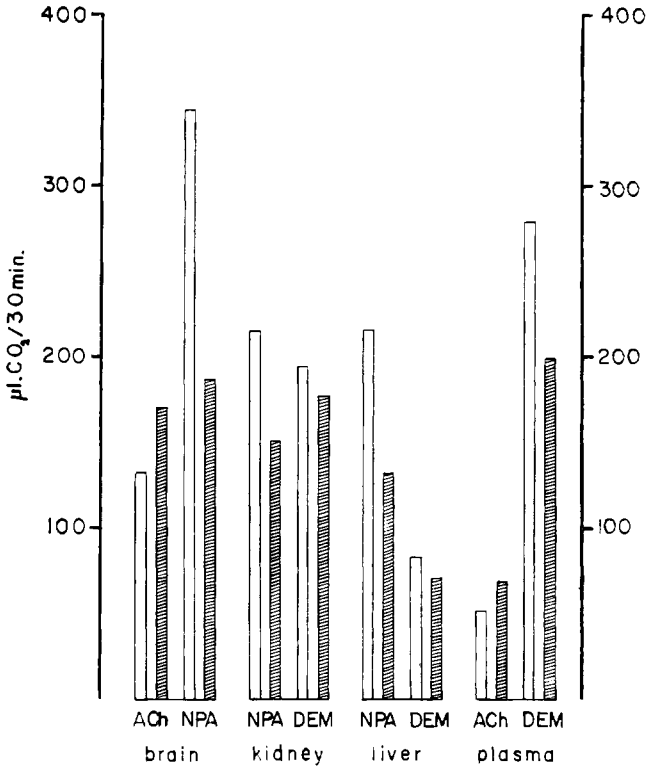

Figure 3. Comparison of unprotective and protective homogenization techniques

Left. Unprotective homogenization
Right. Protective homogenization
Tissues from untreated rats (protection of plasma consisted in diluting it to the desired concentration in substrate solution)

Table IV. Modified Homogenization Methods

(ACh; brain from untreated rats)

Conditions	Molarity	μl. CO_2/ 30 Min.
1. Protective homogenization in 0.05*M* substrate		
a. No additional substrate		182
b. Extra substrate added 5 min. before zero time to total	0.06	185
	0.08	173
	0.1	154
2. Protective homogenization in indicated substrate concentration, no additional substrate	0.05	203
	0.08	168
	0.1	127
3. Unprotective homogenization in buffer, substrate concentration for reaction	0.05	139

Table V. Unprotective and Protective Homogenization with Treated Rats

(5 mg./kg. parathion intraperitoneally)

	Time of Exposure to Parathion, Hr.	Protective		Unprotective	
		μl. CO_2/30 min.	Inhibition, %	μl. CO_2/30 min.	Inhibition, %
Brain NPA	0	187	0	343	0
	0.2	170	9	332	3
	1	150	20	201	41
	5	120	36	190	45
	25	152	19	240	30
Brain ACh	0	169	0	132	0
	0.2	172	−2	134	−1
	1	50	70	41	69
	5	37	78	29	78
	25	59	65	48	64

a substrate optimum for both procedures of homogenization at 0.05*M*. In addition to this proof that the substrate concentration in the protective procedure was sufficient and optimal, the rate of carbon dioxide evolution was constant for the 30-minute reaction period (with the exception of NPA as previously mentioned).

After the validity of the methods had been established comparatively with tissue homogenates from untreated rats, experiments were run with tissues from rats treated intraperitoneally with 5 mg. per kg. of parathion. Two examples of the generally analogous results are given in Table V. The differences of the absolute activity values were noted above. It is clear that in the case of ACh as substrate, the method of homogenization—whether protective or unprotective—does not have any influence upon the inhibition values, if these are based upon the definition "per cent inhibition of normal activity" (columns 4 and 6, Table V). However, in the case of NPA as substrate, some protection was observed, the maximal difference between both methods being at 1 hour of exposure to parathion. The patterns of NPA inhibition were, however, substantially the same with both methods, in spite of the greatly reduced absolute activity which resulted from the protective technique in tissue homogenates containing no free inhibitor.

The differences between the activity values depending merely upon the method of preparation of tissues from unpoisoned animals cannot be explained from the data presented.

The difference between van Asperen's (*4*) and these experimental results might be explained by the fact that he has worked with an insecticide [DDVP (*O,O*-dimethyl *O*-2,2-dichlorovinyl phosphate)] which is a potent inhibitor per se whereas the thiono compound used in this study (parathion) needs conversion into its oxygen analog in order to become a potent ChE inhibitor. As this conversion requires time, thus giving an opportunity for metabolic detoxification, free esterase inhibitor may not have accumulated in amounts sufficient to be affected by competition with the substrate added in the protective method.

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HERBICIDE TOXICOLOGY

Toxicology of Dalapon Sodium (2,2-Dichloropropionic Acid, Sodium Salt)

O. E. PAYNTER and T. W. TUSING

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Dalapon sodium, a plant growth regulator, is low in acute oral toxicity to laboratory animals, and cattle tolerate large doses without serious effect. Dogs receiving 100 mg. per kg. per day for 1 year and rats given 50 mg. per kg. per day for 2 years exhibited only slight, statistically significant increases in average kidney weights. Rats receiving 15 mg. per kg. per day for 2 years and dogs at 50 mg. per kg. per day for 1 year showed no significant differences from controls. There were small amounts of dalapon found in rat and dog tissues, and in the milk of lactating female rats as determined by chemical analyses. Reproduction and lactation were not affected in rats receiving diets equivalent to 150 mg. per kg. per day. Dalapon sodium presents no unusual handling problems in the field. These data provide a basis for judging the safety of the various residues likely to occur when this herbicide is used as directed on edible produce, or on soil in which food crops are grown.

DALAPON SODIUM is herbicidally active on grasses and cattails at relatively low rates. Controlled laboratory investigation has establishd that this herbicide is readily absorbed and translocated by active grass foliage. It is also absorbed by roots following soil application (*1*). As dalapon sodium may be used effectively for selective control of grasses in certain crops, the following studies were designed to obtain information from experimental animals upon which to base conclusions regarding the safety of any residues occurring in or on edible portions of such crops. Data were desired also for the purpose of making recommendations for safe handling of the material.

Material

Dalapon sodium is a white to tan-white, free-flowing, water-soluble powder with a melting point range of 193° to 197° C. The technical grade material was used in the acute oral studies. By chemical and infrared analyses of the original acid, and by hydrolysis assay techniques on the sodium salt, it was determined that this preparation had the following approximate composition:

83 to 85% 2,2-dichloropropionic acid, sodium salt
6% related chloropropionic acids, sodium salts
1% pyruvic acid, sodium salt
8% sodium chloride
2% undetermined

The sample used in both the subacute and chronic feeding studies was obtained from a large batch produced in May 1953. Twelve 1-pound bottles were sealed and stored under refrigeration, and a particular bottle was not opened until needed. Each time, a 25-gram sample was analyzed. Special attention was given to moisture content as an index of hygroscopy and to sodium pyruvate as an index of hydrolysis. These analytical results indicated that the 12 pounds of material had the following approximate composition and did not change significantly during the storage period:

65% 2,2-dichloropropionic acid, sodium salt
16% related chloropropionic acids, sodium salt
2% pyruvic acid, sodium salt
5% sodium chloride
5% water
7% undetermined

Acute Toxicity

Single oral doses of dalapon sodium dissolved in water were administered by intubation to five animal species in the following concentrations: rats: 10 and 50%; mice: 16%; guinea pigs: 10 and 50%; rabbits: 20%; New Hampshire red chickens: 30%. A total of 130 rats, 15 mice, 18 guinea pigs, 16 rabbits, and 10 chicks were divided into groups and received single doses ranging from 1.0 to 15.8 grams per kg. of body weight. All surviving animals were observed until it was certain that they had fully recovered—usually about 2 weeks. The acute oral LD_{50} with its 19/20 confidence limits was calculated for the five different animal species according to the "Moving Average" method of Thompson (*5*). The results are presented in Table I. Animals that died succumbed within a period of 2 hours to 1 day following administration. No gross pathological changes were found and the principal autopsy findings were a large quantity

Table I. Acute Oral LD_{50} Values for Dalapon Sodium

Species	Sex	LD_{50} 19/20 Confidence Limits, G./Kg.
Rat	Male	9.33 (8.46 to 10.3)
	Female	7.57 (6.88 to 8.35)
Mouse	Female	>4.6
Guinea pig	Female	3.86 (2.76 to 5.43)
Rabbit	Female	3.86 (2.30 to 6.50)
Chick	Mixed	5.66

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Chick	Mixed	5.66

of fluid and gas in the gastrointestinal tract. Autopsies of the surviving animals revealed no gross pathology which could be attributed to dalapon sodium.

Dermal and Eye Irritation Studies

Rabbits. Dalapon sodium and its solutions were bandaged repeatedly to the shaven abdomen—10 continuous applications in 14 days. The undiluted material produced moderate hyperemia and slight superficial necrosis after three or four applications. Healing was complete, without scarring, 1 week following the final application. On the abraded skin, prolonged contact resulted in necrosis. A 10% solution in water (equivalent to 25 pounds in 30 gallons) was only very slightly irritating to the intact or abraded skin, and a 1% solution in water (equivalent to 2.5 pounds in 30 gallons) caused no irritation whatsoever even after prolonged and repeated contact with either intact or abraded skin.

Observations of appearance, behavior, and body weight records made upon the rabbits during the various skin irritation tests revealed no evidence that dalapon sodium penetrated the intact skin in acutely or subacutely toxic amounts.

The dry, powdered material was placed in the eye of a rabbit. The immediate response was one of moderate pain and slight irritation of the conjunctival membranes. An hour later moderately severe conjunctivitis and corneal injury were observed. Healing was complete after several days with no indication of permanent damage. Washing the rabbit's eye immediately with a copious amount of water greatly alleviated the amount of irritation produced. A 10% aqueous solution caused only very slight pain and conjunctivitis when placed in the rabbit eye, whereas a 1% solution in water produced no reaction whatsoever.

Subacute Toxicity

Cattle. A heifer weighing 252 kg. and a bull suckling calf, approximately 3 weeks old and weighing 59.1 kg., were maintained under normal animal husbandry conditions. Each animal was given 1.0 gram per kg. of dalapon sodium dissolved in water by intubation on each of 10 successive days.

During the latter half and at the end of the experimental period, the heifer exhibited some abnormality, as evidenced by general lassitude, diarrhea, roughness of coat, loss of appetite, slight loss of weight, slowed pulse rate, mild cyanosis of the mucous membranes, and some discharge from the eyes. Four days after the last dose, the heifer appeared to be in good condition. The symptoms noted above were all absent except that the slowed pulse rate persisted and the discharge from the eyes was still excessive. At this time, the animal was sacrificed and autopsied. During the course of the study, the bull calf failed to exhibit any evidence of adverse effects and actually gained 6.8 kg. Twenty-four hours after the last dose, the calf was sacrificed and autopsied.

The organs and viscera of both animals appeared normal upon gross examination. Microscopic examination of sections from the rumen, reticulum, omasum, abomasum, lymph nodes, adrenals, duodenum, thyroid, heart, kidneys, liver, spleen, pancreas, lung, bladder, and gall bladder revealed these tissues to be within normal limits. The only possible exceptions were the kidneys of the bull calf, which showed slight cloudy swelling of the proximal convoluted tubules and hypertrophy or swelling of the glomerular cells with decreased glomerular spaces.

It is notable in the case of the heifer that recovery from the general nonspecific symptoms observed was prompt upon cessation of the dosing and that no evidence of organic injury was apparent at autopsy. The calf seemed to be particularly resistant to effects of the material.

Dogs. One male and one female mongrel dog were immunized, pre-experimentally, against rabies, given distemper serum, and were allowed to adjust to laboratory conditions and diet. Each animal was individually housed and observed daily for signs of systemic toxicity. Body weight and compound consumption were recorded at weekly intervals. Food was offered once a day and water was available at all times. Each animal was given dalapon sodium orally, by capsule, 5 days a week during an 80-day period. The dogs were weighed once a week and the doses for the week were adjusted according to their weight at that time. During the first 2 weeks, each dog received 50 mg. per kg. per day. Beginning with the third week, the dose for each dog was adjusted upward at weekly intervals during the remainder of the study until a maximum dosage of 1000 mg. per kg. per day was reached.

During the tenth week, the total daily dose of dalapon sodium was administered in two divided doses in an attempt to reduce the occurrence of vomiting; however, vomiting occurred in both dogs two or three times during the eleventh week while receiving the 1000 mg. per kg. per day dosage level. To prevent marked disturbances in nutrition and secondary metabolic changes associated with frequent vomiting, the study was terminated.

Complete blood counts, sedimentation rates, cell volumes, blood urea nitrogen determinations, bromosulphalein liver function tests, and urinalyses were conducted on each dog initially and at termination. At 81 days the dogs were sacrificed by exsanguination under Pentothal anesthesia and autopsied. Liver, kidney, spleen, and testes were weighed.

The total amount of dalapon sodium administered to the male and female dogs was 199.58 and 161.0 grams, respectively. Both dogs maintained good appetites and exhibited good nutrition during the study. Except for vomiting by both dogs, there were no gross signs of systemic toxicity associated with the oral administration of dalapon sodium in doses ranging from 50 to 1000 mg. per kg. per day.

The results of hematological and biochemical studies and urinalyses obtained prior to sacrifice revealed no specific changes or alterations in values as compared with values from similar studies obtained initially from both dogs.

On autopsy, gross inspection of the viscera and body cavities revealed no specific findings in either dog which could be associated with the oral administration of dalapon sodium. Organ–body weight ratios of liver, kidneys, spleen, or testes for both dogs were within normal limits.

Rats. Groups of 10 male and 10 female rats (Wistar-Dow strain) each were maintained for 97 days on diets containing 0.0 (control), 0.0115, 0.0346, 0.115, 0.346, or 1.15% of dalapon sodium. In the male rats, there was no effect, whatsoever, at the 0.115% level—equivalent to an average of about 115 mg. per kg. per day for young growing animals—or below. In the female rats, there were slight, statistically significant increases in average kidney weights at the 0.115 and 0.0346% dietary levels, but no other differences from the control values were indicated by any criterion, including histopathological examination. At the higher concentrations, 0.346 and 1.15%, there was no evidence of adverse effect as judged by mortality and food consumption records, hematological examination, or average weights and microscopic examination of the lungs, heart, and spleen. Growth retardation, increases in average weight of liver and kidneys, and slight histopathological changes in the liver and kidneys were the principal findings of significance.

One-Year Oral Administration

Dogs. Twelve mongrel dogs were selected at random in December, 1953, and immunized, pre-experimentally, against rabies, given distemper serum and a vermifuge, and allowed to adapt to laboratory conditions and diet. They were then divided into four groups of two males and one female, each group receiving either 0, 15, 50, or 100 mg. per kg. per day of dalapon sodium. The compound was administered by capsule, 5 days a week, for 52 weeks.

The dogs were housed in individual cages and observed closely for signs of

systemic toxicity. Body weights were recorded weekly and compound consumption was recorded daily. Food was offered once a day, with water available at all times.

Complete blood counts, hematocrits, sedimentation rates, blood urea nitrogen determinations, bromosulphalein liver function tests, and urinalyses were conducted on each dog initially and at the end of the thirteenth, twenty-sixth, and fifty-second week.

Following ether anesthesia, each dog was sacrificed by exsanguination and gross autopsies were performed. Weights of the lungs, heart, liver, spleen, and kidneys were determined for each animal. The weights of the testes were also obtained. Sections from thyroid, lung, heart, liver, kidney, adrenal, pancreas, spleen, lymph nodes, bone marrow, urinary bladder, and gonads from each animal were examined microscopically.

The test dogs exhibited normal behavior, gained weight, and exhibited no untoward reactions attributable to the test material. The results of hematological and biochemical studies and urinalyses indicated no significant alterations. Average organ-body weight ratios (Table II) were normal when compared to the controls except for a statistically significant increase in average kidney weight in those dogs that received the 100 mg. per kg. per day dosages of the chemical. Histopathological examination of the tissues from the control dogs and from the dogs that had received dalapon sodium revealed no significant difference, even in the slightly heavy kidneys of the dogs on the high dosage level.

Table II. Average Weights of Various Organs, as Per Cent of Terminal Body Weights with Standard Deviations, Taken from Dogs Which Received Dalapon Sodium for 1 Year

Level, Mg./Kg./Day	Weight, Kg.	Lungs, %	Heart, %	Liver, %	Spleen, %	Kidneys, %	Testes, %
Control	9.33 ±0.82	0.67 ±0.12	0.64 ±0.05	2.51 ±0.23	0.16 ±0.02	0.42 ±0.03	0.17 ±0.03
15	9.87 ±1.85	0.70 ±0.18	0.77 ±0.07	2.91 ±0.24	0.20 ±0.03	0.43 ±0.02	0.16 ±0.00
50	8.43 ±1.79	0.62 ±0.06	0.88 ±0.23	2.80 ±0.09	0.20 ±0.07	0.46 ±0.01	0.18 ±0.00
100	9.60 ±1.21	0.73 ±0.15	0.85 ±0.22	2.83 ±0.15	0.23 ±0.05	0.56 ±0.03	0.21 ±0.00

Table III. Average Weights of Various Organs, as Per Cent of Terminal Body Weights with Standard Deviations, Taken from Rats Maintained for 2 Years on Diets Containing Dalapon Sodium

Diet Level, %	Sex	Number of Rats	Body Weight, G.	Liver, %	Kidneys, %	Testes, %
Control	M	15	488 ±60	2.88 ±0.37	0.64 ±0.06	0.73 ±0.10
0.01	M	16	491 ±65	2.79 ±0.38	0.66 ±0.10	0.64 ±0.15
0.03	M	15	463 ±94	2.59 ±0.33	0.68 ±0.14	0.60 ±0.15
0.10	M	15	473 ±58	2.91 ±0.25	0.71[a] ±0.20	0.69 ±0.10
Control	F	12	336 ±39	3.54 ±0.46	0.68 ±0.09	... ...
0.01	F	15	326 ±66	3.35 ±0.43	0.80 ±0.27	... ...
0.03	F	13	353 ±64	3.51 ±0.10	0.70 ±0.14	... ...

[a] Statistically significant difference P = <0.05

Two-Year Feeding

Rats. Albino rats of the Carworth Farm strain were randomized in June 1953, and housed individually in wire mesh cages. Water and the appropriate diet, consisting of Purina Lab Chow, were available at all times. Weekly records were kept of the body weights, food consumption, general appearance, condition, and behavior of each rat.

The preliminary 97-day studies indicated that dietary levels of about 0.01%, 0.03%, and 0.1% were tolerated and that such levels would be suitable for long-term feeding studies. Mature rats of the Carworth strain receiving these dietary levels would be consuming approximately 5, 15, and 50 mg. per kg. per day, respectively. On this basis, the design for the first 13 weeks consisted of feeding weekly adjusted diets to approximate the above levels. Beginning with the fourteenth experimental week and continuing through the 104th week, dalapon sodium was added to the diet on a constant percentage basis as follows:

	Number of Rats		Dietary Level	
Group	Male	Female	%	P.p.m.
1	24	20	Control	0
2	24	20	0.01	100
3	24	20	0.03	300
4	24	..	0.1	1000

Complete blood counts and hemoglobin determinations were conducted on animals from each group at the following intervals: initially, five males and five females; at 13 weeks, three males and three females; at 26 weeks, two males and two females; at 52 weeks, three males and three females; and at 104 weeks, three males and three females. At the end of 26 weeks, two male and two female rats from each group were sacrificed, and after 52 weeks, two male rats from each group were sacrificed for histological study of representative tissue. At the end of 104 weeks, all surviving animals were sacrificed and gross autopsies were performed. All rats were sacrificed by exsanguination. At each interval when rats were sacrificed, organ weights of the liver, kidneys, and testes from the males, and the liver and kidneys from the females were determined. The histological findings in the tissues from representative male and female animals from each group were evaluated at the end of 104 weeks.

Growth, food consumption, and survival of the male and female rats at each dietary level were comparable to that of the control rats of corresponding sex during the 2-year period. The results of hematological studies conducted on the rats at intervals during the 2 years revealed no significant alterations in hemoglobin, cell counts, or peripheral blood smears. Gross examination of test and control rats sacrificed at 26, 52, and 104 weeks revealed no characteristic findings that could be attributed to the dietary feeding of dalapon sodium. The average kidney weight of the male test rats receiving the 0.1%—50 mg. per kg.—level showed a statistically significant increase when compared to the male control rats at the end of 2 years (Table III). The average liver and testes weights of these rats were not significantly different from the controls. Microscopic examination of the tissues, including the slightly heavier kidneys, revealed no evidence of adverse effect. In the groups of male and female rats maintained for 2 years on diets containing

Table IV. Summary of Results of Reproduction Study on Three Generations (2 Litters Each) of Rats Receiving Diets Containing Dalapon Sodium

Diet, %	Group	Fertility Index[a]	Gestation Index[b]	Viability Index[c]	Lactation Index[d]
Control	F_0	100	100	96	95
0.03	F_0	86	100	95	86
0.1	F_0	100	100	95	96
0.3	F_0	100	100	93	94
Control	F_{1b}	100	100	88	92
0.03	F_{1b}	100	100	86	100
0.1	F_{1b}	96	100	86	98
0.3	F_{1b}	100	100	89	99
Control	F_{2b}	100	96	87	82
0.03	F_{2b}	95	95	69	95
0.1	F_{2b}	100	100	81	99
0.3	F_{2b}	100	100	85	99

[a] Percentage of females achieving two pregnancies.
[b] Percentage of pregnancies yielding live litters.
[c] Percentage of rats born that survived for 5 days.
[d] Percentage of rats alive at 5 days that survived the 21 day lactation period.

0.03 or 0.01%, there were no differences whatsoever from control rats when judged by any criterion.

Reproduction Studies—Rats

Procedure. Groups of young albino rats (four male and 12 females per group) were started on diets containing 0.0 (control), 0.03, 0.1, or 0.3%. These animals were selected when approximately 90 days of age and each sex maintained in separate cages until the age of 110 days was reached. At this time, the males and females from each dietary group were placed together in large breeding cages. This comprised the parent generation (F_0). Parent rats and all descendant animals were maintained on the diets containing dalapon sodium throughout the entire experimental period.

When pregnancies were determined visually, or by palpation, the females were transferred to individual cages.

Records were kept of the number of young cast, including number born alive, born dead, or killed by mother. After 5 days, the number of pups per litter was reduced to eight if necessary, and the lactation period continued until 21 days. All of the pups were weighed at weaning. After 10 days, the females were returned to the breeding cage.

All pups from the F_{1a} litter (F_0 parents) were discarded at weaning. However, a sufficient number of rats was saved from the F_{1b} litters (F_0 parents) and maintained on their respective experimental diets for selection of four males and 12 females to be mated at 110 days to obtain generations F_{2a} and F_{2b}. F_{2a} animals were discarded when weaned. When the F_{2b} animals reached 110 days of age, four males and 12 females were mated to produce the third generation progeny (F_{3a} and F_{3b}) which then were discarded after weaning.

Results. Records for this experiment were kept in such a manner that indices of reproductive and lactating efficiency similar to those outlined by Oser and Oser (*2*) could be utilized. These included measures of fertility, gestation, viability, and lactation. In all, 2476 rats in 261 litters were involved in this experiment over a period of approximately 18 months. The results are presented in Table IV.

These show that continuous administration of dalapon sodium in the diet of rats through three generations of two litters each in concentrations as high as 0.3%—3000 p.p.m.—had no effect upon reproduction or lactation in these animals. Body weight records obtained at weaning and weekly thereafter for those rats allowed to mature for mating likewise did not indicate any evidence of adverse effects.

Table V. Summary of Tissue Analyses from Male and Female Rats Maintained for 2 Years on Diets Containing Dalapon Sodium and of Milk from Lactating Female Rats

Organ	Dosage, P.P.M. in Diet: 100	300	1000
	2,2-Dichloropropionic Acid, P.P.M.[a]		
Kidney	4.0	9.7	28.2
Liver	1.0	3.5	10.7
Muscle		2.9	7.5
Brain		2.6	5.4
Fat		0.5	1.4
Milk[b]		6.0	19.1

[a] Average net values after correction for blanks and recovery.
[b] From third generation lactating female rats. Milk samples for the rats maintained on diets containing 3000 p.p.m. of dalapon contained 29.3 p.p.m. of acid.

Tissue and Milk Analyses

Terminal tissues of dogs and rats from the long-term feeding studies were selected for analysis of dalapon sodium. The tissues were taken immediately upon autopsy, frozen immediately at dry ice temperature, and held at 0° C. or lower until analysis. In order to obtain enough tissues for the analyses, it was necessary to pool samples from several rats in most cases. The analyses were carried out by a modification of the method of Smith, Getzendaner, and Kutschinski (*4*) in which the compound is ether extracted, isolated chromatographically, hydrolyzed to pyruvic acid, converted to its 2,4-dinitrophenylhydrazone, and determined colorimetrically. The data are then expressed in terms of 2,2-dichloropropionic acid.

During the latter part of the reproduction studies, pooled samples of milk were obtained from lactating female rats that had been separated from their litters for a 24-hour period. Gentle suction from a pulsating pump was applied to the rat nipples through the flared end of a capillary tube, the milk being trapped in a small Erlenmeyer flask. The analyses were made using an isotope dilution method (*3*).

The results of the analyses of rat tissues and milk are given in Table V. Results of the dog tissue analyses are given in Table VI. On a dietary percentage basis, the repeated oral doses of 15, 50, or 100 mg. per kg. are equivalent for adult dogs to about 500, 1750, or 3500 p.p.m. of the total diet. The

Table VI. Summary of Tissue Analyses from Dogs Given Daily Doses (5 Days a Week) by Capsule of Dalapon Sodium for 1 Year

Organ	Sex	Dosage,[a] Mg./Kg./Day: 15	50	100
		2,2-Dichloropropionic Acid, P.P.M.[b]		
Kidney	M	23	21	78
	F	17	32	60
Liver	M	27	16	48
	F	17	27	57
Muscle	M	15	13	51
	F	12	27	35
Brain	M	11	9	25
	F	7	13	18
Fat	M	2	2	15
	F	1	4	5

[a] Equivalent to about 500, 1750, or 3500 p.p.m. in diet.
[b] Net parts per million corrected for blank and recovery.

2-year rats received diets containing 100, 300, or 1000 p.p.m. Comparison of tissue levels as parts per million of 2,2-dichloropropionic acid with dietary levels expressed as parts per million of dalapon sodium shows the results for the two species to be of the same order of magnitude. Quantitatively, the amounts occurring in the meat or other tissues were quite low in relation to the levels administered.

Practical Considerations for Handling and Use

The results of the studies reported here indicate that dalapon sodium is low in acute and subacute oral toxicity. The likelihood of human subjects, livestock, or wildlife ingesting sufficient amounts of the material to cause serious toxic effects is therefore extremely remote. However, salt-hungry stock have been known to ingest lethal amounts of sodium chloride, so it is recommended that dalapon sodium or strong solutions of it be kept in closed containers where it is unavailable to stock or wildlife.

Undiluted dalapon sodium may cause skin irritation, if it is allowed to remain on the skin for a prolonged period. Exposures of short durations are not likely to be injurious. Contact with dust, particularly when sweating, may cause a mild burning sensation. Prolonged contact with dilute aqueous solutions is not likely to cause any appreciable effect. Systemic effects due to absorption through the skin are not likely to occur. Because the ability of the material to cause irritation is markedly reduced by dilution with water, the prompt flooding of exposed areas with water will practically eliminate the possibility of irritation occurring as a result of skin contact.

The material in the solid form or in concentrated solutions is capable of causing appreciable pain, and irritation of the eyes, but is not likely to cause serious damage. Nevertheless, eye protection, such as safety glasses, should be worn when handling the salt or concentrated solutions. Solutions of less than 10% concentration are not likely to cause more than transient pain and inflammation. Prompt washing of contaminated eyes will markedly reduce the severity of any effect caused by the solid or strong solutions and practically eliminate the effect from dilute solutions.

Experience in the manufacturing, handling, and application of dalapon sodium has indicated that it presents no unusual problems. No incidences of irritation or other adverse effects have come to the attention of the authors thus far in the use of this product.

The results of the 2-year, life-span dietary feeding studies to rats, the reproduction and lactation studies in rats, and the 1-year repeated oral administration to dogs also show a low order of toxicity for this herbicidal material. Thus, these data provide a basis for judging the safety of the small amounts of dalapon sodium residues likely to occur when this herbicide is used as directed on edible crops, or on soil in which food crops are grown.

Acknowledgment

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INSECTICIDE RESIDUES

Colorimetric Determination of Residues of Phorate and Its Insecticidally Active Metabolites

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A colorimetric method, based on the chromatropic acid procedure for formaldehyde, has been developed for the estimation of Thimet (phorate) and its insecticidally active metabolites. The limit of detection of the method as indicated by results from analysis of 100-gram samples of treated plant leaves is about 0.10 p.p.m. and with larger samples it is even less. Although the method was developed for the determination of residues of phorate and related materials, it can also be applied with some minor modifications to the analysis of residues of any organic phosphorus insecticide that has a methylene linkage present in its molecule which will produce formaldehyde on hydrolysis.

PHORATE [the recently assigned generic name for *O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate, the major constituent of the commercial product, Thimet] has recently attracted wide interest as a systemic insecticide (*2, 7, 9*), although it also has considerable contact activity. Like many of the other organophosphorus insecticides, phorate is toxic to warm-blooded animals, being comparable to parathion in this respect (*2*).

In studies of the fate of phorate in plants, Bowman and Casida (*3*) and Metcalf, Fukuto, and March (*8*) found five oxidation products which are insecticidally active and which have high mammalian toxicity. These are the phorate oxygen analog, phorate sulfoxide, the phorate oxygen analog of the sulfoxide, phorate sulfone, and the phorate oxygen analog of sulfone.

Hydrolysis or any further oxidation results only in decomposition products that have little toxicity to insects or mammals. These authors found from in vitro enzymatic studies, that the sul-

2-year rats received diets containing 100, 300, or 1000 p.p.m. Comparison of tissue levels as parts per million of 2,2-dichloropropionic acid with dietary levels expressed as parts per million of dalapon sodium shows the results for the two species to be of the same order of magnitude. Quantitatively, the amounts occurring in the meat or other tissues were quite low in relation to the levels administered.

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Hydrolysis or any further oxidation results only in decomposition products that have little toxicity to insects or mammals. These authors found from in vitro enzymatic studies, that the sul-

Table I. Recoveries of Phorate and Its Oxidation Products Added to 100 Grams of Cabbage without Perbenzoic Acid Oxidation

Chemical	Added, γ	Recovered γ	Recovered %
Phorate	87	47	54
	44	22	50
Phorate sulfoxide	102	86	84
	51	42	82
Phorate sulfone	78	73	94
	39	37	94
Oxygen analog	85	55	65
	42	25	60
Oxygen analog of sulfone	91	83	91
	46	43	93
Mixture of compounds	78	60	77
	39	32	82

fones are ten times more potent as cholinesterase inhibitors than the respective sulfoxides, and that pure phorate itself is a weak inhibitor.

Currently, the only available analytical method for phorate residues is a cholinesterase inhibition method (*2*), which is also used for the determination of residues of many of the other organophosphorus insecticides. Oxidation products of phorate are found in plants in varying amounts depending on time, temperature, light, soil conditions, etc. As each oxidation product possesses a different degree of anticholinesterase activity, the results obtained by this method are difficult to interpret, unless the phorate residue is oxidized (*8*). It is important that any method for the analysis of phorate residues include not only phorate itself, but also all its metabolic products that have high anticholinesterase activity.

The method described herein is based on the observation that, under appropriate conditions, certain types of organophosphorus compounds will yield formaldehyde (*6*), which can be determined by a colorimetric chromotropic acid procedure. Phorate, its oxygen analog, and their sulfoxides produce a red-violet color with this reagent due to the production of formaldehyde. These compounds produce the color whether they have been hydrolyzed by alkali or not, whereas the respective sulfones must be hydrolyzed by alkali before they will produce a color. However, all these compounds can be easily oxidized to their respective sulfones by perbenzoic acid. In experiments on the recovery of each of the compounds without including an oxidation step, the sulfones gave the highest recoveries (over 90%), the sulfoxides about 80 to 85%, and phorate and its oxygen analog about 50 to 60% (Table I). Thus, it is advantageous to include an oxidation step, with perbenzoic acid to convert the compounds to their sulfones, in order to obtain maximum recoveries.

In the method described, the plant material is extracted with chloroform, the extract is oxidized with perbenzoic acid, and then chromatographed through a Nuchar–Hyflo Super-cel column. The eluate is washed with sodium metabisulfite and saturated sodium chloride solutions, and the solvent is evaporated completely. The residue is taken up in water and chilled, and the waxes are removed by filtering through a layer of Attaclay–Hyflo Super-cel mixture. The filtrate is extracted with chloroform, the solvent is again evaporated, and the sodium hydroxide solution is added. The color is developed by heating with chromotropic acid reagent and is read in a spectrophotometer at 570 mμ.

Apparatus

Borosilicate glass test tubes, 20 × 150 mm., with ground-glass stoppers. Scratch a mark on each tube at 6-ml. volume.

Gooch crucible with a perforated bottom, top about 29 mm. and bottom about 18 mm. in diameter.

Reagents

Chloroform, technical grade. Wash the solvent four times with an equal volume of water, dry over anhydrous calcium chloride, decant, and distill.

Perbenzoic acid in benzene. Prepare and analyze as outlined by Adams (*1*) and Braun (*4*). For use in this method, dilute 5 ml. of the prepared benzene solution to 50 ml. with chloroform.

Adsorbent mixture. Boil some Nuchar in a large flask with concentrated hydrochloric acid for 20 minutes, wash by decantation with distilled water, filter, and wash until neutral to litmus. Then wash with two 200-ml. portions of 95% ethyl alcohol, and dry in the hood and finally in a 120° C. oven overnight. Mix 3 parts of this activated Nuchar with 1 part of Hyflo Super-cel.

Sodium metabisulfite ($Na_2S_2O_5$), 0.5% solution, freshly prepared.

Saturated sodium chloride (C.P. grade) solution in distilled water.

Filter mixture. Mix 3 parts of Attaclay with 1 part of Hyflo Super-cel.

Chromotropic acid reagent. With the aid of heat, dissolve 16 grams of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid sodium salt, practical) in concentrated sulfuric acid (C.P. grade), cool to room temperature, dilute to 1 liter with sulfuric acid, and store in a brown bottle. Prepare fresh reagent every 2 weeks. The reagent is conveniently dispensed from an automatic buret.

Mixed solvent. Using redistilled solvents, add 5 parts of chloroform, 5 parts of carbon tetrachloride, and 7 parts of diethyl ether. Mix the solvents just before use.

Procedure

Preparation of Standard Curve. Transfer an accurately weighed sample of 50 mg. of phorate to a 500-ml. volumetric flask and make up to the mark with chloroform. After mixing, pipet 20 ml. of the solution into a 100-ml. volumetric flask, make up to the mark with chloroform, and mix well. Pipet 0-, 1-, 2-, 3-, 4-, and 5-ml. aliquots of this solution into borosilicate glass test tubes. Add to each tube 1 ml. of the diluted perbenzoic acid solution. Stopper, shake well, and place the tube in a 50° C. water bath for 15 minutes. Then immediately transfer the contents to a 60-ml. separatory funnel. Rinse the tube with two 5-ml. portions of chloroform and add the rinses to the material in the separatory funnel. Extract the chloroform solution with 10 ml. of freshly prepared 0.5% sodium metabisulfite and then twice with 10-ml. portions of saturated sodium chloride solution. Place a small plug of cotton and some anhydrous sodium sulfate (about 3 to 5 grams) in a Gooch crucible holder, and filter the washed chloroform solution into a clean borosilicate glass test tube. Evaporate the solvent carefully in a 60 to 70° C. water bath in the hood. Heat no longer than necessary, to avoid loss of the sulfones.

To develop the color, pipet 3 ml. of 0.5*N* sodium hydroxide solution into each tube, rinse the sides of the tube well, and heat in a hot-water bath for 1 minute. Cool, add 3 ml. of the chromotropic acid reagent, and mix by stoppering the tube tightly and shaking well. Loosen the stopper carefully, place the tube in a boiling-water bath for 45 minutes, and shake occasionally. Remove the tube from the bath and cool at once in a beaker of cold water. Make up to the 6-ml. mark with water and mix the contents well. Fill cuvettes with the solution, cover, and measure in the photometer (Beckman Model B or equivalent) at 570 mμ against a blank solution carried through the same procedure. Prepare the standard curve by plotting the absorbance readings against micrograms of insecticide. The curve follows Beer's law up to about 100 γ, having a slope of 11.6 γ of phorate per 0.100 unit of absorbance on the Beckman Model B spectrophotometer.

Analysis of Plant Samples. Cut a weighed plant sample into small pieces and macerate with a measured volume of chloroform (about 2 ml. per gram of leafy vegetable) in the blender for about 3 minutes. Strain the extract through a piece of cheesecloth, and wash the chloroform with about half its volume of saturated sodium chloride solution. If the chloroform and aqueous layers do not separate readily, centrifugation may be

used; otherwise, filter the chloroform layer through a Gooch crucible holder containing cotton and anhydrous sodium sulfate. Measure the volume collected, and evaporate to about 100 ml. in an Erlenmeyer flask.

For the oxidation add 5 ml. of the diluted perbenzoic acid to the extract, stopper tightly, mix thoroughly, and let stand for 10 minutes. The oxidation should be carried out just before percolation of the extract through the chromatographic column.

For adsorption chromatography use 10 grams of the Nuchar–Hyflo Super-cel mixture for each 100 grams of plant sample. Slurry the mixture with chloroform, pour into the chromatographic tube (about 20 × 400 mm.), and pack the column with a little air pressure. Add approximately 2 inches of anhydrous sodium sulfate at the top of the column followed by more chloroform (50 ml. for each 10 grams of Nuchar mixture).

When the chloroform level reaches the top of the sodium sulfate, quantitatively transfer the oxidized chloroform extract to the column, and apply a little pressure if necessary, so that the speed of percolation will be not more than 60 drops per minute. When the level of the chloroform extract reaches the top of the column, carefully add more chloroform (100 ml. for each 10 grams of Nuchar mixture). Transfer the eluate into a 500-ml. separatory funnel, wash first with 100 ml. of 0.5% freshly prepared sodium metabisulfite solution and then with three 150-ml. portions of saturated sodium chloride solution, and filter through anhydrous sodium sulfate in a Gooch crucible holder.

For distillation of the solvent, place the flask on the steam bath and attach a three-bulb Snyder column. Evaporate the last traces of solvent at room temperature by inserting a glass tube connected to a vacuum line into the neck of the flask while it is being rotated. Heat the waxy residue with 20 ml. of water by swirling on the steam bath until the wax is melted. Chill thoroughly in an ice bath, and then filter with vacuum through a 0.5- to 1-cm. layer of Attaclay mixture on a filter-paper disk in a Gooch crucible. Rinse the flask with two 10-ml. portions of water and filter each through the same filter. At no time during the filtration should the Attaclay filter cake be allowed to dry or crack.

Extract the combined filtrate with 50 ml. of chloroform by vigorously shaking for at least 1 minute. When the two layers have completely separated, filter the chloroform layer through anhydrous sodium sulfate. Extract the aqueous layer again with another 25 ml. of chloroform and filter through the same sodium sulfate. Distill the chloroform extract on the steam bath through a three-bulb Snyder column until about 5 to 10 ml. are left. Quantitatively transfer this extract to a borosilicate glass test tube with two successive 5-ml. chloroform rinsings. Evaporate the solution, almost to dryness, in a water bath at 60° to 70° C. Remove the last traces of solvent with slight vacuum at room temperature.

Add 3 ml. of 0.5*N* sodium hydroxide solution to the material in the tube and finish the determination as described under Preparation of the Standard Curve. Occasionally, the colored solution shows some turbidity, especially when the sample is large and the Nuchar mixture does not remove impurities completely or the Attaclay mixture packed in the Gooch crucible is not thick enough or cracks during filtration. This turbidity can be remedied by cooling the solution, extracting with 5 ml. of the mixed solvent, and centrifuging.

A control sample of untreated plant should be analyzed along with the treated samples. The residues of phorate and all its active metabolites are expressed in terms of phorate equivalents.

Recovery Tests. The percentage recovery of phorate and of each of its oxidation products added to cabbage was determined in the following manner: Known amounts were added to a series of 100-gram samples of cut-up cabbage, extracted with 200 ml. of chloroform in a blender, and analyzed, without the perbenzoic acid oxidation step. The results are shown in Table I.

Additional experiments on the application of the method to each of the metabolites, with the inclusion of the oxidation step, showed that in each case over 90% recovery was obtained. Table II shows results of recoveries of phorate added to a variety of plants when the oxidation step is included.

Table II. Recovery of Phorate Added to 100 Grams of Plant Material with Perbenzoic Acid Oxidation

Plant	Added, γ	Recovered γ	Recovered %
String beans	19	17	89
	38	37	97
	56	54	96
Red cabbage	19	18	95
	38	35	92
	56	53	95
Spinach	19	18	95
	38	36	95
	56	52	93
Kale	11	10	91
	23	22	96
	45	43	95
	91	82	90
Tomatoes	11	10	91
	23	20	87
	45	42	93
Potatoes	23	21	91
	45	40	89
	90	83	92
Beet leaves and roots	19	16	84
	38	33	87

Discussion

For chromatographic cleanup, the charcoal-Celite column of Fukuto *et al.* (*5*) for demeton was adopted. In order to minimize the volume of solvent to be evaporated, only chloroform was used instead of the solvent mixtures used by them.

For their preparation of sulfone and sulfoxide derivatives, Metcalf, Fukuto and March (*8*) employed perbenzoic acid in chloroform overnight in a refrigerator. In the method described herein, however, 10 to 15 minutes with slight heating was found to be sufficient for oxidation of phorate and related compounds for the preparation of the standard curves. For residue determinations, heating is not necessary, because of the long time taken for the chromatography step.

The amount of perbenzoic acid needed in the phorate oxidation procedure is not critical. For the preparation of the standard curve, the use of 0.5 to 1.0 ml. of the chloroform-benzene solution of perbenzoic acid is sufficient. For small samples (about 50 grams), 2 ml. of the diluted perbenzoic acid solution may be used, but for larger samples (100 to 200 grams), 5 ml. are preferred.

In the cleanup procedure, the chromatography with Nuchar–Hyflo Super-cel as the adsorbent would remove most of the plant pigments and interferences other than plant waxes. Washing with sodium metabisulfite is necessary to remove the yellow or brown color produced by the perbenzoic acid with control samples of plant crops. After distillation of the chloroform, plant waxes are removed by dissolving the residue in water, and filtering through the Attaclay–Hyflo Super-cel mixture.

Although practically no interference has thus far been encountered from runs on plant materials not treated with phorate, it is always important to run such control analyses. Occasionally, the results of an analysis can be too high if a suitable control sample is not run or if the cleanup procedure is not carefully followed. Absorbance readings on control samples of various vegetables measured against a reagent blank in the reference beam of the spectrophotometer were in the range of 0.02 to 0.08 for the vegetables shown in Table II.

Many insecticides—such as DDT, BHC, chlordan, aldrin, dieldrin, malathion, parathion, demeton, schradan, and Phosdrin—do not interfere with this colorimetric analysis. However, some of them—such as Guthion, Trithion, and ethion—do liberate formaldehyde in this procedure and therefore should be considered interferences. A procedure based on the liberation of formaldehyde has been developed for Guthion (*6*), and further work is in progress on Trithion and ethion.

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INSECTICIDE-MITICIDE RESIDUES

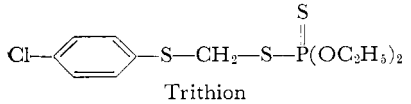
Determination of Trithion Crop Residues by Cholinesterase Inhibition Measurement

G. G. PATCHETT and G. H. BATCHELDER

Stauffer Research Laboratory, Richmond, Calif.

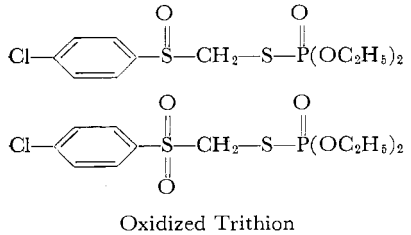
A two-phase hydrogen peroxide–acetic acid–benzene system has been developed for oxidizing Trithion to strong cholinesterase inhibitors with subsequent determination by measuring its inhibition of cholinesterase in human blood plasma. The method is sensitive to 0.01 γ of Trithion and will detect 0.005 p.p.m. residue in crops having low background values. Derived anticholinesterase metabolites which may occur as a spray residue are also determined. Interference levels of some other cholinesterase-inhibiting pesticides are also discussed.

TRITHION (formerly Stauffer R-1303), or *S*-(*p*-chlorophenylthio)methyl *O*,*O*-diethyl phosphorodithioate, is an insecticide-miticide exhibiting moderately long residual activity.



Trithion

It is a poor cholinesterase inhibitor, but is converted into strong inhibitors by oxidation in a two-phase hydrogen peroxide–acetic acid–benzene system. The products of oxidation have been determined by column chromatography and ultraviolet spectroscopy to be a mixture of *S*-(*p*-chlorophenylsulfinyl)methyl *O*,*O*-diethyl phosphorothioate and *S*-(*p*-chlorophenylsulfonyl)methyl *O*,*O*-diethyl phosphorothioate which result from oxidation at two sites on the Trithion molecule.



Oxidized Trithion

Trithion, the two above oxidation products, and the three other possible intermediate oxidation products effect a similar cholinesterase inhibition when the pure materials are subjected to the oxidation procedure. The detection of the oxidation products which may be present in aged spray residues is the chief advantage of the cholinesterase method over the previously employed colorimetric method (*9*) which is specific for the parent compound. In the latter method the chromatographically isolated Trithion is hydrolyzed to *p*-chlorothiophenol and reacted with 2,6-dibromo-*N*-chloro-*p*-quinoneimine at pH 6.6 to form an orange color ($\lambda_{max.}$ 480 mμ). A total chloride procedure employed by Gunther and Blinn (*5*) has been used to determine Trithion residues in oranges and lemons (*6*), but residue values tend to be high because of the detection of hydrolyzed as well as oxidized forms of Trithion. The high sensitivity of the cholinesterase method greatly facilitates sample extraction by permitting the processing of smaller samples.

Earlier attempts to oxidize Trithion crop extracts with dilute bromine water by the method of Fallscheer and Cook (*2*) were not successful. Trithion could be satisfactorily oxidized with extremely small concentrations of bromine, but not in the presence of bromine-reacting crop extractives. Use of higher concentrations of bromine, such as those successfully employed with parathion, etc., resulted in the destructive oxidation of Trithion. The use of *n*-bromosuccinimide as described by Cock (*1*) and of bromine in carbon tetrachloride did not result in the desired conversion of Trithion to strong inhibitors.

A less destructive oxidizing agent was sought which could be used in excess to minimize interference from crop extractives. Also required was the incorporation of an organic solvent which would dissolve the extracted fats and waxes and eliminate the anticipated mechanical problems. The organic solvent would preferably be one that could be employed for crop extraction. These requirements were met by the development of the oxidation system employing hydrogen peroxide, acetic acid, and benzene which has been proved reliable during 3 years of application.

The cholinesterase procedure employed is an adaptation of the method of Giang and Hall (*3*) as modified by Hensel *et al.* (*7*).

Analytical Procedures

Apparatus. Beckman Model G pH meter or equivalent which has a precision of ±0.01 pH unit.

Glass-stoppered test tubes. 160 × 16 mm. in outside diameter with ⅌ 14 flat-head stoppers. These tubes must be made to order.

Constant temperature bath maintained at 37.5° ± 0.5° C. equipped with racks for supporting 5- and 20-ml. beakers.

Constant temperature bath maintained at 75° ± 1° C. equipped with a rack for holding glass-stoppered test tubes in a vertical position and immersed to a depth of 4 to 5 cm.

Solvent evaporator. A manifold of air jets suspended with rubber couplings directly over a hot plate maintained at about 40° to 60° C. Means are provided for adjusting the height of the jets above the hot plate. The air line should contain a trap and filter.

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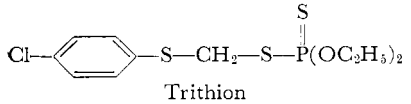

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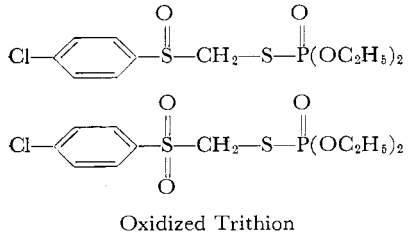

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Solvent evaporator. A manifold of air jets suspended with rubber couplings directly over a hot plate maintained at about 40° to 60° C. Means are provided for adjusting the height of the jets above the hot plate. The air line should contain a trap and filter.

Waring Blendor or suitable substitute for macerating samples.

Rotary tumbling device similar to that described by Gunther and Blinn (*4*), geared to revolve at 29 r.p.m. Compartments are made to hold 1-gallon wide-mouthed glass jars, and also combinations of half-gallon, quart, or pint jars with the aid of cardboard separators.

Reagents. Pooled human blood plasma (as source of cholinesterase). Purchase plasma at local blood banks and keep it constantly refrigerated or in an ice bath. Test individual batches and mix them so that a 1-ml. aliquot will effect an acidity between 1.8 and 2.0 pH units. Plasma can be stored in sterile screw-cap bottles for several months under refrigeration without detectable change in activity. Frozen plasma can be stored for more than a year.

Buffer Solution. Add 2.67 grams of barbital, 0.545 gram of potassium dihydrogen phosphate, 44.7 grams of potassium chloride, and 12 ml. of 1.0*N* sodium hydroxide to a 500-ml. glass-stoppered, volumetric flask and dissolve in about 480 ml. of distilled water. Shake vigorously if necessary to dissolve the barbital, and remove any flocculent material by filtration. Adjust the pH to 7.9 to 8.0 with about 1 ml. of 1*N* hydrochloric acid and dilute to the 500-ml. mark. Add a drop of toluene as preservative and store in the refrigerator when not in use.

Acetylcholine Solution. Transfer 12.0 grams of acetylcholine chloride (Matheson, Coleman, and Bell) into a 100-ml. volumetric flask, dissolve in distilled water, and bring to mark. Add a drop of toluene as preservative and store in the refrigerator when not in use.

Acetic Acid–Peroxide Solution. Mix 1 volume of 30% hydrogen peroxide with 5 volumes of glacial acetic acid. Prepare this mixture just before use and discard the excess.

Trithion Stock Solution. Prepare a solution containing 1.00 γ of Trithion per ml. of benzene and store in the absence of direct light in a tightly stoppered container

Nujol Solution. Weigh 1.0 gram of Nujol or other refined mineral oil into a 60-ml. dropper bottle and dissolve in 50 ml. of benzene.

Boiling Chips. Hengar granules or equivalent—preferably crushed and screened to about 10- to 15-mesh.

Sample Extraction. Weigh 100 grams of sample, selected so as to be representative, into a 1-quart Waring Blendor cup. Add 200 ml. of benzene, blend intimately with pulp for 5 minutes, and transfer to a 1-pint, wide-mouthed jar. Tumble for 1 hour in a mechanical tumbler and filter a portion of the benzene extract into a suitable container.

Trithion Oxidation. Transfer 5-ml. portions of the benzene extract of the sample to glass-stoppered test tubes. Add 3 ml. of acetic acid–peroxide solution, and a boiling chip to each tube. Stopper the test tubes, shake briefly, loosen the stoppers and place in a 75° C. bath for 20 ± 0.5 minutes. At the end of the heating period, remove the test tubes and cool them in an ice bath. Add 5 ml. of distilled water to the tubes, stopper, and shake well. When the benzene layer (top) has cleared, transfer measured aliquots into 20-ml. beakers with the aid of a serological pipet. (Aliquots should contain between 0.02 and 0.15 γ of Trithion. Use a Propipette to assist in taking 4-ml. aliquots.) Add 4 drops (about 0.1 ml.) of Nujol solution to each 20-ml. beaker and mix with the sample aliquot. (The Nujol keeps waxy extractives soft and facilitates the subsequent water extraction.) Remove the benzene with solvent evaporator. Allow a few minutes after the beakers appear dry to remove traces of acetic acid which may remain (can be detected by odor). Add 9.0 ml. of water to each beaker and heat for 10 minutes on a hot plate set at 110° to 130° C. Cover the beakers tightly with a piece of aluminum foil during the heating period to minimize evaporation (heating to near boiling temperature is necessary to extract the oxidized Trithion from sample waxes and oils). After the heating period, remove the foil and cool the beakers in a water bath. Samples may be capped and stored overnight at this point, if necessary.

Cholinesterase Inhibition. Determinations are conveniently run in sets of 24, typically including two blanks and several standards or fortified control samples.

Add 1.0 ml. of plasma to the 20-ml. beakers containing the oxidized Trithion samples. Swirl to effect homogeneous solutions and place in a 37.5° C. bath for 70 minutes. (Beakers need not be individually covered, as a loose cover over the water bath minimizes evaporation and prevents contamination.) After the heating period, remove the samples from the bath and transfer a 1.0-ml. aliquot from the 20-ml. beakers to 5-ml. beakers to which has been added 1.0 ml. of buffer solution. Take the pH of the beaker solutions and record them as the initial pH. Carefully add exactly 5 drops (0.2 ml.) of the acetylcholine solution to the 5-ml. beakers. Use the same dropper for each sample. Place the beakers in a 37.5° C. bath for 120 minutes. At the end of this period, remove the beakers, allow them to cool for 5 minutes, and take the pH of the solutions without unnecessary delay and record them as the final pH. Calculate the per cent inhibition as follows:

$$\% \text{ cholinesterase inhibition} = 100\left[1 - \left(\frac{\text{sample } \Delta\text{pH}}{\text{blank } \Delta\text{pH}}\right)\right]$$

The ΔpH is the difference between the initial and final pH, and the blank is a sample of distilled water (containing no Trithion), which is processed in a manner identical to the standards and samples. The ΔpH of the blank should range from 1.8 to 2.0 pH units. A higher ΔpH may decrease the sensitivity of the method, so it should be lowered by diluting the plasma. The ΔpH can also be adjusted by changing the acetylcholine chloride concentration.

If two blanks are run as the first and the last sample of a 24-sample run, the last blank will average about 0.15 pH unit lower than the first because of the continuing, but slower, hydrolysis of acetylcholine at the lower temperature. For maximum accuracy this effect is compensated for in the calculations.

Standard Curve Preparation. Oxidize a 5-ml. portion of Trithion stock solution containing 1 γ per ml. of benzene as described under Trithion oxidation. Determine the cholinesterase inhibition of an appropriate number of aliquots of the oxidized Trithion solution and plot per cent inhibition *vs.* amount of Trithion in the aliquot. The curve approximates a straight line between 75 and 20% inhibition when the per cent inhibition is plotted on the linear scale of semilog graph paper. The slope of the curve increases below 20% inhibition, but is still considered useful. Typical values are listed in Table I.

Sample Analysis. Dilute the sample extract with benzene if the Trithion concentration exceeds 2 γ per ml. and proceed as in the preparation of the standard curve. Refer the resulting per cent inhibition values to the standard curve to determine the Trithion content of each sample aliquot.

Special Oil-Removal Procedure. The presence of excessive quantities of oil and/or wax in the extract tends to decrease the extractability of the oxidized Trithion with hot water. Crops such as almonds, walnuts, and cottonseed must therefore be subjected to an oil-removal step. The acetonitrile extraction of Jones and Riddick (*8*) is employed to separate the oils and waxes from the Trithion and derived anticholinesterase products in the following procedure:

Table I. Typical Cholinesterase Inhibition Values

Trithion, γ	*Inhibition*, %
0.000	0
0.010	4
0.015	7
0.020	10
0.030	18
0.050	33
0.080	47
0.120	58
0.190	71

Table II. Cholinesterase Inhibition of Some Other Pesticides

Pesticide	γ Required to Effect 50% Inhibition
Delnav	1.0
Demeton	4.3
Diazinon	0.08
Di-syston	4.2
EPN	0.2
Ethion	0.09
Guthion	4.4
Malathion	4.3
Methyl parathion	3.1
Methyl trithion	15.5
OMPA	50.0
Parathion	0.03
Phosdrin	1.5
Sevin	77.0
TEPP	0.06
Thimet	5.5
Trithion	0.09

Table III. Recovery of Trithion Added to Crop Extracts

Crop	Added, P.P.M.	Recovered, %
Almond hulls	5.0	102
Apples	1.0	90
Beans, fresh	0.10	81
Beans, dried	0.05	95
Beets, red	0.05	110
Beets, sugar	0.05	116
Eggplant	0.06	109
Grapes	1.0	95
Hops, dried	10.0	30
Lemon peel	10.0	90
Lemon juice	0.10	98
Olives	2.0	95
Orange peel	8.0	100
Orange juice	0.10	92
Peaches	1.0	87
Pears	0.10	99
Plums	0.50	96
Sorghum	1.0	89
Almonds[a]	0.10	66
Cottonseed[a]	0.10	84
Walnuts[a]	0.05	70

[a] These crops were subjected to the special oil-removal procedure and are calculated on the basis of 80% theoretical recovery from the acetonitrile partition.

Transfer 10 ml. of the sample to a 100-ml. beaker and remove the benzene on the solvent evaporator. Dissolve the residual material in 10 ml. of iso-octane and transfer to a centrifuge tube with the aid of 10 ml. of acetonitrile. Stopper the tube and shake well. Stable emulsions form in most cases and the sample must be centrifuged. Addition of mineral acids to prevent emulsions can cause interferences. Transfer 5.0 ml. of the acetonitrile phase (lower) to a glass-stoppered test tube (the Propipette pipet filler will aid in this operation). Immerse the test tube in a beaker of warm water at 40° to 50° C. and remove the acetonitrile under a gentle stream of air. Dissolve the residual material with 5 ml. of benzene and oxidize as previously described.

The partition coefficient for Trithion is 80% into the acetonitrile, and is greater than 87% for the five oxygen analogs.

Other Pesticides. Table II lists the cholinesterase-inhibiting pesticides which have been subjected to the oxidation procedure. Many of these could seriously interfere with the determination of Trithion residues unless selectively removed by reduction, hydrolysis, etc. These possibilities are being investigated.

Discussion

Oxidation Conditions. When the proportions of benzene, acetic acid, and peroxide are adjusted to effect a miscible system, the conversion to good cholinesterase inhibitors is greatly reduced. In the oxidation procedure a small interfacial area between the organic and inorganic phases is essential, as exemplified by the poor results obtained in an Erlenmeyer flask as compared to a test tube. Optimum time and temperature for the two-phase system have been determined. Excessive or insufficient oxidation will occur at the rate of 2 to 3% per minute of deviation from the 20-minute oxidation time. Change in temperature is less critical, unless it reaches about 78–79° C., where the solutions begin to boil with subsequent increased oxidation rate. This is to be avoided, because of the inability to maintain uniform boiling times. Originally, the oxidation system employed an 80° C. bath without addition of boiling chips. The solutions did not boil and were shaken by hand at intervals. Shaking is now unnecessary, because of uniform evolution of small bubbles from the boiling chips which result in sufficient mixing as they rise through the benzene phase.

Extraction Efficiency. Trithion and its five oxygen analogs will all partition completely into benzene from water or aqueous fruit pulp, and very good recovery has been effected when these compounds were added to pulverized samples. Sample types tested include aqueous and oily fruits, green and dried beans, grain crops, and nuts.

Extensive tests on pippin apples showed the absence of any potential cholinesterase inhibitor which was not extracted by benzene. These tests were based upon water extractions of the samples which were checked for cholinesterase activity before and after bromine oxidation.

Analytical Recovery. Table III lists some typical values for the recovery of Trithion which had been added to a variety of crop extracts in the indicated amounts. Many other crops have resulted in similar recoveries. Interferences are generally small and mostly of a mechanical nature. The recovery percentage is usually lower for crops having a high oil or wax content, although the acetonitrile partition reduces this interference markedly. Dried hops gave a very low recovery, although extractives in the final aliquot were low.

Natural inhibitors are suspected to be present in hops and celery.

Stability of Trithion Solutions. Trithion and its five oxygen analogs have exhibited no decomposition, when stored for 7 weeks in the absence of direct light as dilute solutions in benzene. A dilute solution of Trithion in iso-octane was stored under similar conditions for 19 weeks without detectable decomposition as determined by ultraviolet spectroscopy and anticholinesterase activity. Dilute solutions of Trithion in iso-octane and methanol are not stable when subjected to strong ultraviolet radiation.

The half life of Trithion in distilled water at laboratory temperatures is 102 days (solubility is 0.33 p.p.m.), whereas the half life for some of the more water-soluble oxidation products is about 30 days, as determined by the cholinesterase method.

Tests on sample extracts stored at laboratory temperatures in clear glass containers have exhibited some changes after 1 to 2 months. The magnitude of the change appears to be dependent upon the type of crop. It is thus recommended that samples be analyzed soon after extraction. If this is not possible, the extracts should be refrigerated, along with controls fortified with known quantities of Trithion, so that storage stability of the experimental samples can be verified.

Accuracy, Precision, and Sensitivity. The maximum precision of any single cholinesterase inhibition determination is estimated to be ±0.005 γ Trithion in the 30 to 60% inhibition range. Precision is determined by the limitations for making rapid and accurate volumetric measurements of some of the reagents, and by the precision of the pH meter. Increased precision is attained by averaging the results from multiple determinations.

Accuracy of the analysis is dependent upon reliability of the reference curve. Greatest accuracy is attained when a working curve prepared from fortified control samples is determined simultaneously with actual samples.

Quantities of Trithion down to 0.01 γ can be satisfactorily estimated in samples with low background and low interference levels. This represents 0.005 p.p.m. for a 2-gram aliquot (in 4 ml. of benzene) which is the practical limit of the procedure.

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INSECTICIDES AND FLAVOR

Flavor of Cantaloups as Affected by Treatment with Lindane

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The flavor of cantaloups treated with lindane as a dust, wettable powder, or emulsion concentrate at the rate of 0.35 pound active ingredient per acre in each of four applications was compared to the flavor of untreated cantaloups. Results of these palatability evaluations indicate that lindane applied as a dust had no detrimental effect on flavor. The effect of lindane wettable powder on flavor of melons was less definite, but its use may contribute to off-flavor. Treatment with lindane emulsion concentrate resulted in an adverse effect on flavor of cantaloups.

THE USE OF AGRICULTURAL CHEMICALS has greatly reduced insect damage to food crops and thus has increased yield and quality of these crops, although in certain foods off-flavors have been produced.

A possible residual in the soil by lindane treatments may affect the flavor of subsequent crops grown in the same soil. Present U. S. Department of Agriculture recommendations (*4*) for the control of the pickleworm include the use of lindane applied as dust or wettable powder with the restriction that it not be applied within 1 day before a harvest and not be applied in fields to be planted later to potatoes or other root crops, as it may adversely affect their flavor.

Reported here are the results of a study conducted by the Human Nutrition Research Division in cooperation with the Entomology Research Division to determine whether cantaloups treated with lindane in the form of dust, wettable powder, or emulsion concentrate developed off-flavors or off-odors.

Production and Harvesting

Cantaloups of the Hearts of Gold variety were grown at the Plant Industry Station, Beltsville, Md., by the Crops Research Division, and were treated and harvested by the Entomology Research Division. The melons were grown in three field blocks, each of which contained one plot for the untreated cantaloups and one plot for each of the three treatments. Two rows of cantaloups were grown in each plot.

The insecticide treatment was lindane applied to the vines and melons as dust, wettable powder, or emulsion concentrate at the rate of 0.35 pound of active ingredient per acre. Cantaloups received the first application of lindane treatment the week of July 25, 1957, and treatment was continued at weekly intervals for 4 weeks. The last application of lindane was made the day preceding harvest to allow the greatest possible influence on flavor. This is in accordance with commercial practice in growing the relatively new fall crop. All samples, including the control, were sprayed with a fungicide made with 3 pounds of tribasic copper sulfate and 2 pounds of Dithane to 100 gallons of water per acre.

The cantaloups were harvested from August 26 to September 4 at the "full-slip" stage of maturity in order to obtain melons of uniform ripeness. However, upon cutting the cantaloups, some variation was found in degree of ripeness regardless of the care exercised in selection. At the time of harvest the top of each melon was marked with an X to distinguish it from the bottom, which was unmarked. The top of the cantaloup was considered that portion which did not touch the ground during growth. This procedure made it possible to separate the two portions for palatability evaluation and to ascertain whether both portions were similarly affected by the lindane treatment.

Two melons from each plot (one from each row) of a single field block were harvested and delivered daily to the Human Nutrition Research Division laboratory. The cantaloups, which were of good quality when received, were held overnight at 40° F. They were judged the day following harvest to avoid long holding, as flavor loss in full-slip, yellow melons is rapid in storage (*3*).

Procedures for Palatability Evaluations

The two melons from each of the untreated plots and each of the treated plots were cut in half; top halves were prepared as one sample and bottom halves as another. The rind was removed, any damage from cracks or bruises was carefully trimmed away, and the cantaloups were then cut into 1-inch cubes. To provide a representative sample of cut pieces and juice, the cantaloup was prepared in an electric blender. To avoid development of oxidized flavor, only one eighth of the cubes at one time were chopped for 15 to 20 seconds on low speed. All eight aliquots of a sample were transferred to a glass bowl and any pieces uncut by the blender were cut by hand with a stainless steel paring knife. The bowl was covered with aluminum foil and the melon was chilled in a refrigerator until served—no longer than 20 minutes.

Individual portions of the melon samples, approximately two tablespoons, were served in coded, white procelain dishes to the panel members.

Palatability testing was done at individual tables in a room especially equipped for panel testing and separate from that in which samples were prepared. Five members of the staff, in no way connected with preparation of the samples, evaluated the cantaloups for flavor and odor, and were asked to identify any detectable off-flavors and/or off-odors. Separate 5-point scoring scales were used for flavor and odor: for flavor, 5 represented natural flavor or no off-flavor; 4, slight off-flavor; 3, moderate off-flavor; 2, strong off-flavor; 1, very strong off-flavor. A similar scoring scale was set up for odor.

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INSECTICIDES AND FLAVOR

Flavor of Cantaloups as Affected by Treatment with Lindane

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The flavor of cantaloups treated with lindane as a dust, wettable powder, or emulsion concentrate at the rate of 0.35 pound active ingredient per acre in each of four applications was compared to the flavor of untreated cantaloups. Results of these palatability evaluations indicate that lindane applied as a dust had no detrimental effect on flavor. The effect of lindane wettable powder on flavor of melons was less definite, but its use may contribute to off-flavor. Treatment with lindane emulsion concentrate resulted in an adverse effect on flavor of cantaloups.

THE USE OF AGRICULTURAL CHEMICALS has greatly reduced insect damage to food crops and thus has increased yield and quality of these crops, although in certain foods off-flavors have been produced.

A possible residual in the soil by lindane treatments may affect the flavor of subsequent crops grown in the same soil. Present U. S. Department of Agriculture recommendations (*4*) for the control of the pickleworm include the use of lindane applied as dust or wettable powder with the restriction that it not be applied within 1 day before a harvest and not be applied in fields to be planted later to potatoes or other root crops, as it may adversely affect their flavor.

Reported here are the results of a study conducted by the Human Nutrition Research Division in cooperation with the Entomology Research Division to determine whether cantaloups treated with lindane in the form of dust, wettable powder, or emulsion concentrate developed off-flavors or off-odors.

Production and Harvesting

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Table I. Mean[a] Panel Scores for Cantaloups Treated with Lindane Dust, Wettable Powder, or Emulsion Concentrate

Treatment	Flavor[b]		Odor[b]	
	Top half	Bottom half	Top half	Bottom half
Untreated, control	4.3	4.7	4.5	4.9
Lindane				
Dust	4.4	4.5	4.9	4.8
Wettable powder	3.8	3.6	4.5	4.5
Emulsion concentrate	2.8	3.0	3.5	3.7
Test difference[c]	0.8	0.6	0.4	0.5

[a] Mean for flavor of top halves of untreated cantaloups is based on 25 scores (5 judges, 5 scores per judge). All other means are based on 30 scores (5 judges, 6 scores per judge).
[b] 5 represents no off-flavor or off-odor; 1, very strong off-flavor or off-odor.
[c] Difference between two means is significant at 5% level when it equals or exceeds test difference [Duncan and Bonner test (*2*)].

Two days of preliminary training familiarized the judges with the natural flavor of the cantaloups and the form in which they were served.

The experimental design was a randomized block plan (*1*) which provided for two replications of both top halves and bottom halves of the four samples from each of the three field blocks. The design provided, therefore, six scores per judge for cantaloup top halves and six scores for cantaloup bottom halves for each treatment and for the untreated melons.

Taste-testing sessions were held in midmorning and mid-afternoon. At each session both top halves and bottom halves of the three treated samples and one untreated sample of melons representing a single field block, a total of eight samples, were rated. The samples were divided into two groups, top halves and bottom halves. The serving order was randomized, so that each group was served first four times. A 10-minute interval between the serving of the two groups of four samples allowed for recovery of the judges' sensory acuity.

Results and Discussion

Mean scores for flavor of untreated cantaloups and lindane-treated cantaloups show that development of off-flavors may be influenced by the form of lindane treatment, whether dust, wettable powder, or emulsion concentrate (Table I).

No significant difference was found between the flavor scores for the lindane dust-treated sample and the control sample. The mean flavor score for bottom halves of melons treated with lindane dust was significantly higher (flavor more natural) than the mean score for flavor of cantaloups treated with either emulsion concentrate or wettable powder. Also the mean score for top halves of melons treated with lindane dust was significantly higher than that for which emulsion concentrate was used. Both top and bottom halves of cantaloups grown with the use of lindane emulsion concentrate were scored significantly lower for flavor and odor than the untreated sample and melons receiving other forms of lindane treatment. Cantaloups treated with lindane emulsion concentrate were said by panel members to have a "musty" flavor more frequently than other treated samples as shown at right above:

Frequency of Occurrence of "Musty" Flavor

Control, %	Dust, %	Wettable Powder, %	Emulsion Concentrate, %
14	10	44	58

The effect of lindane wettable powder on the flavor of melons was less definite. Bottom halves of samples treated with lindane-wettable powder were significantly poorer in flavor than bottom halves of untreated samples. The top halves of lindane-wettable powder-treated melons also received a mean flavor score which was lower, although not significantly so, than the mean flavor score received by the top halves of untreated cantaloups.

Mean scores for top and bottom halves of the three treated samples were in agreement, indicating that the two portions of the melons were similarly affected by the lindane treatment.

Acknowledgment

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(4) U. S. Dept. Agr. Handbook **120,** p. 46, 1959.

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COFFEE CONSTITUENTS

Carbohydrates of the Coffee Bean

M. L. WOLFROM, R. A. PLUNKETT, and M. L. LAVER

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THE chemistry of the coffee bean roasting process is not well understood, but is undoubtedly concerned in part with its carbohydrate content, because this amounts to 50 to 60% of the green bean. The literature on the carbohydrates of the coffee bean is incomplete and is often conflicting.

In the present work the authors (*14, 17–19*) have determined the free sugars present in green and roasted coffee. The mixed polysaccharide fractions of green and roasted coffee have been isolated. Derivatives of the sugars present in the polysaccharide fractions of green coffee have been isolated and a quantitative analysis of the sugars present is reported.

Green Coffee Bean

Coffee beans used in this investigation were grown on Fazenda Limeira (210, 000 trees), Morais Sales, Brazil, at an altitude of 631 meters, in Massapé soil (clayey earth), and were classified as Santos 4's, the mildest or least harsh to taste of the Brazilian coffees.

Chromatographic Methods

Flowing-type chromatography on clay columns was employed for the crude fractionation of the ethanol–water (80 to 20) extracts of green and roasted coffee.

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In the present work the authors (*14, 17–19*) have determined the free sugars present in green and roasted coffee. The mixed polysaccharide fractions of green and roasted coffee have been isolated. Derivatives of the sugars present in the polysaccharide fractions of green coffee have been isolated and a quantitative analysis of the sugars present is reported.

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Chromatographic Methods

Flowing-type chromatography on clay columns was employed for the crude fractionation of the ethanol–water (80 to 20) extracts of green and roasted coffee.

The carbohydrates of green and roasted Santos coffee were identified and measured. The soluble sugars of green coffee were sucrose, 6 to 7%, and a trace of glucose; those of roasted coffeee were sucrose, glucose, and fructose. The holocellulose fractions of green and roasted coffee were isolated. The 10% potassium hydroxide–insoluble holocellulose of green coffee was hydrolyzed by a new method employing anhydrous sulfuric acid. It contained the following ratio of sugars: L-arabinose, 1; D-galactose, 2; D-glucose, 2; D-mannose, 6. Little, if any, lignin, cellulose, or pectin was found. The holocellulose was partially solubilized on roasting. The arabinose was easily acid-hydrolyzable. Other coffee constituents identified and estimated were caffeine, trigonelline, caffeic acid, chlorogenic acid, "isochlorogenic" acid, and 10 amino acids. The free amino acids disappeared on roasting. A method was developed for locating caffeine on chromatograms.

For paper chromatography of simple sugars, the descending method was used, according to Jermyn and Isherwood (*7*) or Quick (*15*). The spray reagent was either *p*-anisidine hydrochloride in water-saturated 1-butanol (2%, w./v.) or aniline (0.93 gram), and phthalic acid (1.66 grams) in water-saturated 1-butanol (100 ml.).

Electrochromatography was accomplished in borate buffer (ca. pH 10) at 800 volts and 10 to 15 ma. The same spray reagents were used as in paper chromatography.

Paper chromatography of the amino acids was effected by the two-dimensional, ascending method (*5*) using 0.2% of ninhydrin in water-saturated 1-butanol as a spray reagent.

Techniques were developed for the identification of caffeine on paper chromatograms and clay columns. The paper or electrochromatogram was dried and dipped in an acidified ether solution (5 drops of concentrated hydrochloric acid per 100 ml.) of pyrene (0.01%). Under ultraviolet light, the caffeine appeared as a spot of quenched fluorescence on a yellow-white background. The limit of sensitivity was about 20 γ of caffeine in a 15-mm.-diameter spot. It has been found (*25*) that a large number of purines quench the fluorescence of acidified aqueous solutions of various polynuclear aromatic substances. To detect caffeine on clay, the column was streaked with slightly acidified bromothymol blue indicator. The caffeine zone appeared as a blue section on the yellow streak.

Extraction of Soluble Sugars of Green Coffee Bean. Ground, green coffee bean (500 grams, 10% moisture, 80-mesh) was placed in quart Mason jars (50.0 grams per jar) and covered with 100 ml. of absolute ethanol. The contents of the jars were boiled for 2 to 3 minutes and the following additions were made to each jar: 150 ml. of ethanol-water (40 to 20 weight ratio), 20 ml. of water, 29 ml. of ethanol, and 0.5 gram of calcium carbonate. This made a total of 300 ml. of ethanol-water (80 to 20) per 50-gram sample. The contents of each jar were boiled 1 to 2 minutes and the jars were sealed and stored for 70 hours at 20° to 30° C. to effect extraction.

The mixture was filtered and the residue washed with 1 liter of 80% ethanol. The filtrate was concentrated to 100 ml. under reduced pressure at 40° to 45° C. The viscous amber sirup was dissolved in 800 ml. of methanol-ethanol (3 to 1) and adjusted to 2000 ml. with absolute methanol; only a trace of material did not dissolve.

ANALYSIS. Nonvolatile solids, made up 90.08 grams or 20% of dry weight of the original bean.

Fractionation of Ethanol-Water (80 to 20) Extract of Green Coffee Bean. An amount of 1900 ml. of the adjusted extract was added to the top of an 11 to 12 × 7 to 9 cm. diameter bed of 250 grams of a mixture of Florex XXX (Floridin Co., Warren, Pa.) and Celite (No. 535, Johns-Manville Co., New York, N. Y.) contained in a 2-liter pharmaceutical percolator. The adsorbent bed was prewashed with 1 liter of 95% ethanol and the column was developed by successive percolations of the developers shown in Table I.

The ethanol-water (95 to 5) fraction was concentrated slightly and then diluted to 2000 ml. Crystallization of crude sucrose was induced in four 200-ml. aliquots of this fraction. The product was recrystallized from ethanol; melting point 189–90° C., $[\alpha]_D^{27}$ + 67° [*c* (concentration, grams per 100 ml. of solution), 6.468, water] accepted values: 188° C. and +66.5°, respectively.

The supernatant liquid from the direct crystallization of sucrose was concentrated to a thick sirup and transferred with water to a continuous liquid-liquid extractor. The aqueous solution was extracted for 20 hours with ethyl ether. The aqueous solution which remained was freed of ether by heating at 45° to 50° C. under reduced pressure and was then lyophilized. The residue was a nearly white, crystalline material; yield 13.7 grams. Five grams of this substance were dissolved in 100 ml. of water and extracted for 20 hours with benzene in a continuous liquid-liquid extractor. The extract was freed of benzene by heating at 60° to 70° C. under reduced pressure and lyophilized. The white, crystalline residue was dissolved in 10 ml. of 95% ethanol by heating. On cooling to 30° C., white needles of caffeine formed; yield 0.124 gram, melting point 234–6° C., accepted value: 235° C. On further cooling to −25° C. a second crop of crystals was recovered; yield 0.305 gram, melting point 231–5° C.

The ether extract was concentrated to a brown fatty residue; yield 4.89 grams.

A 1000-ml. aliquot of the ethanol-water (95 to 5) fraction was concentrated to 150 ml. and extracted for 8 hours with benzene in a liquid-liquid extractor. The aqueous solution remaining after extraction was heated at 45° to 48° C. under reduced pressure to remove dissolved benzene and was then adjusted to 250 ml. with distilled water. Two 70-ml. aliquots of this solution were lyophilized. The resulting solids were dissolved in 15 ml. of absolute methanol to effect crystallization of sucrose; yield 4.2 grams for each 70-ml. aliquot; melting point 174° to 195° C., $[\alpha]_D^{32}$ +45.16° (*c* 4.53, water). The physical constants indicated that the crystals were impure. Electrochromatograms showed the presence of sucrose, chlorogenic acid, and a trace of caffeic acid. Calculations based on the assumption that chlorogenic acid, $[\alpha]_D$ −38°, was the only impurity and that the rotations were additive indicated that the crystals were 79% sucrose and 21% chlorogenic acid.

The mother liquors from the crystallization of sucrose were diluted to 200 ml. with 95% ethanol. A 10-ml. aliquot of this solution, diluted 1000 times, showed an absorbance at 324 mμ of 0.818 on a Beckman Model DU spectrophotometer. The chlorogenic acid content was calculated by the method of Moores, McDermott, and Wood (*13*).

Additional substances present in the ethanol-water (95 to 5) fraction were

Table I. Fractionation of Material from an Ethanol-Water (80:20) Extract of Green and Roasted Coffee Beans on Florex XXX[a] with Successive Ethanol-Water Developers

Substances Identified	Green Coffee			Roasted Coffee	
	Developer 1[b]	Developer 2[c]	Developer 3[d,e]	Developer 1[b]	Developer 2[c,f]
Sucrose	5.5[g]	+		+	
Chlorogenic acid	5.1	1.0		+	
Caffeine	0.7			+	+
Fats	2.9				
Amino acids	Trace[h]				
Glucose	Trace			+	
"Isochlorogenic" acid	+[i]	+		+[i]	+[i]
Caffeic acid	+	+		+[i]	+[i]
Trigonelline		+	+		+
Glycine		+			
α-Alanine		+	Trace		
γ-Aminobutyric acid		+	Trace		
Proline		+			
Valine		+			
Leucine		+			
Tyrosine		+			
Aspartic acid			+		
Glutamic acid			+		
Serine			+		
Asparagine			+		
Fructose				+	

[a] Fuller's earth clay, 5 parts Florex XXX to 1 part Celite No. 535 (weight ratio).
[b] Developer 1. 5 liters of ethanol-water (95 to 5); eluent contained 17.8% of the dry, green coffee and 12.6% of the dry, roasted coffee.
[c] Developer 2. 6 liters of ethanol-water (80 to 20); eluent contained 2.3% of the dry, green coffee and 2.4% of the dry, roasted coffee.
[d] Developer 3. 5 liters of ethanol-water (50 to 50); eluent contained 0.6% of the dry, green coffee and 1.3% of polymeric material from the dry, roasted coffee.
[e] Developing the column of green coffee finally with 4 liters of water (eluted 0.3%) followed by 3 liters of 0.1*N* hydrochloric acid (eluted 0.1%), and 4 liters of 0.5*N* hydrochloric acid, resulted in material which could not be separated by chromatography.
[f] Developing the column of roasted coffee finally with 4 liters of water (eluted 0.4%) followed by 6 liters of 0.1*N* hydrochloric acid resulted in material which could not be separated chromatographically.
[g] Percentage basis dry bean.
[h] Faint spot on a paper chromatogram.
[i] Definite spot on a paper chromatogram.
[j] Either one or both were present.

dentified by paper chromatography (Table I). Attempts to prepare a concentrate of glucose by precipitation of the chlorogenic acid with lead acetate resulted in the complete loss of glucose.

The 6 liters of the ethanol-water (80 to 20) fraction of the green coffee bean were concentrated and then diluted to 100 ml. A 60-ml. aliquot of this solution was lyophilized. An amount of 200 mg. of this lyophilized material was analyzed for chlorogenic acid by the ultraviolet absorption method (*13*). The measured absorbance was 0.458 at 324 mμ.

The material of the ethanol-water (80 to 20) fraction was chromatographed on paper simultaneously with known substances (Table I). Employing the two-dimensional, ascending method of Consden, Gordon, and Martin (*5*), the amino acids listed in Table I were identified—comparisons were made with simultaneously run known substances.

Chlorogenic acid was crystallized from 4.6 grams of solids from the ethanol-water (80 to 20) fraction by the method of Moores, McDermott, and Wood (*13*); yield 48 mg.; melting point 203° C., $[\alpha]_D^{26}$ −47.7° (*c* 0.2, water), accepted values: 206–8° C., and −38°, respectively.

Paper chromatography of the ethanol-water (50 to 50) fraction indicated the presence of trigonelline and several amino acids which were rechromatographed and identified as described above (Table I). Paper chromatograms of the water and the acid fractions gave poor resolution and no compounds could be identified. "Isochlorogenic" acid was isolated as a sirup from the green coffee bean by the method of Barnes, Feldman, and White (*1*) and was characterized chromatographically.

Roasted Coffee Bean

Green coffee beans of the type described above and which had been given a light roast were used in this part of the work.

Extraction of Soluble Sugars of Roasted Bean. Ground, roasted coffee beans (500 grams, 5.4% moisture, 80-mesh) were shaken with 1 liter of petroleum ether (boiling point 30° to 60° C.) and filtered. The residue was extracted with ethanol-water (80 to 20) at 25° to 35° C. for 6 days in the dark. The extract was filtered, concentrated, and diluted to 2000 ml. with methanol and ethanol as with the green coffee extract.

Analysis. Nonvolatile solids made up

Table II. Compilation of Data on Fractionation of Green and Roasted Coffee, Dry Weight Basis

Fraction Extracted[a]	Green Coffee, %	Roasted Coffee, %
Ethanol-water (80:20)	15.9	16.2
Benzene-ethanol (2:1)	17.4	16.0
Water	15.7	12.8
Ammonium oxalate	3.0	3.7
Acidified sodium chlorite	6.3	8.2
KOH, 10%	4.9	8.6
Alkali-insoluble holocellulose, final residue	36.8	34.5
Total	100.0[b]	100.0

[a] Extractions were made successively from top to bottom.
[b] Adjusted from a 97.6% actual summation for comparative purposes.

80.0 grams or 16.9% of the dry weight of the roasted bean.

Fractionation of Ethanol-Water (80 to 20) Extract of Roasted Coffee Bean. An amount of 1900 ml. of the 2000-ml. roasted coffee extract was fractionated on a clay column, analogous to that used for the green coffee extract with the developers shown in Table I. The hydrochloric acid fraction was light yellow-brown; all the other fractions were dark brown.

The ethanol-water (95 to 5) fraction was adjusted to 2000 ml. with absolute ethanol. Four 200-ml. aliquots were used in an unsuccessful attempt to crystallize sucrose.

A paper chromatogram of the ethanol-water (95 to 5) fraction was developed and observed under ultraviolet light. The presence of caffeine was shown by developing a chromatogram for 11 hours and was detected as described above.

The ethanol-water (80 to 20) fraction was concentrated to 200 ml. A chromatogram of this solution was developed and viewed under ultraviolet light to indicate the presence of trigonelline, chlorogenic and "isochlorogenic" acids (or caffeic acid or both).

No amino acids were found in the ethanol-water (80 to 20) fraction of roasted coffee bean by the method (*5*) used to identify the amino acids in the green coffee bean.

Paper chromatograms of the ethanol-water (50 to 50), water, and acid fractions showed no resolution. These fractions were not further investigated.

Polysaccharide Fractions of Green Coffee

Ground, green coffee beans (2400 grams, 5.6% moisture) were extracted with ethanol-water (80 to 20), as described above. The insoluble residue

was then extracted successively with benzene-ethanol, water, three times at 20° to 25° C., and twice with 12 liters of 0.5% ammonium oxalate at 90° C. (Table II).

The investigation of the ethanol-water (80 to 20) extract has been described above. The benzene-ethanol extract contained mostly lipides and was not examined further.

An amount of 9.5 liters of the first water extract (neutral to litmus) was poured with stirring into 22 liters of absolute ethanol (final concentration 70% ethanol). The flocculent cream-colored precipitate formed was recovered by filtration and lyophilized; yield 72.7 grams of a gray powder (3.8% ash as sulfate) or 3.5% of the original bean on a dry weight basis. The 70% alcohol-insoluble powder gave a negative ferric chloride test (for phenols), a positive ninhydrin test, and a positive Molisch test. Hydrolysis with 1*N* sulfuric acid for 8 hours (homogeneous solution) yielded arabinose and galactose by paper chromatography.

The filtrate from the 70% ethanol precipitation was concentrated and lyophilized; yield 213.0 grams of dense yellow solids which were not further examined. This amounted to 10.2% of the original bean on a dry weight basis. The work of Underwood and Deatherage (*23*) would indicate that this fraction was largely protein in nature.

This water extraction was repeated twice in the same manner; yield 21.8 grams (1.1% of tan colored) and 8.7 grams (0.4% of light green) solids, respectively. The products gave positive ferric chloride, ninhydrin, and Molisch tests.

The material from the first 0.5% ammonium oxalate extract gave a negative ferric chloride test, a strongly positive ninhydrin test, and a positive Molisch test. The residue from the first ammonium oxalate extraction was extracted once more with 0.5% of ammonium oxalate at 90° ± 3° C. The combined ammonium oxalate extracts were concentrated under reduced pressure and lyophilized; yield 21.4 grams after subtraction of its ammonium oxalate content. This light fluffy solid gave a negative ferric chloride test, a strongly positive ninhydrin test, and a positive Molisch test.

An amount of 150 mg. of material from the first ammonium oxalate extract was hydrolyzed for 2 hours with 1% sulfuric acid. A paper chromatogram of the hydrolyzate indicated galactose, mannose, arabinose, rhamnose or apiose [apiose is almost identical to rhamnose in chromatographic behavior in ordinary solvents (*2*)], and two, slow-moving spots, one of which was probably galacturonic acid. Comparisons were made with simultaneously run known substances. Mannose and arabinose have nearly identical R_f values in this developer and further confirmation of these two sugars was obtained by electrochromatography, by which method mannose and arabinose are readily separated.

Coffee Holocellulose

The holocellulose was prepared essentially according to the modification by Whistler and associates (*26*) of the procedure of Wise and coworkers (*27*). The holocellulose, a nearly white powder, gave a negative ferric chloride test for tannin, a negative ninhydrin test, and a strong positive Molisch test.

Extraction of Holocellulose with Alkali. Coffee holocellulose (25.0 grams) was stirred for 24 hours in 250 grams of 10% potassium hydroxide at 25° ± 1° C. under nitrogen. The mixture was filtered through a glass filter and the residue was washed with 250 grams of 10% potassium hydroxide and 300 ml. of water. The combined filtrate and wash liquors were adjusted to pH 5 with 50% acetic acid and centrifuged; yield 0.15 grams of gray solids, hemicellulose-A fraction. The centrifugate was diluted with three volumes of absolute ethanol and centrifuged; yield 1.77 grams of a cream-white powder, hemicellulose-B fraction. The hemicellulose-A and -B fractions were each hydrolyzed with 1*N* sulfuric acid and the neutralized hydrolyzates were paper-chromatogrammed. Rhamnose (or apiose) (*2*), xylose, arabinose, galactose, a trace of mannose, and several slow-moving, inconclusively identified, spots were detected in the hydrolyzate of hemicellulose-B, whereas no sugar spots could be detected in the hydrolyzate of hemicellulose-A.

The 10% potassium hydroxide–insoluble residue was an almost pure white, granular powder; yield 20.3 grams.

The amounts of apparent lignin in several of the coffee fractions were determined by the method of Ritter and Barbour (*16*). The results were: original bean, 2.40%; holocellulose, 1.80%; 10% potassium hydroxide–insoluble holocellulose, 0.35%. It is doubtful whether these values represent true lignin

Polysaccharide Fractions of Roasted Coffee

For comparison purposes a quantity of roasted coffee was carried through the same fractionation procedure as that described above for the isolation of the polysaccharide fractions of green coffee (Table II).

Characterization of 10% Potassium Hydroxide–Insoluble Holocellulose of Green Coffee Bean

One gram of 10% potassium hydroxide–insoluble holocellulose was hydrolyzed with 25 ml. of 72% sulfuric acid, according to the procedure of Monier-Williams (*12*). The sirup resulting from the hydrolysis was chromatographed on paper using the method of Quick (*15*). The paper was sprayed with aniline phthalate reagent and four well-resolved spots corresponding to glucose, galactose, mannose, and arabinose were detected.

Extraction with Cuprammonium Solution. Ten grams of 10% potassium hydroxide–insoluble holocellulose were stirred into 250 ml. of cuprammonium solution, prepared by the method of Launer and Wilson (*9*). The flask was tightly stoppered and kept for 8 hours at 10° to 20° C. and for 20 hours at 25° to 30° C. The mixture was filtered and the residue was neutralized with 18*N* sulfuric acid, washed with water, and dried at room temperature; yield 7.0 grams. The filtrate was neutralized with 18*N* sulfuric acid in an ice bath and yielded a white, flocculent precipitate which was collected by filtration and dried in air at room temperature; yield 1.1 grams.

An amount of 15 mg. of cuprammonium-soluble material was refluxed as a homogeneous solution for 24 hours in 1*N* sulfuric acid. Electrochromatography of the resulting hydrolyzate showed a strong mannose spot, a glucose spot, and a weak galactose spot.

Partial Hydrolysis with 1.5% Sulfuric Acid. One gram of 10% potassium hydroxide–insoluble holocellulose was mixed with 50 ml. of boiling 1.5% sulfuric acid, refluxed for 5 minutes, and rapidly filtered through a glass filter.

The unhydrolyzed residue was returned to the reflux flask with a fresh portion of boiling 1.5% sulfuric acid. The hydrolysis procedure was repeated several times, gradually increasing the contact time.

Paper chromatograms of the resulting hydrolysis sirups showed that only arabinose was removed upon refluxing for 5 minutes. All of the arabinose was removed after 15 minutes of reflux, but in this aliquot some galactose was also present. No mannose or glucose was removed even after a cumulative refluxing time of 6 hours.

The 10% potassium hydroxide–insoluble holocellulose of roasted coffee was also selectively hydrolyzed with 1.5% sulfuric acid, as described above. The arabinose was again readily removed from this material along with a trace of mannose and a small amount of galactose.

Derivatives of Constituent Sugars

A 1.0200-gram sample of 10% potassium hydroxide–insoluble holocellulose of green coffee was refluxed for 5 minutes

in 1.5% sulfuric acid and filtered quickly. The filtrate was neutralized with Duolite A-4 ion exchange resin (Chemical Process Co., Redwood City, Calif.). An electrochromatogram of the resulting sirup showed only the presence of arabinose.

The tetra-*O*-acetyl-L-arabinose diethyl dithioacetal derivative was then synthesized from the sirup by the procedure of Wolfrom and Karabinos (*28*); melting point 76.0–6.2° C. corrected, unchanged on admixture with authentic material, $[\alpha]_D^{22}$ −26.4° (*c* 1.54, chloroform); accepted values: 79–80° C. and −29.9°, x-ray powder diffraction data (interplanar spacing, CuK_α radiation; relative intensity, estimated visually; vs, very strong; m, medium; w, weak; vw, very weak) (identical with that of authentic material): 13.59 vw, 9.61w, 7.13vs, 6.19vw, 5.54vw, 4.82m, 4.51vw, 4.09s, 3.88m, 3.72vw, 3.59w, 3.40vw, 3.22vw, 2.96vw, 2.83vw, 2.67vw, 2.51vw, 2.42vw.

Ten grams of 10% potassium hydroxide–insoluble holocellulose of green coffee were refluxed for 15 minutes in 250 ml. of boiling 1.5% sulfuric acid and filtered. The undissolved residue was hydrolyzed with 72% sulfuric acid according to the procedure of Monier-Williams (*12*).

A 5.2-gram sample of the brown hydrolysis sirup was acetylated with anhydrous sodium acetate and hot acetic anhydride and poured into water. A chloroform extract of the water was evaporated to a sirup. A 1.0-gram sample of this acetate sirup in 100 ml. of benzene was added at the top of a column (250 × 75 mm. in diameter) of 5 parts of Magnesol (Westvaco Chemical Division, Food Machinery and Chemical Corp., South Charleston, W. Va.) and 1 part of Celite No. 535 (weight ratio), prewet with 150 ml. of benzene. The column was developed with 2500 ml. of benzene–*tert*-butyl alcohol (200 to 1 volume ratio). Two zones were located by streaking the extruded column with permanganate indicator (1% potassium permanganate in 2.5*N* sodium hydroxide). The two zones were numbered from top to bottom and sectioned. The sugar acetate in each was removed by extraction with acetone and evaporated to sirups.

The material from zone 2 crystallized from ethanol as penta-*O*-acetyl-β-D-glucopyranose; melting point 129–30° C. unchanged on admixture with authentic material $[\alpha]_D^{30}$ +6.6° (*c* 3.4, chloroform); accepted values: 135° C. and +3.8°, respectively.

The material from zone 1 crystallized from ethanol as penta-*O*-acetyl-β-D-mannopyranose; melting point 112–13° C. unchanged on admixture with authentic material, $[\alpha]_D^{31}$ −21° (*c* 3.3, chloroform); accepted values: 117–18° C. and −25.2°. D-Mannose was also isolated from the hydrolyzate sirup of 10% potassium hydroxide–insoluble holocellulose of green coffee as D-mannose phenylhydrazone; melting point 186–8° C. unchanged on admixture with authentic material, $[\alpha]_D^{27.5}$+23° ± 4° C. (*c* 0.1, pyridine); accepted values: 199–200° C. and +26.3°, respectively.

A major portion of the sirup from the hydrolysis of 10% potassium hydroxide–insoluble holocellulose of green coffee was added in a streak to an 18.25 × 22.5 inch Whatman No. 3 filter paper. Nine such papers were prepared and developed with 1-butanol–pyridine–water (10:3:3) for 98 hours. Indicator strips were cut from each side and from the center, and sprayed with aniline phthalate. The paper chromatograms were pieced together and the zones of the unsprayed portions corresponding to the movement of galactose were cut out and pulverized in 1 liter of distilled water. The mixture was filtered through Whatman No. 1 filter paper and the filtrate was evaporated to a sirup. A very small portion of the sirup was electrochromatographed and showed only the presence of galactose. The remainder of the sirup was acetylated with anhydrous sodium acetate and hot acetic anhydride and poured into 100 ml. of ice and water. A chloroform extract was concentrated to a sirup. The sirup was dissolved in hot, absolute ethanol and upon cooling and seeding with penta-*O*-acetyl-β-D-galactopyranose, crystallization occurred; melting point 140.9–1.3° C. unchanged on admixture with authentic material, $[\alpha]_D^{24}$+26° ± 4° C. (*c* 0.97, chloroform); accepted values: 142° C. and +25°, respectively. X-ray powder diffraction data, identical with that of authentic material, were as reported by Wolfrom and associates (*30*).

Quantitative Determination of Constituent Sugars in 10% Potassium Hydroxide–Insoluble Holocellulose

The amount of pentose (6.7%) was first determined by the 2-furaldehyde method.

A direct quantitative determination of mannose was obtained by hydrolyzing with 72% sulfuric acid, according to the procedure of Monier-Williams (*12*) and precipitating the mannose as phenylhydrazone. A value of 46.3% mannose was so obtained.

Considerable difficulty was encountered in attempting to determine the amounts of galactose and glucose in this fraction of the green coffee bean by direct chemical procedures. It was also evident that the 72% sulfuric acid method of Monier-Williams (*12*) did not result in complete hydrolysis, because the material did not dissolve completely without considerable degradation. After neutralization and concentration of the hydrolyzate, polysaccharide material was precipitated from the sirup by the addition of absolute methanol. A quantitative determination of the constituent sugars was finally obtained as described below.

A 3.0-gram sample of holocellulose of the green coffee bean was extracted with 10% potassium hydroxide for 24 hours at 25° ± 2° C. The mixture was filtered and the residue washed free of base with water. The 10% potassium hydroxide–insoluble holocellulose was lyophilized.

A 2.0295-gram sample of this freshly lyophilized material was added to 45.0 grams (24.5 ml.) of anhydrous sulfuric acid (melting point 7° to 9°) in a round-bottomed flask submerged in a sodium chloride–ice-water bath at −2° C. After 54 hours the 10% potassium hydroxide–insoluble holocellulose had dissolved with the exception of a few, very small particles. The solution was dark in color. Water was then added dropwise from a capillary tube at such a rate that the temperature was kept below 10° C. The solution was strongly stirred during the addition of water. The hydrolysis solution was diluted to 1500 ml. to give a 3% concentration of sulfuric acid. The 1500-ml. solution was light amber in color and no insoluble material could be detected.

The hydrolysis solution was refluxed and 2-ml. aliquots were removed after 6, 12, 15, and 18 hours. Because the Somogyi (*21*) reducing values of the 15- and 18-hour aliquots were constant, it was considered that hydrolysis was complete. The solution was neutralized with a saturated aqueous solution of barium hydroxide and the resulting precipitate of barium sulfate was removed by centrifugation. The supernatant liquid was made slightly acid to methyl red with 0.1*N* sulfuric acid and concentrated to 600 ml. under reduced pressure at 40° to 50° C. This 600-ml. portion was carefully neutralized to litmus paper with barium hydroxide and filtered under gravity. The filtrate was decolorized with a very small amount of carbon and after filtration to remove the carbon, was concentrated somewhat and then diluted to 250 ml. A 200-ml. aliquot was concentrated as above to a sirup which was refluxed with 200 ml. of absolute methanol. This resulted in a white precipitate of inorganic salts that was removed by filtration. The methanol filtrate was concentrated to a sirup which dissolved in cold, absolute methanol with no resulting precipitate. The solution was again concentrated to a sirup which was dissolved in water and diluted to 200 ml.

Quantities of this solution ranging from 0.015 to 0.030 ml. were applied from a microburet (Aloe Scientific Division, A. S. Aloe Co., St. Louis, Mo.) to paper chromatograms which were

developed according to the procedure of Quick (*15*). The chromatograms were sprayed with aniline phthalate reagent, air-dried for 15 minutes, and then heated in an oven at 110° to 115° C. for 15 minutes. The densities of the resulting spots were read as quickly as possible with a Photovolt Electronic Densitometer, Model 525 (Photovolt Corp., 95 Madison Ave., New York 16, N. Y.) using ordinary white light and a slit width of 6 mm. The amounts of sugars corresponding to the densities of the spots were then determined from standard curves prepared from known sugar solutions in which logarithms of the concentrations were plotted *vs.* densities. This technique closely followed that of McFarren, Brand, and Rutkowski (*11*) for the quantitative determination of sugars on filter paper chromatograms by direct photometry.

The results of the quantitative determinations in percentage of the theoretical hydrolysis yield were as follows: D-glucose, 17.8%; D-mannose, 48.5%; D-galactose, 14.8%; L-arabinose, 2.8%; total yield 83.9%. This sum of the separate sugar percentages compares favorably with the 85.4% yield of a total solids analysis of the same solution.

Because the strongly acidic conditions used for hydrolysis degraded arabinose, this sugar was quantitatively determined in a separate sample by refluxing 1.0297 grams of 10% potassium hydroxide–insoluble holocellulose with 50 ml. of 1.5% sulfuric acid for 20 minutes. The mixture was filtered and the filtrate was neutralized and concentrated to a sirup, as described above. The amount of arabinose in the sirup was quantitatively determined by paper chromatography and direct photometry as described above; yield 6.0%.

The results of the quantitative determinations in percentage of the theoretical hydrolysis yield, using 6.0% for arabinose, were as follows: D-glucose, 17.8%; D-galactose, 14.8%; D-mannose, 48.5%; L-arabinose, 6.0%. Total recovery, 87.1% of the theoretical. Correcting these values to 100.0% resulted in: D-glucose, 20.4%; D-galactose, 17.0%; D-mannose, 55.7%; L-arabinose, 6.9%. Considering arabinose as 1 this resulted in a molar ratio of: L-arabinose, 1.0; D-glucose, 2.2; D-galactose, 1.8; and D-mannose 6.2.

The 10% potassium hydroxide–insoluble holocellulose of roasted coffee was hydrolyzed with anhydrous sulfuric acid, as described above for the hydrolysis of green coffee. Paper chromatograms developed simultaneously with known compounds showed the presence of arabinose, glucose, mannose, and galactose.

Results and Discussion

The procedure used in the fractionation of the ethanol-water (80 to 20) extract of green coffee was similar to the clay column techniques developed by Wolfrom and coworkers (*3, 10*).

Table I includes the data obtained on the fractionation of the ethanol-water (80 to 20) extract of the green coffee bean on a clay column.

Sucrose was identified by crystallization from the ethanol-water (95 to 5) fraction. A small amount of sucrose was also identified in the ethanol-water (80 to 20) eluent from the clay column by paper chromatography, but it resisted crystallization. It may be estimated that the green coffee bean contains 6 to 7% sucrose. This agrees with the work of Ewell (*6*) and of Slotta and Neisser (*20*).

Chlorogenic acid was identified by crystallization from the ethanol-water (80 to 20) eluent from the clay column. The green coffee bean contains a total of 6.1% of chlorogenic acid.

The presence of a trace of glucose in the ethanol-water (95 to 5) fraction of the ethanol-water (80 to 20) extract of green coffee was indicated by paper chromatography. The presence of caffeic acid and "isochlorogenic" acid was indicated by paper chromatography. "Isochlorogenic" acid was identified by comparison with a known preparation isolated by the procedure of Barnes, Feldman, and White (*1*).

The ethanol-water (80 to 20) fraction (green coffee) from the clay column contained the substances indicated in Table I. The amino acids which were found were not in complete agreement with the report of Underwood and Deatherage (*23*). However, the authors extracted the free amino acids with ethanol-water (80 to 20) whereas Underwood and Deatherage undoubtedly hydrolyzed some proteins with their 6*N* hydrochloric acid extraction. The ethanol-water (50 to 50) fraction (green coffee) from the clay column contained the substances shown in Table I.

Table I includes also data obtained on the fractionation of the ethanol-water (80 to 20) extract of the roasted coffee bean on a clay column. The investigation of the roasted coffee extract was complicated by dark brown decomposition products. There was no evident fractionation of the brown-colored substances by the clay column, and on paper chromatograms yellow-brown streaks were formed rather than discrete spots. The procedure employed for the crystallization of sucrose from the green coffee bean was unsuccessfully attempted on roasted coffee. However, paper chromatograms showed evidence for sucrose in the ethanol-water (95 to 5) eluent (clay column) of roasted coffee and crystallization was probably restricted by degradation products. Some hydrolysis of sucrose evidently occurred on roasting, because there was only weak evidence for glucose in the green coffee bean and no evidence for fructose. However, both glucose and fructose were identified by paper chromatography in the ethanol-water (95 to 5) eluent (clay column) of roasted coffee.

No attempt was made to determine the amount of chlorogenic acid found in this fraction quantitatively, as had been done for the green coffee bean.

Caffeine was not crystallized from roasted coffee bean, as it was from the green coffee bean, but was identified by paper chromatography using the new technique described previously.

The ethanol-water (80 to 20) fraction from the clay column contained the substances indicated in Table I. The paper chromatograms of the ethanol-water (50 to 50) fraction from the clay column showed no resolution, which indicated the possibility of polymeric materials. No free amino acids were found in the roasted coffee bean, which is in agreement with the work of Clements and Deatherage (*4*).

Table II is a compilation of the data on the fractionation of green and roasted coffee, leading to polysaccharide isolation. Before the polysaccharides of coffee can be separated, the low molecular weight materials must be removed. Some of these materials are easily oxidized and interfere with the holocellulose preparation. Although a clean-cut separation of one type of material from another is not accomplished, each of the fractionation procedures has a definite purpose, the total effect of which is to remove the low molecular weight materials.

Referring to Table II, the percentage of material soluble in ethanol-water (80 to 20) shows no significant difference for green and roasted coffee. The benzene-ethanol (2 to 1) extraction removes the lipides from the plant material. Table II shows a small decrease in the fat content of the roasted coffee as compared to green coffee. Much of this decrease is probably due to decomposition during the roasting process.

After removing the lipides, a water extraction at room temperature is possible. Underwood and Deatherage (*23, 24*) found that most of the protein (3%) which can be extracted from green Santos coffee beans is water-soluble. The water extract at 20° to 25° C. contained polysaccharide material in addition as evidenced by paper chromatography. The present water extractions did not completely remove the proteins, as the residue gave a positive ninhydrin test. The data of Table II show a decrease in water-soluble material from green coffee to roast coffee. Some of the water-soluble part of the green coffee undoubtedly underwent a change during the roasting process with some portions possibly volatilizing.

Dilute ammonium oxalate solution

removes the water-insoluble pectins of plants. There is little variation in the amount of apparent pectin in the green and roasted coffee. This extract probably contained trace amounts of pectic substances grossly contaminated with other polysaccharide materials.

The strong oxidizing ability of chlorine dioxide removes the lignin and leaves the carbohydrate material behind. The apparent lignin content—acidified sodium chlorite extract—rises from green to roasted coffee. This is probably due to decomposition of the carbohydrate material during roasting and extracting, thus making it soluble in the acidified sodium chlorite solution. An apparent lignin value of 2.4% was found in the green bean by the 72% sulfuric acid residue method (*16*). While some lignin may be present, it is certainly grossly contaminated by polysaccharide material.

The 10% potassium hydroxide–soluble portion constitutes the "hemicellulose" of the coffee. The increase in the hemicellulose portion of the roasted coffee is probably due to a change in the holocellulose portion during the roasting process.

Table II shows that there is a slight decrease in the 10% potassium hydroxide–insoluble holocellulose in roasted coffee as compared to green coffee. This indicates water-solubilization of the polysaccharide material by the roasting process. It is well established that pyrolysis of starches results in water-solubilization with the final production of 1,6-anhydro-β-D-glucopyranose (levoglucosan). Hudson and coworkers (*8*) report that the mannan of tagua palm seeds undergoes pyrolysis to form the water-soluble 1,6-anhydro-β-D-mannopyranose. It is probable that the holocellulose of green coffee undergoes some pyrolysis, with probable shifting (*22*) of linkages, during roasting, resulting in water-extractable materials. The increase in the acidified sodium chlorite and 10% potassium hydroxide extracts for roasted coffee, as compared to green coffee, probably arises from this decrease in the alkali-insoluble holocellulose fraction.

Because only 11.0% of the 10% potassium hydroxide–insoluble holocellulose of green coffee dissolved in cuprammonium hydroxide solution, and as this fraction was primarily mannose as shown by electrochromatography, it appears that there is very little, if any, cellulose in the green coffee bean.

Hydrolysis of the 10% potassium hydroxide–insoluble holocellulose of green coffee yielded D-glucose, D-galactose, D-mannose, and L-arabinose as indicated by paper chromatography and proved by the isolation of crystalline derivatives of each. The arabinose was readily split out by refluxing with 1.5% sulfuric acid. This result was not unexpected, because arabinose commonly occurs in nature in the labile furanose ring form.

Schulze (*17*) attempted to hydrolyze the carbohydrate component of coffee with 75% sulfuric acid, but a small residue of undissolved material always remained. As was indicated above, the authors encountered the same difficulty when attempts were made to hydrolyze the 10% potassium hydroxide–insoluble holocellulose of green coffee quantitatively by the 72% sulfuric acid treatment of Monier-Williams (*12*). Because the 10% potassium hydroxide–insoluble holocellulose of green coffee apparently did not completely hydrolyze in 72% sulfuric acid, it was decided to attempt the hydrolysis by using anhydrous sulfuric acid. Anhydrous sulfuric acid is known to be a powerful solvent for organic compounds and was applied to polysaccharides by Wolfrom and Montgomery (*29*). The technique used herein was to lyophilize the 10% potassium hydroxide–insoluble holocellulose to give better surface properties and then to dissolve the material in anhydrous sulfuric acid (hydrogen sulfate). After solution was complete, water was slowly added in the cold without effecting precipitation. The dilute acid solution was then heated to produce complete hydrolysis. As this method constituted a new procedure for hydrolyzing polysaccharides, it was decided to experiment with cellulose to determine if the method were feasible. Hydrolysis of cellulose by the procedure described in the experimental portion of this paper for the quantitative determination of the constituent sugars of 10% potassium hydroxide–insoluble holocellulose of green coffee resulted in a glucose yield of 90.3% of the theoretical as determined by the Somogyi reduction procedure (*21*). This result compared favorably with the yield of 90.7% recorded by Monier-Williams (*12*) for the hydrolysis of cellulose with 72% sulfuric acid. Thus, the anhydrous sulfuric acid appeared to be of comparable hydrolyzing ability to 72% sulfuric acid with the added advantage that it was a very powerful dissolving medium which would dissolve compounds not completely soluble in 72% sulfuric acid. The total recovery of 87.1% from the anhydrous sulfuric acid hydrolysis of the 10% potassium hydroxide–insoluble holocellulose of green coffee appears favorable, because of the difficulty of hydrolyzing the material. The value of 87.1% has been corrected for the arabinose loss occurring in the absolute sulfuric acid method by evaluating the arabinose released by mild acidity.

The value of 6.0% L-arabinose obtained by direct photometry of paper chromatograms compared favorably with the direct pentose value of 6.7% obtained by the authors and that reported by Schulze (*18*). This shows that all the pentose is L-arabinose and that it is easily acid-hydrolyzable. The use of 6.7% for arabinose makes no significant change in the ratios of the sugars reported above.

The value of 46.3% D-mannose obtained by precipitation as phenylhydrazone is slightly below the value of 48.5% obtained by direct photometry of paper chromatograms. This is probably due to the incomplete hydrolysis with the 72% sulfuric acid utilized in obtaining the former. The close agreement obtained for the L-arabinose and D-mannose values by direct methods and by paper chromatographic methods substantiates the use of direct photometry of paper chromatograms as a procedure for quantitative determinations of sugar mixtures and supports the values obtained for the sugars in the 10% potassium hydroxide–insoluble holocellulose of green coffee.

The chemical nature of the coffee polysaccharides is under further investigation.

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FLAVOR CHEMISTRY

Constituents of Meat Flavor: Beef

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The flavor precursors of cooked beef are water-soluble. Lyophilization of a water extract yields a powder concentrate that on heating develops a flavor similar to that of cooked beef. A standard technique for the heat treatment and fractionation of the flavor constituents in this powder is described. Small amounts of carbonyls, ammonia, and hydrogen sulfide have been found in the most volatile fraction. An oily, viscous, liquid mixture of very low vapor pressure, but with a strong aroma, has also been isolated.

ALTHOUGH AN UNDERSTANDING of the chemical composition of meat flavor is basic to many aspects of meat technology, an actual knowledge of meat flavor constituents is nearly nonexistent. Bouthilet (*1*, *2*) and Pippen and coworkers (*10*, *11*) studied the flavors of chicken broth and separated and identified several of the volatile constituents of chicken broth distillate. Classical studies of beef flavor chemistry (*9*) dealt largely with the analysis of beef extract, a product which is formed by the subsequent concentration of beef tissue hydrolyzates and is thus different from beef per se.

The present investigation was undertaken as part of a program on fundamental studies of meat composition. The flavor aspects of this program are expected to result eventually in identifying the naturally occurring substances which are responsible for flavor in beef, pork, and lamb, and in various products made from these meats. This paper deals with the methods used to fractionate fresh beef into potentially flavorful and nonflavored portions and with the preliminary chemical investigation of some of the isolated products.

A beef powder extract separated from raw beef has been heated under vacuum and the total volatiles have been condensed at low temperature. This condensate, in turn, has been analyzed for carbonyls, and the most volatile fraction has been examined for acidic and basic components.

Experimental

Freeze-Dried Beef Powder Extract. Fresh meat was aged for 10 days at 36° to 38° F. Several of the muscles were then dissected and stored at 0° F. As needed, 1.5 kg. of meat was thawed, fat and connective tissue were removed, and the trimmed beef was ground in an electric grinder maintained at 32° F. One part by weight of the ground beef was blended for 1 minute with 1.5 parts by volume of ice-cold, distilled water. The slurry was allowed to stand overnight at 32° F., blended again for 1 minute, and centrifuged at 4000 r.p.m. for 20 minutes in a refrigerated centrifuge kept at 28° F. The supernatant liquid was decanted, mixed with 1% w./v. of Filter-aid, and filtered under vacuum through a Büchner funnel. The filtrate was shell-frozen in a dry ice–isopropyl alcohol bath and lyophilized. The yield of dry powder was approximately 3.5% of the weight of the trimmed meat.

Distillation and Fractionation. A vacuum of better than 10^{-5} mm. of mercury was maintained by means of a two-stage oil diffusion pump backed by a mechanical vacuum pump. The fractionation train consisted of three fraction collectors. The vacuum system is shown schematically in Figure 1. Thirty to 35 grams of dried powder were placed in flask *a*. Vapor from a boiling liquid in flask *b* rose through the jacket, *c*, surrounding *a* and returned to *b* by the condenser. An auxiliary heating tape was wrapped around the jacket to ensure that vapor condensation did not take place around *a*. By the appropriate choice of liquid any desired temperature could be maintained.

In a given experiment, traces of moisture were first removed from the dried powder by evacuating the system ($<10^{-5}$ mm. of mercury) at room temperature for 4 hours. A Dewar flask containing liquid nitrogen was then placed around trap *d*. Maintaining the same vacuum, the solvent in *b* was brought to a boil and heating was continued for 6 hours. At the end of the heating period the vacuum stopcocks at *h* and *i* were turned off, the liquid nitrogen trap was removed from *d* and placed around trap *g*, and a dry ice–isopropyl alcohol freezing mixture placed around trap *e*.

Spontaneous distillation took place as the contents of trap *d* came to room

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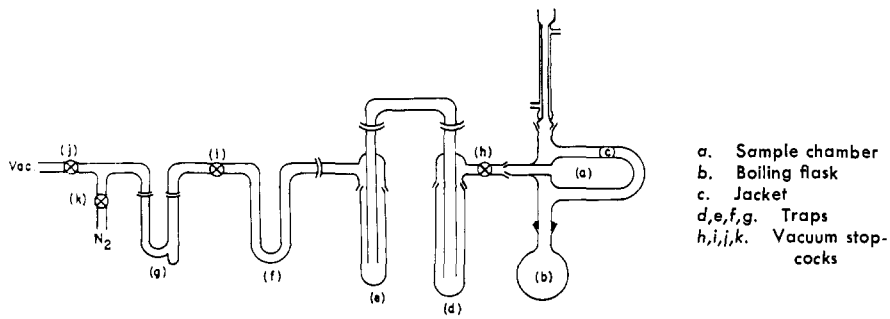

Figure 1. Vacuum system for fractionation of volatile flavor components

temperature. After 16 hours, dry nitrogen was admitted into the system.

A series of tests was first conducted to select an optimum operating temperature. At room temperature no odor products were trapped out in liquid nitrogen at 10^{-5} mm. of mercury. At 78° C. (ethyl alcohol) the over-all odor profile was essentially pleasant and fruitlike. At 100° C. (water) a similar, but more accentuated odor was noted. At 157° C. (hexyl alcohol) the predominant odor pattern was ammoniacal and unpleasant. At 188° C. (propylene glycol) the over-all odor impression was strongly ammoniacal and decidedly unpleasant; in addition, yellow crystals, not further investigated, sublimed to the top of the beef powder. At higher temperatures (200° C.) massive decomposition took place and odors characteristic of burned meat were obtained. As a result of these observations an operating temperature of 100° C. was selected and adhered to in all further experiments.

Chemical Identifications. Carbonyls in *d*, *e*, and *g* were converted to their respective 2,4-dinitrophenylhydrazones by the addition of a 2*N* hydrochloric acid solution saturated with 2,4-dinitrophenylhydrazine. In a separate experiment the acidic compounds in the most volatile fraction—trap *g*—were converted to their ammonium salts by the addition of 5 ml. of 5% ammonium hydroxide, and, in another run, basic compounds were converted to their hydrochlorides by the addition of 5 ml. of 2*N* hydrochloric acid. The residue in trap *d* consisted of a very small amount of a colorless viscous liquid, which was water-soluble and of a pleasant fruity odor. On standing exposed to air, this liquid slowly darkened and assumed a desirable meaty aroma. This material has been separated into at least two constituents by ascending paper chromatography using butyl alcohol saturated with water as the solvent. Two spots were detected by spraying the dried paper with aqueous potassium permanganate. Identification of the compounds in this mixture is under way at present.

Determination of Carbonyls. Carbonyls were identified by the paper chromatographic technique of Gaddis and Ellis (*5*, *6*). The combined phenylhydrazones were extracted from the aqueous phase with carbon tetrachloride. The solution was evaporated almost to dryness and the residue washed with carbon tetrachloride onto a column of chromatographic alumina. The chromatogram was developed with benzene and a monocarbonyl fraction eluted. The benzene was removed by distillation and the residue taken up in a minimum of carbon tetrachloride spotted on No. 3 Whatman paper. The chromatogram was developed with petroleum ether (boiling point 37° C.). Two spots appeared; these were rechromatographed on paper treated with propylene glycol using Skellysolve C (essentially heptane) as the mobile phase. The R_F values for these spots corresponded to those for authentic samples of acetone, acetaldehyde, and formaldehyde. Less than 0.05 mg. of each of these carbonyls was recovered for each gram of dried beef powder heated.

Analysis of Acid Volatiles. A portion of the fraction trapped in ammonium hydroxide on acidification evolved a gas, the major constituent of which was carbon dioxide. The remainder of the fraction was analyzed for free sulfide ion. The analytical procedure was essentially the standard reaction between hydrogen sulfide and *N*,*N*-dimethyl-*p*-phenylenediamine to form methylene blue. Recent modifications of this procedure by Marbach and Doty (*8*) and by Sastry (*12*) have been used to estimate sulfides released after zinc reduction from gamma irradiated beef and from plant materials. In the present procedure, zinc reduction was omitted, and free sulfide (H_2S) was determined on a 1-ml. aliquot of the total acid fraction, trapped in ammonium hydroxide.

Absorption was measured at 667 mμ, using a Beckman DU spectrophotometer. The actual concentration of hydrogen sulfide could be read from a standard curve prepared from known concentrations of sulfide ion obtained from sodium sulfide. Approximately 0.1 mg. of hydrogen sulfide was recovered per gram of dried beef powder.

Analysis of Basic Volatiles. The entire volatile fraction trapped in 2*N* hydrochloric acid was lyophilized to yield approximately 150 mg. of a white crystalline salt. The addition of one drop of sodium hydroxide to a few crystals of this material gave the characteristic odor of ammonia. To determine whether or not any other hydrochlorides, presumably of low boiling aliphatic primary amines, were present, 1 mg. of the salt was spotted on No. 1 Whatman paper and the chromatogram was developed with methanol. Spots were detected by spraying with an indicator similar to the one used by Davies, Wolfe, and Perry (*4*) to detect primary amines. The indicator contained 0.5% ninhydrin in 70% ethyl alcohol to which 20% pyridine v./v. had been added. Ammonium chloride did not give a spot with this indicator. However, known mixtures of ammonium chloride containing 1% of the primary methyl- and ethylamine hydrochlorides gave characteristic purple spots.

A very faint purple spot of the R_F for methylamine hydrochloride was obtained for the unknown. This spot was far less intense than the corresponding spot for the control containing 1% of the primary amines. When similar chromatograms of the unknown were sprayed with methyl orange as an indicator, an acid spot with the R_F value corresponding to ammonium chloride was obtained.

Assuming that the salt was essentially ammonium chloride, approximately 1.7 mg. of ammonia were obtained from each gram of dried beef powder heated.

Reproducibility of Results. Most of this work was done using the *longissimus dorsi* muscle. To check the reproducibility of the results from animal to animal and also from muscle to muscle, the infrared spectra of the least volatile, and, by coincidence, the most appetizing, odor constituent were obtained for several of the batches run. These spectra were similar in nature, and the general odor characteristics were identical, an indication that good reproducibility could be achieved in experimental work by the use of the isolation techniques described.

Results and Discussion

The distribution of flavors and their precursors between beef solids and juices has been studied by Crocker (*3*), and more recently by Kramlich and Pearson (*7*); their results indicated that flavor contributors were water-soluble. Preliminary experiments confirmed these findings. For example, hamburgers prepared from water-extracted ground beef were essentially tasteless and odorless; the water extract, on the other hand,

developed a "beef" aroma when heated. Freeze-drying of the unheated extract yielded a water-soluble, friable powder. This dried powder, when heated, developed an odor reminiscent of roast beef; a water solution of the powder, upon heating, evolved a boiled beef aroma. As the extraction and lyophilization procedure concentrated the flavor precursors and simultaneously simplified the system under study by the removal of fat, water, and water-insoluble matter, this concentrate has been the starting point for this work, with the realization, however, that under usual cooking conditions where fat and moisture are present, the isolated products may differ in some respects from those obtained in the present study.

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MEAT AGING AND FREEZING

Post-Mortem Changes in the Water-Soluble Proteins of Bovine Skeletal Muscle during Aging and Freezing

M. J. KRONMAN and R. J. WINTERBOTTOM

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Alteration of the water soluble proteins of bovine skeletal muscle is of particular interest, because these proteins have enzymatic character and are involved in biochemical processes occurring in meat. The effect of aging meat for 7 days and of freezing at −20° C. for 5 weeks was studied by electrophoresis and ultracentrifugation, as well as by several chemical methods. Systematic differences in protein content and in the content of the enzyme aldolase were noted from muscle to muscle. Both aging and freezing of meat were shown to result in a decreased extractability of water soluble proteins, as well as in a loss of specific electrophoretic and ultracentrifugal components.

POST-MORTEM CHANGES occurring in meat are known to be rather profound. Without doubt, a number of biochemical changes identified with alterations in tissue properties are associated with the salt extractable, structural proteins such as actin and myosin. On the other hand, many processes are controlled by enzymes found in the water-soluble myogen fraction—e.g., lactic acid production. While such protein enzymes may comprise only a small weight percentage of meat, the significance of the reactions that they catalyze may render their alteration during aging or freezing of tissue of the greatest importance.

The effect of aging and freezing on the extractability of the enzyme aldolase and the other water-soluble proteins has been studied. These data, as well as more specific information as to the alteration of the water soluble proteins by ultracentrifugal and electrophoretic analysis of muscle extracts and subfractions derived from these extracts by fractional salt precipitation, are presented.

Experimental

Materials and Methods. Details of analytical and preparative methods have been described previously (*8*).

Four animals were used in this study. Animal 6 was a 5-year-old cow, while animals 7, 8, and 9 were 18-month-old steers of choice grade. Muscle dissected from the left side of the animal approximately 20 minutes post mortem—fresh muscle—was compared with muscle taken from the right side of the same animal held at 3° C. for 7 days. For the sake of brevity the latter muscle has been referred to as aged. Samples of the fresh muscle, which had been chilled in ice for transportation to the laboratory, were frozen in a deep freeze unit about 3 hours post mortem. Frozen muscle was stored at −20° C. for 5 weeks after which it was thawed for about 24 hours at about 5° C. prior to extraction. Aqueous extracts were prepared in the manner described previously (*8*).

In the case of animal 6, an average muscle sample was taken over much of its length. In the case of animals 7, 8, and 9, sections were made according to published diagrams (*4*) and individual muscles were then removed from the proper section (Table I). Shown in the remaining columns of Table I are the locations at which sections were made, the illustration plate number of the reference cited, and the commercial cut of meat corresponding to this section.

Moving Boundary Electrophoresis and Ultracentrifuge Measurements. The methods and equipment employed have been described previously (*8*). Both types of measurements were carried out on protein solutions in pH 8.17, 0.05 ionic strength tris(*N*,*N*,*N*-hydroxymethyl) methylamine (Tris) buffer. Prior to measurement, solutions were centrifuged in the Spinco Model L at 60,000 × G in order to remove trace amounts of insoluble protein.

Because it was virtually impossible to carry out electrophoresis or ultracentrifuge measurements at identical concentrations, it was necessary to normalize the curves for purposes of calculation. The areas of projected traced

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Table I. Individual Muscles

Muscle No.	Muscle Name	Location of Section	Commercial Cut Corresponding to Section	Illustration Plate No. (4)
1	*Longissimus dorsi*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
2	*Psoas major*	4th, 5th lumbar vertebrae	Porterhouse or T bone steak	XXVII
3	*Semimembranosis*	Proximal extremity of femur	Round steak	IX
4	*Serratus ventralis*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
5	*Rectus abdominus*		Flank steak	XXI
6	*Semitendinosis*	Proximal extremity of femur	Round steak	IX
7	*Latissimus dorsi*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
8	*Trapezius*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV

Table II. Absolute Aldolase and Soluble Protein Content of Fresh, Aged, and Frozen Bovine Muscle

Animal	Aldolase Units/100 G. of Wet Tissue × 10^{-6}			Grams of Protein/100 G. of Wet Tissue		
No.	Fresh[a]	Aged	Frozen	Fresh	Aged	Frozen
6	2.1 ± 0.6	...	2.5 ± 1.1	3.55 ± 0.45	...	2.82 ± 0.28
7	2.9 ± 1.0	2.8 ± 1.0	1.6 ± 0.4	2.60 ± 0.51	2.29 ± 0.47	2.81 ± 0.28
8	2.8 ± 0.9	3.3 ± 0.9	2.8 ± 0.8	3.65 ± 0.27	2.76 ± 0.32	2.62 ± 0.22
9	2.8 ± 0.6	2.9 ± 0.8	3.9 ± 0.6	3.19 ± 0.42	2.47 ± 0.29	2.90 ± 0.26
Av.	2.6	3.0	2.7	3.25 ± 0.27	2.51 ± 0.17	2.79 ± 0.08

[a] (*8*).

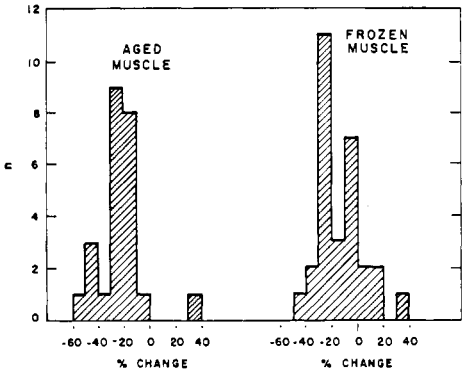

Figure 1. Per cent change in water-extractable protein for aged and frozen muscle

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

patterns were determined with the planimeter. In the case of ultracentrifuge patterns, suitable solvent base line corrections were applied. All curves shown in this paper were constructed from the tracings using a pair of proportional dividers to reduce the patterns to the same area. In each case the standard area was that of the fresh muscle pattern. For calculation of per cent change in ultracentrifugal components, use was made of a normalized peak height—i.e., the height of the peak, h, divided by the area of that pattern, A. The per cent change in this ratio, h/A, was taken as the per cent change in the amount of that component.

Results and Discussion

Protein and Aldolase Content. Table II shows average values of the protein content for eight muscles with the exception of muscle 8, animal 6, and muscles 4 and 8, animal 9, which were not studied. Figure 1 illustrates the magnitude and variation in the change in protein contents as compared to fresh muscle. As Table II and Figure 1 indicate, aging beef muscle for 7 days renders the previously soluble protein less liable to extraction from the tissue in agreement with the results of Wierbicki *et al.* (*14*). Using solubility as a criterion of denaturation, one would conclude that from 10 to 30% of the soluble protein may be denatured during the 7 days of aging.

The fact that the aged values shown in Figure 1 are somewhat larger than those reported by Wierbicki *et al.* (*13, 14*) for 13 days of aging is probably due to differences in analytical methods. The biuret procedure employed in this study will distinguish highly degraded protein from that in the native state in contrast with the analysis for total nitrogen used by Wierbicki *et al.* (*13, 14*). Furthermore, the basis for their changes was a 3-day old sample in contrast with one of 3-hours employed in this study. The extent of protein alteration during this initial 3-day period may be sufficient to account for the observed differences.

As Table II and Figure 1 indicate, 5 weeks of freezing of the fresh muscle lead likewise to a decrease in the amount of extractable protein. These results are in marked contrast with those obtained by Artyukh (*2*), and Sadikov and Khaletzkaya (*11*) who reported that freezing had no effect on the extractability of the water-soluble proteins. Loss of protein solubility due to denaturation would appear to be a more reasonable explanation in the case of freezing than degradation of protein by proteolysis, because the latter process would probably be rather slow at −20° C.

The effect of aging or freezing of tissue on extractability of aldolase is not at all clear-cut. Examination of Table II might lead one to believe that, with the exception of animal 7—frozen tissue—these processes have no effect, or if anything, lead to an increase in extractability. This is, however, only a consequence of data averaging. Shown in Figure 2 are plots of the per cent change in aldolase content *vs.* the number of samples, n, exhibiting this change. The extreme variation observed for these changes cannot be due to assaying errors and must be regarded as being due to differences in extractability of different muscle samples. No systematic variation from muscle to muscle or animal to animal was observed with the exception of animal 7—frozen tissue—which showed a consistent decrease.

This apparent wide variation in extractability of aldolase from beef skeletal muscle may be related to the question of the extractability of aldolase from rat liver. Roodyn (*10*) found that after complete destruction of liver nuclei by ultrasonic vibrations, aldolase was strongly bound to cellular debris. It may be, therefore, that the extractability of muscle aldolase will depend upon the degree of disruption of the cellular structure during the aging or freezing process and that this disruption is a function of parameters which are not apparent at the present time.

These results, together with those of Roodyn, throw some doubt on the conclusion drawn by Andrews and others (*1*) that aldolase is not a rate-limiting glycolytic enzyme. The question that remains unanswered at present is whether their observed loss of activity is due to inactivation of the enzyme or its adsorption on the homogenized tissue.

Examination of the aldolase and protein content for a given animal under a fixed set of conditions indicates a greater variation of values from muscle to muscle than one would expect from experimental error. The significance of this variation can be more readily demonstrated by comparing relative quantities—e.g., relative protein content is defined as:

$$\frac{\text{(Grams of protein per 100 grams of tissue) animal } a\text{, muscle in question}}{\text{(grams of protein in 100 grams of tissue) animal } a \text{ av.}}$$

Table III. Relative Protein and Aldolase Contents of Individual Muscles

Muscle	Relative Protein Content			Relative Aldolase Content			Specific Activity = Relative Aldolase Content / Relative Protein Content		
	Fresh[a]	Aged	Frozen	Fresh[a]	Aged	Frozen	Fresh[a]	Aged	Frozen
1	1.2 ± 0.08	1.1 ± 0.06	1.2 ± 0.15	1.4 ± 0.05	1.1 ± 0.05	1.4 ± 0.12	1.2	1.0	1.2
2	1.0 ± 0.10	0.82 ± 0.13	1.0 ± 0.07	0.72 ± 0.09	0.92 ± 0.08	1.1 ± 0.05	0.72	1.1	1.1
3	1.2 ± 0.10	1.3 ± 0.07	1.1 ± 0.08	1.3 ± 0.10	1.5 ± 0.04	1.2 ± 0.13	1.1	1.3	1.1
4	0.86 ± 0.04	0.68 ± 0.07	0.96 ± 0.14	0.65 ± 0.28	0.38 ± 0.18	0.57 ± 0.41	0.76	0.56	0.59
5	0.86 ± 0.10	1.1 ± 0.07	0.90 ± 0.08	0.71 ± 0.07	0.92 ± 0.03	0.69 ± 0.30	0.83	0.84	0.77
6	1.1 ± 0.03	1.0 ± 0.04	0.94 ± 0.11	1.4 ± 0.15	1.4 ± 0.08	1.3 ± 0.15	1.3	1.4	1.4
7	0.96 ± 0.06	1.1 ± 0.08	0.96 ± 0.04	1.1 ± 0.16	1.0 ± 0.08	0.99 ± 0.17	1.1	0.91	1.0
8	0.87 ± 0.07	0.92 ± 0.05	0.93 ± 0.02	0.75 ± 0.13	0.64 ± 0.17	0.65 ± 0.10	0.86	0.70	0.70

[a] (*8*).

Use of this type of parameter tends to eliminate variations characteristic of a given animal. Shown in Table III are values of relative aldolase and protein contents and specific activities. Shown also are the values for fresh tissue taken from a previous paper (*8*).

The fresh muscles 4, 5, and 8 differ from the others on the basis of protein and aldolase contents (*8*). In spite of the wide variation of the magnitude of the change in protein and aldolase contents on aging and freezing, these data substantiate this previous observation. The specific activities for fresh, aged, and frozen tissue all indicate that for muscles 4, 5, and 8 aldolase makes up a smaller percentage of the total water soluble protein fraction. The differences in soluble protein content are not merely a consequence of differences in aldolase contents. Assumption of a specific activity of 5.4 units per γ (*8*) for pure aldolase, permits calculation of the aldolase protein content for each sample. When such a calculation is carried out and the aldolase content is subtracted from the total soluble protein content, the differentiation of muscles 4, 5, and 8 is not only maintained, but is made even more extreme.

pH Changes. The pH that a muscle attains post mortem is a very important factor in determining its characteristics as meat. Grau and coworkers (*6*) have shown that the water-holding capacity of meat can be modified by changing its pH. More recently, Swift and Berman (*12*) demonstrated for the same eight muscles studied that the higher the ultimate pH attained by the muscle, the higher the water-holding capacity.

Shown in Table IV are relative pH values at present obtained for the extracts of aged and frozen tissue. As for protein and aldolase content, the pH values serve to differentiate muscles 4, 5, and 8 from the others—i.e., these muscles have higher pH values than the others. Similar differences in pH have also been observed by Swift and Berman (*12*) for the same group of eight muscles. They also found that the total protein nitrogen contents of muscles 4, 5, and 8 were lower than those of the other muscles.

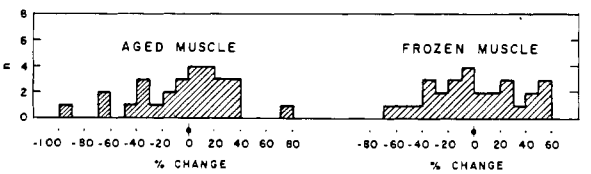

Figure 2. Per cent change in the amount of aldolase for aged and frozen muscle

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

The post-mortem pH of a muscle will be determined chiefly by the rate of lactic acid production, the buffering capacity of the muscle, and the rate of inactivation of the significant glycolytic enzymes. The latter factor may be taken to be the same for all muscles. Thus, the fact that muscles such as 4, 5, and 8 yield relatively high pH values means either that the rate of glycolysis—lactic acid production—is slower in such muscles or that their total buffering capacity is higher. While there is no information as to the total buffering capacity, the protein buffering capacities of muscles 4, 5, and 8 are lower than the others (*12*). Furthermore, if one takes the total phosphorus content of such muscles as a measure of phosphate content, muscles 4, 5, and 8 have phosphate buffering capacities lower than or comparable to the other muscles (*12*).

While it must be conceded that the relatively higher pH values of muscles 4, 5, and 8 could be a consequence of higher anserine and carnosine contents (*3*), they may, on the contrary, be due to lower rates of lactic acid production. The fact that these three muscles have lower amounts of water-extractable protein—the enzyme rich protein—suggests that the cellular concentration of rate-limiting glycolytic enzymes is lower in these muscles. Aldolase, one of the important enzymes of glycolysis, makes up a smaller percentage of the soluble proteins in these three muscles than in the others. Until the question of the rate-limiting character of aldolase or other glycolytic enzymes is answered, it cannot be certain that the final pH value a muscle can attain will be a function of its soluble protein content.

Table IV. Relative pH, Average

Muscle No.	Aged Muscle[a]	Frozen Muscle[b]
1	0.996 ± 0.014	0.981 ± 0.021
2	0.989 ± 0.010	0.995 ± 0.006
3	0.976 ± 0.008	0.988 ± 0.012
4	1.056 ± 0.023	1.031 ± 0.026
5	1.008 ± 0.003	1.019 ± 0.005
6	0.981 ± 0.003	0.992 ± 0.008
7	0.987 ± 0.005	0.993 ± 0.005
8	1.007 ± 0.003	1.012 ± 0.016
Av. Absolute	5.51 ± 0.10	5.44 ± 0.09

[a] Av. for animals 7, 8, and 9.
[b] Av. for animals 6, 7, 8, and 9 with the exception of muscle 8, animal 6, and muscles 4 and 8, animal 9 which were not studied.

Furthermore, gross variations in glycogen levels could also be responsible for differences in rates of lactic acid production. Small differences in glycogen concentration apparently do not have much effect on ultimate pH of beef muscle (*7*). Until more detailed analytical data become available on the composition of these eight muscles, none of the factors considered can really be excluded.

Fractionation of Muscle Extracts. Solubility has commonly been taken as a criterion of protein denaturation. In order to determine what effect the aging and freezing of muscle has on the water-extractable proteins, such extracts were fractionated with ammonium sulfate in the manner described previously (*8*). Shown in Table V are the protein distributions obtained for fresh, aged, and frozen *longissimus dorsi*—rib eye.

As was true for the fresh muscle, a relatively large percentage of the pro-

Table V. Protein Distribution in *Longissimus Dorsi*, Rib Eye

Fraction No.	$(NH_4)_2SO_4$, Molarity	Total Soluble Protein, %											
		Animal 7			Animal 8			Animal 9			Average		
		Fresh	Aged	Frozen	Fresh	Aged	Frozen	Fresh	Aged	Frozen	Fresh	Aged	Frozen
FI	0–1.75	5.4	5.0	7.2	5.5	3.5	8.2	4.8	3.5	10.6	5.2 ± 0.3	4.0 ± 0.7	8.7 ± 1.3
FII	1.75–2.40	8.5	8.7	12.4	3.5	8.9	9.5	2.2	8.9	17.2	4.7 ± 1.8	8.8 ± 0.1	13.0 ± 2.8
FIII	2.40–2.65	10.1	12.4	8.2	4.9	10.4	14.9	8.9	5.2	17.9	8.0 ± 2.0	9.3 ± 2.8	13.7 ± 3.6
FIV	2.65–3.35	25.7	22.7	17.2	30.0	34.0	18.0	29.7	34.0	11.2	28.5 ± 1.8	30.2 ± 5.0	15.5 ± 2.8
SIV	>3.35	17.9	16.5	20.5	11.8	8.0	16.7	4.1	8.0	17.5	11.3 ± 4.8	10.8 ± 3.8	18.2 ± 1.5
	Unrecovered protein	32.4	35.0	34.4	44.3	33.0	32.6	50.3	40.6	36.1	42.3 ± 6.7	36.2 ± 2.9	34.3 ± 1.2

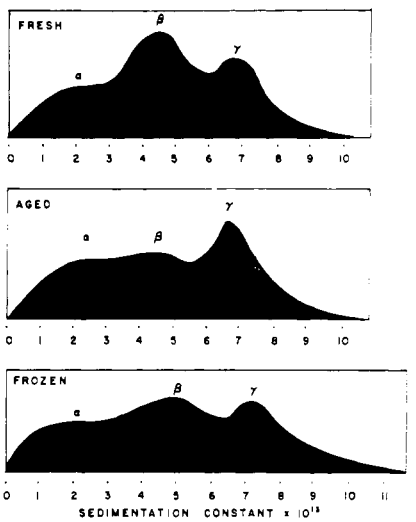

Figure 3. Normalized sedimentation patterns for extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Speed. 59,780 r.p.m.
Temperature. 10° C.
Time. 80 minutes
Bar angle. 50°
Solvent. pH 8.17 Tris buffer, $\gamma/2 = 0.05$
Protein concentration. 1.3 grams/100 ml. for fresh; 1.2 grams/100 ml. for aged; 1.1 grams/100 ml. for frozen

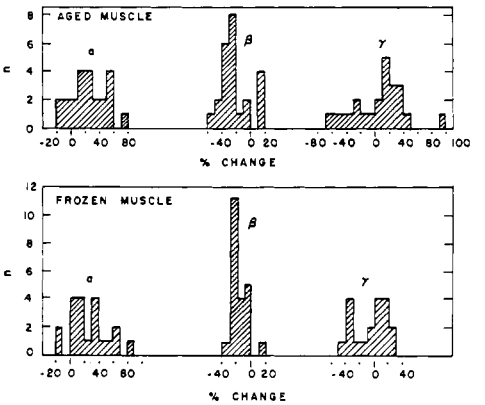

Figure 4. Per cent change in ultracentrifugal components, α, β, and γ aged and frozen tissue

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

tein is not recovered in the fractions and presumably corresponds to the insoluble protein removed during the course of the fractionation. Whether this unrecovered protein was denatured before addition of ammonium sulfate or became so during the course of the fractionation cannot be answered with certainty.

The total amount of protein precipitating in fractions FI, FII, and FIII from frozen tissue is significantly greater than that derived from fresh tissue (Table V). Presumably, this increased amount of protein precipitating at lower ammonium sulfate concentrations is protein which has been altered during the storage at −20° C. so as to decrease its solubility. This increase in the first three fractions was obtained at the expense of a decrease in the amount of fraction FIV derived from frozen tissue.

As Table V illustrates, aging of tissue had little effect on the solubility of the myogen proteins in ammonium sulfate solutions. Nonetheless, as will be shown in a subsequent section, a rather drastic change in the myogen protein fraction has occurred.

Sedimentation and Electrophoretic Analysis. In order to obtain information as to the extent and freezing of muscle tissue, electrophoretic and sedimentation analysis was carried out on extracts and ammonium sulfate fractions. Both electrophoresis and sedimentation patterns can serve as a kind of fingerprint for protein components in complex mixtures, if the pure components have been previously studied. This approach has been considered in a previous paper (*8*).

Figure 3 shows three representative ultracentrifuge patterns obtained for extracts of muscle 1, *longissimus dorsi*. These patterns have been normalized in the manner described in the experimental section. The sedimentation patterns of extracts of aged and frozen muscle are qualitatively similar to those of fresh tissue in that they exhibit the characteristic α, β, and γ groups having average sedimentation constants of 2.3*S*, 4.7*S*, and 6.8*S*, respectively. Comparison of the patterns in Figure 2 indicates a change in the sedimentation pattern which means that the relative proportions of proteins having different molecular weights has changed.

Summarized in Figure 4 are data derived from ultracentrifuge measurements on extracts of all eight muscles from animals 7, 8, and 9. The muscles noted in the footnote to Table IV were not studied. The percentage change for a given component was calculated by comparison of the normalized peak heights in the manner described under Experimental. Considerable variation in the actual numerical value of the percentage change was observed from sample to sample without any apparent regularity from muscle to muscle as was the case for other properties considered previously. Nonetheless, the direction of change for both component α and β was quite evident.

Component α showed a significant increase on aging and freezing. Component β decreased in both cases, but probably to a greater extent in the case of the aged muscle. The experimental data for component γ are inconclusive, yielding increases apparently as often as decreases.

The average molecular weight of proteins in the α group will almost certainly be less than those of proteins in the β group. It therefore seems reasonable to associate an increase in component α with degradation of larger protein molecules—e.g., proteins of group β.

Examination of ultracentrifuge and electrophoresis patterns obtained from ammonium sulfate fraction FI indicated even more striking changes in protein composition. Sedimentation patterns (Figure 5) obtained for this fraction derived from muscle 1, animal 9 are quite representative of patterns obtained for all three animals. Superficially, the patterns for the fresh muscle fraction FI (Figure 5) and the fresh muscle extract (Figure 3) are comparable. More careful examination of these patterns, however, indicates an enrichment of components having sedimentation constants greater than 7.5*S*. The patterns of Figure 5 show that the bulk of protein having sedimentation

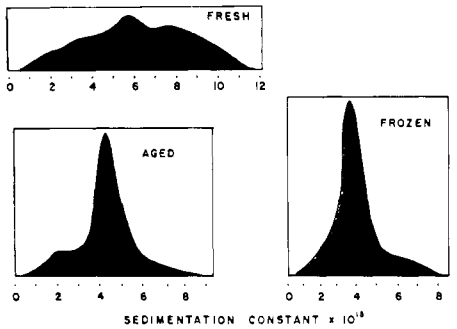

Figure 5. Normalized sedimentation patterns for fraction FI derived from extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Speed. 59,780 r.p.m.
Temperature. 10° C.
Time. 80 minutes
Bar angle. 40°
Solvent. pH 8.17 Tris buffer $\gamma/2 = 0.05$
Protein concentration. 1.5 grams/100 ml. for fresh; 1.0 gram/100 ml. for aged; 1.2 grams/100 ml. for frozen

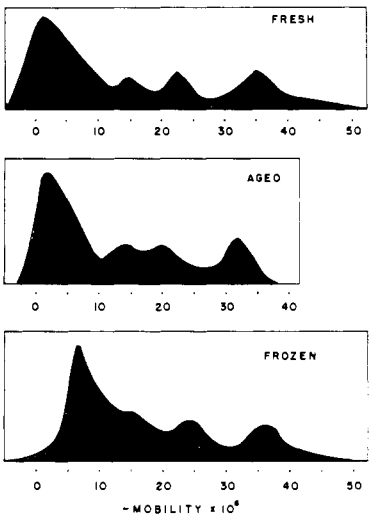

Figure 6. Normalized electrophoretic patterns for extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Field strength. 14.3 volts cm.$^{-1}$
Time. 6000 seconds
Solvent. pH 8.17 $\gamma/2 = 0.05$ Tris buffer
Protein concentration. 1.3 grams/100 ml. for fresh; 1.2 grams/100 ml. for aged; 1.1 grams/100 ml. for frozen

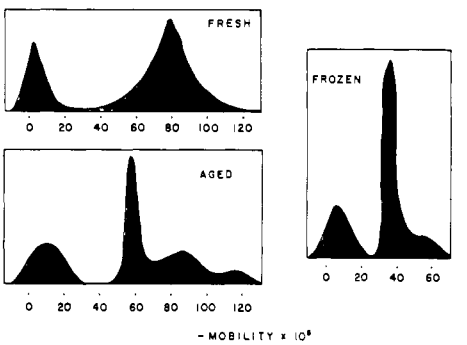

Figure 7. Electrophoretic patterns for fraction FI derived from extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Field strength. 14.3 volts cm.$^{-1}$
Time. 4300 seconds
Solvent. pH 8.17 $\gamma/2 = 0.05$ Tris buffer
Protein concentration. 1.5 grams/100 ml. for fresh; 1.0 gram/100 ml. for aged; 1.2 grams/100 ml. for frozen

constants greater than 7.5*S* has been lost upon aging or freezing.

Changes in electrophoretic patterns usually occurred on aging or freezing, but were less clear-cut than those occurring in sedimentation patterns. Shown in Figure 6 are representative electrophoresis patterns of extracts of muscle 1, animal 9. While changes in the relative amounts of components were noted, these changes were not at all consistent. Similar nonregular changes were observed in electrophoresis patterns of ammonium sulfate fractions FII, FIII, and FIV. The reason for this lack of reproducibility is not clear.

Changes in patterns obtained for fraction FI, however, were quite clear-cut. Shown in Figure 7 are representative curves obtained from muscle 1, animal 9. Proteins having mobilities in excess of 50×10^{-6} are found exclusively in FI (*8*). Comparison of Figures 7, *b* and *c*, with 7, *a*, demonstrates that aging or freezing results in a loss of protein components having mobilities in excess of -65×10^{-6}. This effect is particularly pronounced in the case of the frozen tissue where components having mobilities in excess of -70×10^{-6} are completely absent. These losses are accompanied by an increase in the amount of material of lower mobility.

Identification of protein components which change on aging or freezing tissue is handicapped by the fact that many of the pure components have never been isolated and characterized and are only known to be present on the basis of their enzymatic activity. The situation is further complicated by the fact that the bulk of the work done has been with rabbit muscle proteins. The identification of some of these components of fresh muscle was considered in a previous communication (*8*). The tentative conclusions made are of some value in identifying components which change during the aging or freezing process.

The sedimentation constants of lactic dehydrogenase and phosphorylase *b* are 7.0*S* and 8.2*S*, respectively, and their solubilities are such that one would expect to find them in fraction FI (*8*). Thus, the material lost from FI may be partially composed of these two enzymes. The mobilities of these two enzymes place them in the main peak of Figure 4, *a*. Because this main peak is displaced on aging or freezing (Figure 5, *b* and *c*) there is support for the hypothesis that lactic dehydrogenase and phosphorylase *b* are two of the components lost during aging and freezing. The decrease in the β component in whole extracts (Figures 3 and 4) may be due to a loss of ATP–creatine transphosphorylase, which has a sedimentation constant of 5.0*S* and is probably present in relatively large amounts in beef muscle [10 to 38% in rabbit muscle (*8*)].

The changes in the soluble protein fraction of beef muscle are obviously less profound than those observed by Zender *et al.* (*15*) during the aseptic storage of lamb muscle at 25° and 38° C. In their experiments, 42 days of storage yielded significant amounts of low molecular weight peptides and gross alterations of electrophoretic patterns of extracts. In the experiments reported here, a maximum of 30% of the soluble protein may have been converted to amino acids and low molecular weight peptides on freezing and aging. This amount represents unextractable protein and as has been pointed out may consist of degraded protein undetectable by the biuret procedure.

It seems likely that degradative changes are associated in part with hydrolysis by the naturally occurring proteolytic enzymes of muscle (*15*). It should be emphasized that much more subtle changes in protein structure occur which can so alter a protein-enzyme as to completely halt any process in which it is involved. The splitting of pepsin (*5*) and phosphorylase *a* (*9*) by very gentle chemical treatment are good examples of this type of process. In the former case this is accomplished by increasing the pH beyond 7, while in the latter it occurs on blocking certain sulfhydryl groups. These considerations, as well as the experimental evidence presented, show that extractability or solubility of proteins does not provide a sufficiently sensitive criterion for protein alteration. Measurements of specific properties such as enzymatic activity, acid and base-binding ability, ultracentrifugal and electrophoretic behavior would appear to be more promising methods for elucidating post-mortem changes in the proteins of meat.

Acknowledgment

The authors wish to thank P. S. Dunning and L. E. Weinberger for their very able execution of some of these experiments. The assistance of Harold Moyer, Donald Moyer, Marvin Weisenborn, and Henry Hunsberger of the C. D. Moyer Co., and J. W. Detweiler, Federal Meat Inspection Service, is also gratefully acknowledged.

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FOOD DISCOLORATION

Reddening of White Onion Tissue

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Factors affecting the formation of the water-soluble, red nitrogenous pigment in acidified white onion purée were investigated. The presence and absence in onions of several compounds are reported. Added acetic acid was not necessary for pigment formation, and its great enhancing effect was traced to the 1 to 3 p.p.m. of formaldehyde which occurs in reagent glacial acetic acid as an impurity. Other similar compounds such as diacetyl, acetoin, and glycolonitrile can replace formaldehyde in the reddening reaction, and studies were made in an effort to identify the naturally occurring carbonyl compound. A type-reaction mechanism for the pigment formation is postulated and discussed.

A WATER-SOLUBLE RED pigment is formed in macerates of white onion bulb tissue when the purée is allowed to stand after acidification with acetic acid. In an earlier paper (*5*), it was shown that the pigment formation in purées was affected by variety, storage conditions, and heat and acid treatments.

New evidence affecting pigmentation has been found, and it has been possible to develop the problem considerably. The acetic acid specificity had been fully explained and, along with this, a general type sequence of steps is postulated. These are presented in this paper.

Experimental

Southport White Globe onions obtained from the same sources as previously reported (*5*) were used, as well as some onions from the same lots used in past work. In addition, one lot grown in Washington State was obtained. No significant difference was found between those from Washington and those from the Stockton region of California.

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Methods of preparing the samples, adding reagents, extracting, and measuring the pigment formed were identical with those previously reported.

Properties of the Pigment

The mpure pigment, prepared in the usual manner, could be purified further by adding sodium hydroxide until the aqueous-alcoholic solution reached pH 9.5. The pigment remained in solution, but a colorless, amorphous nitrogen-free precipitate settled out. After filtering rapidly, the clear solution was reacidified to avoid excessive destruction of pigment at the high pH. Acetone was added and the precipitated salt filtered off. Then the pigment was precipitated by ether addition. The solid was redissolved in absolute ethyl alcohol, filtered, and reprecipitated with ether to remove the remaining traces of inorganic salts. The solid preparation was then lyophilized for 24 hours and used immediately.

The infrared absorption spectrum of the pigment, prepared as above, was determined by using the potassium bromide pellet technique. The data obtained are plotted in Figure 1.

The same pigment preparation was used for organic elementary analysis, with the following results:

Element	Per Cent
C	27.96
H	6.70
N	7.88
O	44.06
Ash	13.4

A comparison of the infrared data and the elementary analysis with the previously published data (*5*) shows the relative purity of the two preparations.

The pigment coupled readily with diazotized sulfanilic acid, forming a compound which was orange in acid and red in base. The diazonium salt was prepared in the classical manner by dissolving 10.5 grams of sulfanilic acid in 100 ml. of 2.5% sodium carbonate by boiling. After cooling the solution, 3.7 grams of sodium nitrite were added, stirred until dissolved, and poured into a beaker containing 50 grams of ice and 10 ml. of concentrated hydrochloric acid. For coupling, sodium hydroxide was added to the aqueous solution of the onion pigment, then the diazotized sulfanilic acid suspension was added, stirred, and allowed to stand. The red solid was filtered off, redissolved in 95% ethyl alcohol, giving an orange solution, and precipitated as a yellow oil by ether addition. After redissolving in absolute methanol, the material was precipitated as an orange solid by addition of ether.

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FOOD DISCOLORATION

Reddening of White Onion Tissue

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Factors affecting the formation of the water-soluble, red nitrogenous pigment in acidified white onion purée were investigated. The presence and absence in onions of several compounds are reported. Added acetic acid was not necessary for pigment formation, and its great enhancing effect was traced to the 1 to 3 p.p.m. of formaldehyde which occurs in reagent glacial acetic acid as an impurity. Other similar compounds such as diacetyl, acetoin, and glycolonitrile can replace formaldehyde in the reddening reaction, and studies were made in an effort to identify the naturally occurring carbonyl compound. A type-reaction mechanism for the pigment formation is postulated and discussed.

A WATER-SOLUBLE RED pigment is formed in macerates of white onion bulb tissue when the purée is allowed to stand after acidification with acetic acid. In an earlier paper (*5*), it was shown that the pigment formation in purées was affected by variety, storage conditions, and heat and acid treatments.

New evidence affecting pigmentation has been found, and it has been possible to develop the problem considerably. The acetic acid specificity had been fully explained and, along with this, a general type sequence of steps is postulated. These are presented in this paper.

Experimental

Southport White Globe onions obtained from the same sources as previously reported (*5*) were used, as well as some onions from the same lots used in past work. In addition, one lot grown in Washington State was obtained. No significant difference was found between those from Washington and those from the Stockton region of California.

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Methods of preparing the samples, adding reagents, extracting, and measuring the pigment formed were identical with those previously reported.

Properties of the Pigment

The mpure pigment, prepared in the usual manner, could be purified further by adding sodium hydroxide until the aqueous-alcoholic solution reached pH 9.5. The pigment remained in solution, but a colorless, amorphous nitrogen-free precipitate settled out. After filtering rapidly, the clear solution was reacidified to avoid excessive destruction of pigment at the high pH. Acetone was added and the precipitated salt filtered off. Then the pigment was precipitated by ether addition. The solid was redissolved in absolute ethyl alcohol, filtered, and reprecipitated with ether to remove the remaining traces of inorganic salts. The solid preparation was then lyophilized for 24 hours and used immediately.

The infrared absorption spectrum of the pigment, prepared as above, was determined by using the potassium bromide pellet technique. The data obtained are plotted in Figure 1.

The same pigment preparation was used for organic elementary analysis, with the following results:

Element	Per Cent
C	27.96
H	6.70
N	7.88
O	44.06
Ash	13.4

A comparison of the infrared data and the elementary analysis with the previously published data (*5*) shows the relative purity of the two preparations.

The pigment coupled readily with diazotized sulfanilic acid, forming a compound which was orange in acid and red in base. The diazonium salt was prepared in the classical manner by dissolving 10.5 grams of sulfanilic acid in 100 ml. of 2.5% sodium carbonate by boiling. After cooling the solution, 3.7 grams of sodium nitrite were added, stirred until dissolved, and poured into a beaker containing 50 grams of ice and 10 ml. of concentrated hydrochloric acid. For coupling, sodium hydroxide was added to the aqueous solution of the onion pigment, then the diazotized sulfanilic acid suspension was added, stirred, and allowed to stand. The red solid was filtered off, redissolved in 95% ethyl alcohol, giving an orange solution, and precipitated as a yellow oil by ether addition. After redissolving in absolute methanol, the material was precipitated as an orange solid by addition of ether.

Factors Affecting Pigment Formation

During the first stages of this research, a difference was observed in the rate of pigment formation which depended upon the time elapsed between puréeing and acidifying the tissue. Active precursors of the pigment appeared to form as soon as the tissue was puréed, and the addition of acetic acid caused the precursor already formed to react, forming the red pigment. At the same time, formation of more precursor completely stopped.

Assuming, for simplicity, that there are only two steps involved in pigment formation—the change which occurs immediately after puréeing to form the precursor, and the coupling of the precursor with another compound in the presence of acetic acid—an attempt was made to study the first step.

Sixteen hundred grams of purée were separated into four 400-gram lots. Each was held at 22°, 40°, 50°, or 60° C. for 1¼ hours, cooled to room temperature, and divided into eight 50-gram portions, and varying amounts of acetic acid were added to each. The visual color, pH, and Klett reading were measured after 48 hours at room temperature. In every case the color was increased with increased acetic acid concentration and there was a definite temperature optimum for the first step near 50° C.

Samples of fresh purée were heated rapidly to 50° C. in a hot water bath and stored at 50° C. for varying lengths of time. They were then cooled, acidified to pH 2.7 to 2.8, and allowed to redden at room temperature for 24 hours (Table I).

The initial step was apparently unaffected by calcium ion, but was inhibited by acetic acid and lead. Blending with calcium chloride or addition of calcium salt immediately after puréeing had no effect on color formation, but addition of lead acetate or acetic acid before blending or immediately after puréeing inhibited color formation.

Effect of Enzyme Treatment

The first step might seem to be enzymatic, but none of the usual enzyme inhibitors, except sodium cyanide, had any appreciable effect when added to fresh purée. A number of different enzymes from other sources were added to fresh purées. Whether these were added to fresh purées or to freshly steam-blanched purées, the results were identical. None of the following enzymes added to the purée, at a level of 0.1 gram per 100 grams of purée, had any effect upon pigment formation, when the mixture was incubated for 3 hours at 35° C., followed by acidification and storage at room temperature.

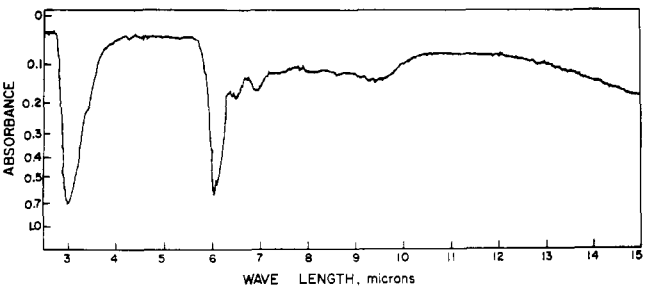


Figure 1. Infrared spectrum of the purified onion pigment

Pectinase	Anthocyanase	Hydralase	Peroxidase
Pectinol	Bromelain	Mylase P	Tyrosinase
Emulsin	Diastase of malt	Rapidase	Acid phosphatase
Clarase	Papain	Taka-diastase	β-Glucosidase
Mylase	Multizyme	Catalase	Pepsin
Trypsin			

The above enzymes were also added to dehydrated onion preparations obtained commercially; none had an appreciable effect on pigment formation.

An attempt was then made to isolate a possible enzyme occurring naturally in the onion tissue which was catalyzing the initial reaction. As reported previously (*5*), the whole pinking system is soluble and when onion juice was dialyzed for 48 hours, then acidified with glacial acetic acid, no pigment formed.

Freshly prepared purée was frozen at −15° C., thawed, and filtered cold. Acetone at 0° C. was mixed with the cold juice to a final concentration of 50%. The precipitate was washed with cold 50% acetone. More material was precipitated at 75% acetone and washed as before. A third solid preparation was obtained by thawing frozen purée directly in acetone solution, to give a final acetone concentration of 50%, and filtering. The various soluble fractions were used in the following manner.

Onions were peeled and sliced, and the slices were steam-blanched for 5 minutes to inactivate all natural enzymes. The material, after being puréed, was divided into samples for mixing with the acetone-precipitated preparations described above. The purées were mixed and stored for 1 hour at 50° C., cooled, acidified to pH 3, and stored at 20° C. for possible reddening. None of the solid preparations added caused reddening. When the 50% acetone solution was evaporated to less than half its volume in vacuo, to remove most of the acetone, it began to redden. With added acetic acid, it became a vivid pink in 2 hours at 20° C.

Acetic Acid Specificity

Figure 2 shows the amount of pigment formed in a typical experiment in relation to the amount of acetic acid added and to the pH of the final solution. The points are corrected for volume changes due to the large amounts of acetic acid required to lower the pH in the lower ranges. Obviously, the acetic acid concentration is the important factor and not the pH. Because previous experiments had shown conclusively that neither acetate concentration nor hydrogen ion concentration per se was limiting, it was suspected that the reddening was caused by an impurity in the acetic acid used.

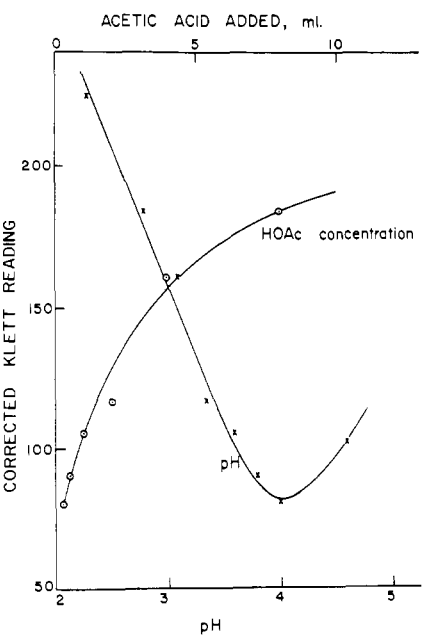


Figure 2. Onion tissue reddening as a function of acetic acid concentration and pH

Table I. Rate of Formation of Precursor

Storage at 50° C., Min.	Klett Reading	Visual Color[a]
0	50	White
10	214	(Pink) ++
20	193	++
40	276	+++
80	284	+++
160	355	++++
320	187	+

[a] Number of plus signs indicates degree of redness.

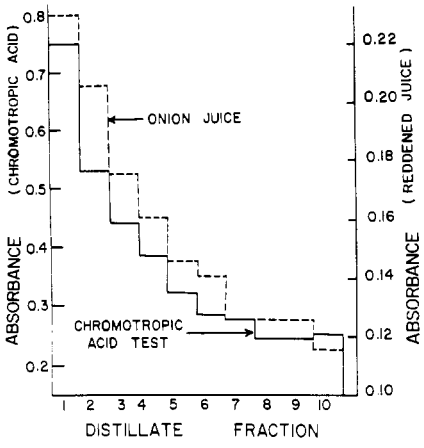


Figure 3. Comparison of onion tissue reddening in the presence of various distillate fractions of acetic acid with the absorbance of chromotropic acid tests of the same acetic acid fractions

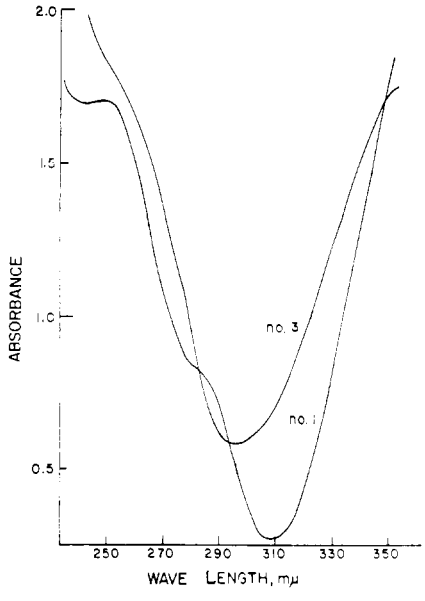


Figure 4. Ultraviolet spectra of DNP derivatives of naturally occurring carbonyl compounds

A "pure" glacial acetic acid was prepared from sodium acetate and sulfuric acid. This was used as a control by adding equal amounts of it and "commercial" reagent glacial acetic acid to identical purée samples. The pure sample did not redden, while the commercial sample did. In addition, the pure acetic acid was found to be chromotropic acid negative—the commercial was positive.

A fractional steam distillation of commercial acetic acid was carried out. One liter was steam-distilled at a rate of about 1 ml. per minute. Ten 25-ml. fractions were collected and 1 ml. of each was mixed with 0.5 ml. of 10% chromotropic acid and 5 ml. of sulfuric acid. After heating at 100° C. for 30 minutes, they were cooled and the absorbance read in 1-cm. tubes at 570 mμ.

Table II. Absorbances of Chromotropic Acid Tests (570 Mμ)

Distillate Fraction	Fresh Purée	Steam-Blanched	Held at 50° C. for 1 Hour	Reddened Purée
1	0.85	0.24	0.58	0.58
2	0.46	0.20	0.44	0.38
3	0.34	0.175	0.36	0.27
4	0.315	0.17	0.27	0.25
5	0.25	0.15	0.22	0.23
6	0.235	0.14	0.17	0.20
7	0.22	0.13	0.16	0.17

Two hundred and fifty milliliters of fresh onion juice were prepared, heated at 50° C. for 1 hour, cooled, brought to pH 3.5 by the addition of sulfuric acid, and divided into ten 25-ml. fractions. Five milliliters of each of the acetic acid distillate fractions were added to the corresponding samples of juice and after reddening, they were filtered and the absorbance was read at 550 mμ. The results are plotted along with the distillate chromotropic acid tests (Figure 3). A complete study of the acetic acid impurity was undertaken and it was shown to be formaldehyde (*12*). An optimum formaldehyde concentration for reddening near 3 p.p.m. was found.

Several other carbonyl compounds, including both diacetyl and acetoin, methyl ethyl ketone (but not acetone), and glycolonitrile also gave red pigments in onion tissue under similar conditions. The compound 5-hexene-2-one yielded a blue-green pigment; other carbonyl compounds (acetaldehyde, acetoacetic ester, and dihydroxyacetone) were inhibitory. Hydrogen peroxide also increased pigment formation during the second step. Hydrogen peroxide was added to the purée after acidification (formaldehyde addition), showing an optimum for pigment formation at 0.002 *M* hydrogen peroxide. Hydrogen peroxide, like acetic acid, inhibited the first step.

Carbonyl Constituents

Onion purée, stored without added formaldehyde for nearly 1 week, formed a very small amount of red pigment. This formaldehyde-free, red onion pigment was isolated in the same manner as the previous pigment. The absorption spectrum differs slightly from that of the formaldehyde pigment in the visible (*14*), and is quite different in the ultraviolet (*11*). Thus, formaldehyde does not occur naturally, even in traces, in these onions. However, the red pigments formed from the addition of diacetyl, etc., to the purées were not studied further and it is assumed that one of these carbonyl compounds occurs naturally and reacts with the precursor formed in the first step to give the red pigment. Lukes (*8*) showed that microbial contamination was not involved in onion reddening. The naturally occurring carbonyl compound was studied by the following procedure.

Two hundred grams of fresh onion purée were diluted with 300 ml. of water and steam-distilled at a rate of 5 ml. per minute. Ten 25-ml. fractions were collected and tested with chromotropic acid as before. Fraction 1 was turbid and had a strong onion odor. Fraction 2 was less turbid, etc., until fraction 7 was clear. The chromotropic acid results were similar; number 1 was a brownish purple, number 2 light brown (no purple), and the others were successively less positive. All of the substances giving a purple chromotropic acid color were in the first fraction. Each fraction was added to 50 ml. of a saturated solution of dinitrophenylhydrazine (DNP) in 2*N* hydrochloric acid and allowed to stand. After 24 hours, the orange precipitates in the first four fractions (no precipitate formed in the others) were filtered off, washed, and dried. The combined solids were then dissolved in a very small amount of benzene–petroleum ether (40 to 60) and the solution was passed over a dry silicic acid–Hyflo (1 to 1) column. Elution with the same solvent mixture gave three bands: number 1, buff colored; 2, yellow; and 3 (at the origin), dark orange.

The column was extruded, the bands were cut off, and the hydrazones present were eluted with acetone, precipitated with water, and recrystallized from hot 95% ethyl alcohol.

Hydrazone 1 formed red-orange needles, melting point 158–9° C. The ultraviolet spectrum is given in Figure 4, although no further identification was made.

Hydrazone 2 formed orange needles, melting point 154–5° C. Mixed melting point with the pure derivative of propionaldehyde showed no depression. The reported existance of propionaldehyde in onion tissue (*7*) is therefore confirmed.

Hydrazone 3 was present in such small amounts that a definite melting point could not be obtained. The ultraviolet absorption spectrum of its dilute solution in 95% ethyl alcohol is given, however.

It is not known whether one of these volatile carbonyl compounds is responsible for the positive chromotropic acid test in onion purée. To determine whether the chromotropic acid positive fraction changed during the process, four stages of reddening were investigated: fresh onions, purée held at 50° C.

for 1 hour, purée held at 50° C. for 1 hour and then allowed to redden, and purée from steam-blanched fresh onions. Two hundred grams of each of these purées were steam-distilled, as before, and after comparing their chromotropic acid tests (Table II), the DNP derivatives of each distillate sample were prepared.

A visual comparison of the curves for the first few distillate fractions indicated that there might be a volatile inhibitor and a nonvolatile reactant present in onion tissue. The inhibitor, being more volatile than the other compounds present, was lost on storage at 50° C. allowing the added formaldehyde or a similarly acting compound to combine with the reactant to give the pink pigment. It is clearly seen that no steam-volatile compound was used up during the reddening reaction, although volatile compounds are present.

Nitrogenous Constituents

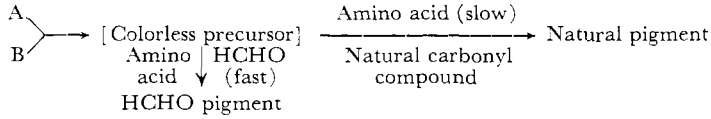
Which amino acids enter into the pinking reaction is not certain. Lukes believes that glycine is the most likely, although other amino acids are able to form similar pigments (*8*). Earlier (*5*, *10*) it was shown that at least three pigments were formed under the present conditions. All the experimental work has dealt with the major pigment.

One kilogram of fresh onion purée was clarified by pectinase treatment, then filtered until a clear juice was obtained. This was divided into three parts; one was passed over a 1 × 10 inch anion exchange column (Duolite A-2), one over a cation exchange resin (Duolite C-3), and the third was given no ion exchange treatment. All three were then acidified with glacial acetic acid and stored at 37° C. for reddening. The cation-free onion juice was colorless, while the anion-free and control samples reddened after 12 hours.

Following the general procedures used by Hulme (*4*), onion juice was passed over a Zeokarb H cation exchange resin. After thorough washing, the column was eluted with 0.5*N* ammonium hydroxide. The ammonia eluate was evaporated to less than 10 ml. on a hot plate, at which point crystals of tyrosine began to form in the neutral solution. The solution was cooled, filtered, and passed over a Dowex 50 × 4 cation exchange column (1 cm. in diameter × 70 cm. long). Elution was carried out by using a 0.2*M* borate buffer at pH 8 and containing 0.1*M* sodium chloride. One-milliliter fractions were collected; Figure 5 shows the ninhydrin reaction plotted *vs.* the fraction number (*15*). In Figure 5, 1 kg. of onion juice was used, the ammonia eluate was reduced to exactly 10 ml. (after removal of excess tyrosine), and 0.5 ml. was used on the final Dowex column.

None of the compounds in the bands was found to react with formaldehyde in acetic acid solution to give a red pigment. Band *A* was shown by usual paper chromatographic methods to contain tyrosine; band *B*, histidine. The actual concentration of these amino acids present was determined by the α-nitroso-2-naphthol method of Ottaway (*9*) for tyrosine and the diazotized sulfanilic acid method of Fraenkel-Conrat and Singer (*3*) for histidine. It was found that 35 mg. of tyrosine and 21 mg. of histidine occur in 1 kg. of the Southport White Globe onions used in this study. The complete absence of arginine was shown by the α-naphthol–hypobromite reaction of Acher and Crocker (*1*). (The original Zeokarb column was eluted with 0.1*N* sodium hydroxide, in this case, to ensure the removal of any arginine present.) Proline and hydroxyproline were also absent. In model systems, no definite effect was detected in any case, although methylhistidine, sarcosine, canavanine, ornithine, citrulline, histamine, tyramine, prolylglycine, anserine, and carnosine were added to the purée in addition to all the "common" amino acids. None gave a red color with formaldehyde in acetic acid solution and none resulted in an increase in pigment formation when added to onion purée, either before or after a 50° C. holding period. None of the proteolytic enzymes (papain, pepsin, trypsin, bromelain) added to onion purées exerted any effect upon pigment formation. Obviously, amino acid concentration is not a limiting factor.

The simplest total system possible is shown below:



The evidence presented above makes it questionable whether any step is enzymatic. If not, compound A or B must be heat-labile. Both the colorless precursor and the naturally occurring carbonyl compound are extracted by ether and neither A nor B is extracted (*8*). Step 1 is inhibited by acetic acid, hydrogen peroxide, and lead salts. The maximum colorless precursor is formed in 2 hours at its optimum temperature, 50° C. The last step must be compound, because Lukes finds the amino acid to be the only component missing from the ether extract. The present authors find formaldehyde to be the only component missing from the incubated purée. Acetic acid and hydrogen peroxide increase the speed of step 2.

Varietal Differences

Differences in reddening tendencies of different varieties of onions have been reported (*5*). Other plants closely related to the onion also undergo spontaneous coloring reactions. It has been known for some time that garlic (*Allium sativum*) bulbs, when puréed and acidified with acetic acid, become green (*2*, *13*). Joslyn and Sano (*6*) showed that the green pigment formed did not behave like chlorophyll and was not formed by the action of a naturally occurring phenolase or peroxidase. In the light of the onion work described here, it became desirable to determine whether formaldehyde entered into the garlic greening system.

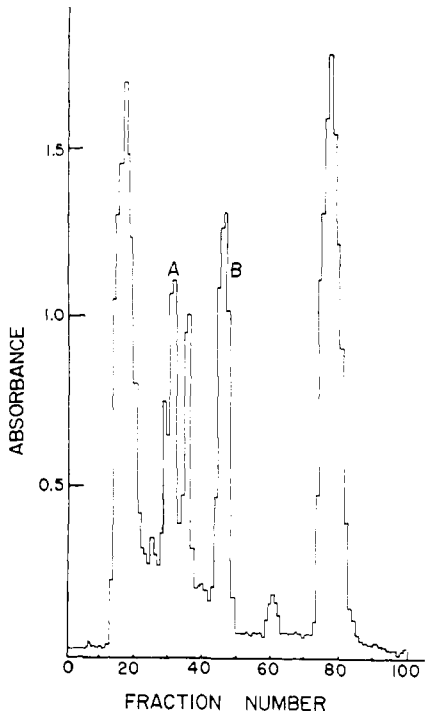


Figure 5. Amino acids in Southport White Globe onions

Two hundred and twenty grams of peeled garlic cloves were puréed and held at 50° C. for 5 minutes. After cooling, 20 ml. of formaldehyde-free acetic acid were added and it was divided into two lots; 0.1 ml. of 0.1*M* formaldehyde was added to one. The dark green pigment formed in both in about 2 hours at 20° C. at the same rate and to the same extent. Fifty grams of fresh garlic juice were treated as above, except that no acetic acid was added to either sample. Twenty-four hours were required for an appreciable amount of green pigment to form in the samples, and again, the formaldehyde sample contained the same amount of pigment as did the blank. The acidified samples were both dark green, while the unacidified samples were both very light green. Formaldehyde alone, or in the presence of acetic acid, had no effect upon garlic greening.

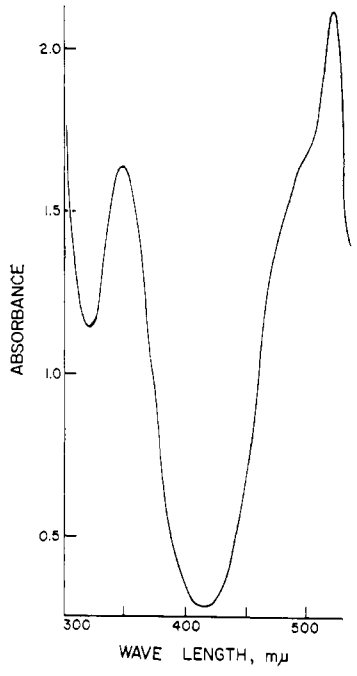


Figure 6. Absorption spectrum of pink solution obtained from *Allium giganteum* juice

Leaf and stem samples of *Allium giganteum* and bulb, stem, and flower samples of *Allium christophii* (both central Asian species) were crushed with and without acetic acid and with and without formaldehyde. The *christophii* samples remained white and no water-soluble pigment formed in the leaf tissue of *giganteum*. A mercaptan odor was given off immediately after cutting the *christophii* bulbs. The flowers are similar but larger than typical onion flowers and contain a small amount of preformed red pigment. This pigment was not identified, and no additional pigment formed after crushing. *Giganteum* leaves yielded a strong manure-like odor when cut. The stems became red-orange in a very few seconds after cutting. The juice came rapidly to the cut surface and reddened almost immediately. This juice was filtered and passed over Magnesol, giving one pink and one orange band. These were eluted readily by acidified 95% ethyl alcohol and the absorption spectrum of the pink solution is shown in Figure 6. A similarity to the onion pigment is noticeable in the visible region, but the ultraviolet absorption is very intense and is probably due largely to impurities. The pink solution turned brown on storage in air at room temperature for a few hours.

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STARCH ANALYSIS

Determination of Amylose in Starch

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Congo red is used colorimetrically for the determination of amylose in starch. Adsorption of the dye is independent of the chain length of the polysaccharide over a wide range. The extinction coefficient of the adsorbed dye is the same for both amylose and dextrin. The results are discussed and compared with those obtained from the usual iodine sorption and colorimetric methods.

THERE ARE SEVERAL METHODS for the determination of amylose in starch. Some of these methods (*4*, *8*) are based on analysis of the hydrolysis products, which procedure is often time-consuming. Iodine has been used colorimetrically (*1*, *6*, *7*). The latter method is based on the fact that amylose binds iodine, whereas amylopectin does not. Optical measurements of the iodine-starch mixtures are usually made at a fixed wave length, in the vicinity of 610 mμ, which is approximately the absorption maximum of the mixture. Iodine interaction depends not only on the degree of branching, but also on chain length, thereby leading to conflicting analytical results (*5*). It seems desirable to obtain another indicator which does not depend on chain length, although responding to changes in the degree of branching in the starch. Congo red appears to be a suitable adsorption indicator for this purpose.

Experimental

Materials. A histological grade of Congo red (National Aniline Division, Allied Chemical and Dye Corp.) was used, without further purification. Buffers were prepared from reagent grade potassium hydrogen phthalate and so-

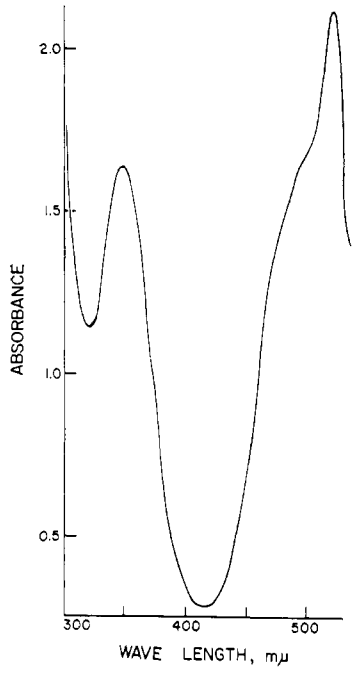

Figure 6. Absorption spectrum of pink solution obtained from *Allium giganteum* juice

Leaf and stem samples of *Allium giganteum* and bulb, stem, and flower samples of *Allium christophii* (both central Asian species) were crushed with and without acetic acid and with and without formaldehyde. The *christophii* samples remained white and no water-soluble pigment formed in the leaf tissue of *giganteum*. A mercaptan odor was given off immediately after cutting the *christophii* bulbs. The flowers are similar but larger than typical onion flowers and contain a small amount of preformed red pigment. This pigment was not identified, and no additional pigment formed after crushing. *Giganteum* leaves yielded a strong manure-like odor when cut. The stems became red-orange in a very few seconds after cutting. The juice came rapidly to the cut surface and reddened almost immediately. This juice was filtered and passed over Magnesol, giving one pink and one orange band. These were eluted readily by acidified 95% ethyl alcohol and the absorption spectrum of the pink solution is shown in Figure 6. A similarity to the onion pigment is noticeable in the visible region, but the ultraviolet absorption is very intense and is probably due largely to impurities. The pink solution turned brown on storage in air at room temperature for a few hours.

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STARCH ANALYSIS

Determination of Amylose in Starch

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Congo red is used colorimetrically for the determination of amylose in starch. Adsorption of the dye is independent of the chain length of the polysaccharide over a wide range. The extinction coefficient of the adsorbed dye is the same for both amylose and dextrin. The results are discussed and compared with those obtained from the usual iodine sorption and colorimetric methods.

THERE ARE SEVERAL METHODS for the determination of amylose in starch. Some of these methods (*4*, *8*) are based on analysis of the hydrolysis products, which procedure is often time-consuming. Iodine has been used colorimetrically (*1*, *6*, *7*). The latter method is based on the fact that amylose binds iodine, whereas amylopectin does not. Optical measurements of the iodine-starch mixtures are usually made at a fixed wave length, in the vicinity of 610 mμ, which is approximately the absorption maximum of the mixture. Iodine interaction depends not only on the degree of branching, but also on chain length, thereby leading to conflicting analytical results (*5*). It seems desirable to obtain another indicator which does not depend on chain length, although responding to changes in the degree of branching in the starch. Congo red appears to be a suitable adsorption indicator for this purpose.

Experimental

Materials. A histological grade of Congo red (National Aniline Division, Allied Chemical and Dye Corp.) was used, without further purification. Buffers were prepared from reagent grade potassium hydrogen phthalate and so-

dium hydroxide for pH ranges 4 to 6, and from monopotassium phosphate and sodium hydroxide for pH ranges 6 to 8.

Materials used as reference substance were corn amylose 14SSP and corn amylopectin 14SSP (Northern Utilization Research and Development Division, Peoria, Ill.). The potato amylose 13SSP and potato amylopectin 13SSP were obtained from the same source. The Superlose (potato amylose), Dutch R-A (native potato), and Ramalin G (potato amylopectin) (Stein Hall & Co.) had been prepared by a salt fractionation process. The defatted corn and potato starches (National Starch & Chemical Corp.) were defatted during the course of this work. The high linear cornstarch and wrinkled pea starch (Corn Products Refining Co.) as well as the other starches were used directly without further purification.

All starch solutions were prepared by dissolving 0.1 gram (dry basis) of the starch in 5 ml. of standardized 1*N* sodium hydroxide. A uniform solution was obtained within 1 hour. The alkaline mixture was then neutralized with an equivalent amount of standardized 1*N* hydrochloric acid, and diluted to 100 ml. In all cases, neutralization was reached within 1.5 hours. The neutralized solution was used within 2 to 3 hours.

Apparatus. A Beckman Model G pH meter was used for all pH measurements and for the potentiometric titrations; calomel-platinum electrodes were used in the latter procedure. All optical measurements were made in a Beckman DU spectrophotometer, equipped with cells of 1-cm. path length.

Procedure. Amylose was determined as follows: Five milliliters of pH 4.2 buffer and 5 ml. of a 0.1% starch solution were introduced into a 50-ml. volumetric flask. Approximately 29 ml. of water were then added, followed by 10 ml. of 0.0004*M* Congo red. The resulting mixture was diluted to volume and the absorbance at 500 mμ was immediately measured. An appropriate starch solution was used as a blank. The final mixture contained $2 \times 10^{-5}M$ Congo red and 0.01% starch. For the iodine mixtures, no buffer was used. The final solution contained 0.003% starch, 0.003% iodine, and 0.03% potassium iodide. The absorbance was measured at 615 mμ. The iodine sorption values were obtained potentiometrically (*2*).

Results and Discussions

Aqueous solutions of Congo red follow Beer's law to within ± 3%, up to a concentration of about 2×10^{-5} *M* (*3*). The addition of starch to the dye causes the extinction at the spectral peak to increase. The absorption spectrum of Congo red in the presence of an excess of starch is shown in Figure 1. This curve shows the spectral property of the bound dye to be independent of the type and size of the substrate. Unlike iodine, which causes a blue color with amylose or native starch, a plum color with amylopectin, and a red color with dextrin, Congo red causes no shift in color with these materials. Such changes in iodine behavior have been attributed to changes in the degree of branching and chain length of the substrate.

Figure 2 shows the effect of variation of the type of substrate upon the affinity of the adsorption process, as carried out at low concentration (0.01%). The starches used were amylose, a native starch, and dextrin. The only difference among these curves is the intensity. The difference in absorbance for these substances is assumed to be, to a large extent, a function of the degree of branching. For mechanical mixtures of solutions of corn amylose and corn amylopectin such a relation has been found to be linear. The relationship can be represented by the equation

$$R_c = (D_s - D_{ap})/(D_{am} - D_{ap}) \times 100$$

where R_c is per cent of amylose in the sample, D_s is the absorbance of the sample, D_{ap} is the absorbance of corn amylopectin, and D_{am} is the absorbance of corn amylose. A series of starches was used to test this equation. The results are shown in Table I. These are compared with results obtained with the usual iodine colorimetric and sorption methods. A fair agreement is seen for most of the samples.

The colorimetric iodine method yields more precise values, because the spread in optical scale between amylose and amylopectin is considerably larger than that for Congo red. However, precision in these determinations may be misleading, as indicated subsequently by the effects of variation in chain length in the starch sample. In this connection, the addition of Congo red to an acid-converted corn dextrin yields an R_c value of 20%, a figure slightly lower than that for native cornstarch.

There is a large discrepancy between the Congo red results and those for iodine for the second and the third samples in Table I. Both were corn amylose and had been heated, one at 150° C. for 1 hour, and the other at 150° C. for 48 hours. Heat treatment usually has two major effects on the starch molecules. One is degradation (the chain length is reduced) and the other is branching. If it is assumed that the binding of Congo red is strongly dependent upon the degree of branching, one could readily explain the considerably lower results with Congo red (see Table I). The heat treatment for the second sample in Table I was relatively mild, so that the reduction of the chain length was probably slight; nevertheless, the degree of branching was sufficient to reduce the Congo red binding.

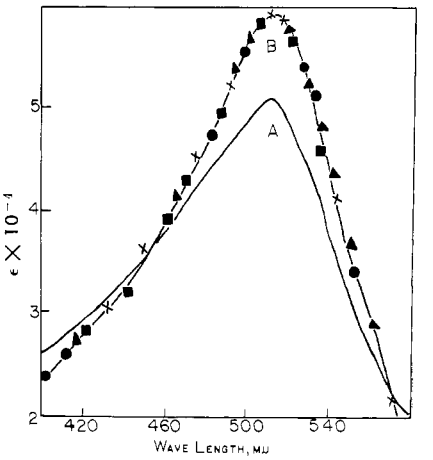

Figure 1. Congo red solution in presence of excess starch

Solutions buffered at pH 5, dye concentration = 2×10^{-5}M, temperature = 25° C.

- A Dye alone
- B. Dye with starch
- ● Corn amylose, 14SSP
- × Potato amylose, 13SSP
- ■ Potato starch
- ▲ Corn dextrin

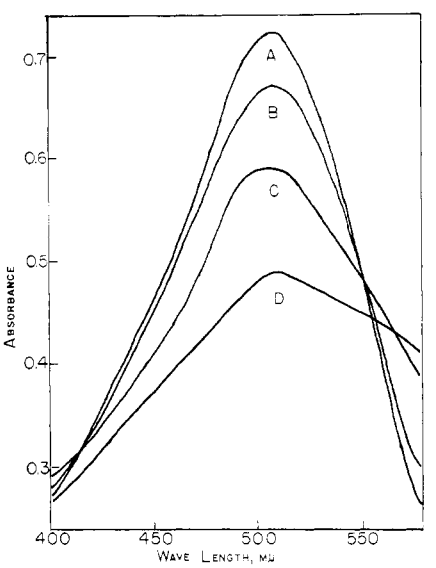

Figure 2. Spectral curves of aqueous Congo red in presence of starch and dextrin

All solutions buffered at pH 4.2, total substrate concentration = 0.01%, dye concentration = 2×10^{-5} M, temperature = 22° C.

- A. Superlose (potato amylose)
- B. Wrinkled pea, containing about 70% amylose Table I
- C. Acid-converted dextrin
- D. Congo red alone

The sample that had been heat-treated for 48 hours at 150° C. was hydrolyzed subsequently in the presence of β-amylase and found to yield only 48% of the theoretical content of maltose. This indicated that linkages other than those in the 1,4 positions were present, and branching was indeed obtained.

Throughout all measurements the concentration of sodium hydroxide used in dissolving the starch was kept constant,

Table I. Amylose Content in Starch

Sample	Iodine Sorption	Amylose, % R_i (iodine)	Amylose, % R_c (Congo red)
Corn amylose, 14SSP	19.4	100.0[a]	100.0[a]
Washed 6 times with methanol, dried at 150° C. for 1 hour	18.7	96.0	73.0
Washed 6 times with methanol, dried at 150° C. for 48 hours	14.6	73.5	54.5
Corn amylopectin, 14SSP	0.8	0.0[a]	0.0[a]
Potato amylose, 13SSP	20.6	104.5	103.9
Superlose	19.0	96.0	105.0
Defatted potato starch, vacuum-dried at 100° C. for 1 hour	3.8	28.0	23.0
Defatted cornstarch, vacuum-dried at 100° C. for 1 hour	5.2	24.5	20.0
Dutch R-A	3.7	29.5	27.0
High linear corn	12.0	46.5	42.0
Wrinkled pea	15.0	76.5	70.0
Ramalin G	1.1	10.0	0.3
Potato amylopectin, 13SSP	1.5	12.0	11.2
Washed 6 times with methanol, dried at 105° C. for 1 hour	0.8	10.1	9.9

[a] Basis of calibration. Congo red results obtained at starch concentration = 0.01%, pH = 4.2, temperature = 32° C., and dye concentration = $2 \times 10^{-5} M$.

Table II. Change in Extinction of Congo Red in Presence of Amylose

pH	$\%\Delta\epsilon_{500m\mu}$	$\%\Delta\epsilon_{600m\mu}$
3.2	32	25
4.2	80	84
4.6	22	66
5.1	9	50

Dye concentration = $2 \times 10^{-5} M$, temperature = 32° C., starch concentration = 0.01% of corn amylose, 14SSP.

and hence the concentration of sodium chloride was fixed at about $0.005M$. To ascertain whether variation of the amount of sodium chloride would affect the optical behavior of the dye itself, measurements were taken over a range of 0.0 to $0.02M$ sodium chloride. The extinction coefficient of Congo red was found to be independent of the sodium chloride concentration.

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SILAGE ANALYSIS

Determination of Sugar in Silages and Forages

H. G. WISEMAN, J. C. MALLACK, and W. C. JACOBSON

Dairy Cattle Research Branch, Animal Husbandry Research Division, ARS, Agricultural Research Center, Beltsville, Md.

A copper reduction method is described for the determination of sugar in silages and forages, whereby the usual sequence of dealcoholization and clarification is reversed. Clarification and deionization are performed on 80% alcoholic extracts, prior to dealcoholization to avoid handling of gummy residues. Somogyi's phosphate sugar reagent has been modified to give equal response to glucose and fructose.

QUANTITATIVE ANALYTICAL methods for sugars in silages and forages have been considerably shortened by the use of Waring Blendors for extraction (*8, 9*), ion exchange resins for clarification (*5, 10*), and Somogyi's copper microreduction reagents for ease of manipulation (*6, 7*).

Although extracts of many types of fresh green plants may be safely analyzed for sugar without clarification (*1, 8*), Williams and coworkers (*10*) have shown that extracts of dehydrated plant material must be clarified to remove the nonsugar reducing substances present. These may be removed by ion exchange resins according to the technique of Williams *et al.* (*10*).

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The latter workers have also demonstrated the reliability of the Somogyi carbonate reagent for sugar analyses in various types of plant materials and the method is listed in the 6th, 7th, and 8th editions of "Methods of Analysis of the

Association of Official Agricultural Chemists." Somogyi's improved phosphate reagent (*7*) is used for the determination of starch (*3*, *4*). An extraction of sugar by 80% alcohol or 80% acetone is common to most methods. It serves to minimize the solubility of protein, pectin, starch, etc. However, the extracts still contain pigments, ionized substances, and gummy materials. The subsequent removal of alcohol before clarification results in a gummy residue which requires scrubbing to suspend it in water. This suspension often filters with extreme slowness and invites occlusion. Furthermore, the sugar has been exposed to interaction with these substances at steam bath temperatures during concentration.

An additional criticism is that the copper-reduction values of glucose and fructose are usually not equal, the yields of the cuprous precipitate in each case varying differently with pH. Thus, with more alkaline copper reagents, glucose has a greater reducing power than fructose, and vice versa (*2*).

The method of analysis of sugar in silages and forages reported here in detail departs from the usual procedures, in the sequence of dealcoholization and clarification. The latter step is completed directly on the 80% ethyl alcohol extract to avoid gummy residues in the ensuing dealcoholization step.

In addition, the Somogyi reagent has been modified in alkalinity to effect equal response by glucose and fructose.

Reagents

Sugar reagent, Somogyi 1945 (*7*) modification. Potassium iodate, 0.3567 gram per liter; disodium phosphate, 56 grams per liter; sodium hydroxide, 82.5 meq. per liter.

Sodium thiosulfate, ACS grade.

Glucose, National Bureau of Standards, sample 41.

Sucrose, National Bureau of Standards, sample 17.

Fructose, National Bureau of Standards, purified sample.

Starch solution, 1%, freshly prepared.

Potassium iodide solution, 2.5% from ACS grade compound.

Sulfuric acid, 1.8*N* and 1.5*N*.

Dowex 50 (Dow Chemical Co., Midland, Mich.) (for activation see company specifications), or Dowex 50-W-X8, 200 to 400-mesh, sold activated.

Duolite A-4 (Chemical Process Co., San Francisco, Calif.) (for activation see company specifications), or Dowex 3, 20 to 50-mesh, sold activated.

Ion exchange resin mixtures. 0.5 gram of Dowex 50 plus 1.75 grams of Duolite A-4 (activated resins) are allowed to soak overnight in 75 to 100 ml. of distilled water. Alternatively, 0.4 gram of Dowex 50-W-X8 plus 1.4 grams of Dowex 3 may be used.

Method

Extraction. Silage or forage materials are chopped by means of a paper cutter into approximately 0.25-inch lengths and are loosened and distributed over a flat surface for sampling. Twenty-gram samples are comminuted for 10 minutes in a Waring Blendor with approximately 400 ml. of 80% ethyl alcohol.

Filtration. The contents of the blender are poured onto a sintered-glass filter containing a filter bed of 60 grams of Darco G-60 and Celite 535 (1 + 3) sandwiched between two layers of Celite 535, each 1 cm. in depth. Three hundred milliliters of 80% ethyl alcohol are used for successive washings. The combined filtrate and washings are made up to 750 ml. in volume.

Deionization. An aliquot, 30 ml., is stirred for 15 minutes (magnetic stirrer) with the ion exchange resin mixture. The deionized solution is decanted and filtered through a glass-wool plug inserted in the neck of a powder funnel placed over a 150-ml. beaker. The resins are rinsed with 80% ethyl alcohol, which is also decanted onto the filter, and the resins are then stirred for 10 minutes with 25 ml. of hot water. The water extract and subsequent rinsing of the resin with approximately 25 ml. of hot water are filtered through the glass wool. The beaker with the filtrate is set on the steam bath. The sugar solution is concentrated to 1 to 2 ml. to remove the alcohol.

Inversion. Hydrochloric acid, 1*N* (25 ml.), is added to the cooled beaker, and the sugar solution is allowed to stand overnight at room temperature to invert.

Neutralization. Sufficient sodium hydroxide (20%) is added to bring the solution nearly to neutrality. Diluted, 2% sodium hydroxide is used to complete the neutralization to pH 7, the adjustment being controlled by use of a pH meter with glass and calomel electrodes. The solution is made to a volume, 50 ml. Aliquot volumes are usually 5 ml. for analysis of the extracts from fresh green material and 10 ml. in the case of silage.

Copper Reduction. Aliquots are pipetted into 25-mm. diameter test tubes as specified by Somogyi. An equal volume of sugar reagent is added to the aliquot. Five milliliters of sugar solution and 5 ml. of reagent are used in the analysis of fresh plant material while 10 ml. of sugar solution and 10 ml. of reagent are taken for silages. The tubes are placed in a rack and immersed in a boiling water bath for 15 minutes. The rack is removed and placed in a pan of cold tap water to cool the solutions.

Table I. Recovery of Glucose from Darco and Resin Treatments

	Recovery, 1.500 Mg. Glucose Treated	
	Darco treatment	Resin treatment
	1.497	1.495
	1.505	1.502
	1.507	1.499
	1.502	1.500
	1.503	1.494
	1.500	1.492
Av.	1.502+	1.497

Titration. Potassium iodide solution (2.5%) is added slowly to form a layer over the solutions in the test tubes; 1 ml. is used for each 5 ml. of sugar solution placed in each tube. Immediately before titration, sulfuric acid is added rapidly to the tubes, 5 ml. of 1.8*N* sulfuric acid for 10-ml. aliquots and 3 ml. of 1.5*N* sulfuric acid for 5-ml. aliquots. A Teflon-covered stirring bar is dropped into the solution and thorough oxidation of the precipitated cuprous oxide by the liberated iodine is obtained by vigorous magnetic stirring for 1 minute. The stirring is continued at a reduced rate, while the excess of iodine is titrated with 0.005*N* sodium thiosulfate to a faint yellow. Four drops of a 1% starch solution are added and the titration is continued until the blue starch-iodine color disappears.

Standardization. Five- or 10-ml. samples of sugar reagent are mixed with equal volumes of distilled water and with equal volumes of freshly prepared standard glucose solutions. Analyses are carried out as described under copper reduction and titration. The difference in titer for the tubes containing water and sugar is divided into the milligrams of sugar present in the aliquot, thereby giving the glucose equivalent for 1 ml. of sodium thiosulfate, *G*.

Calculation. A blank is run to include any contribution to the analysis from reagents or resins. From this value, *B*, is subtracted the titer, *T*, of a silage or forage aliquot. The difference, $B - T$, is multiplied by *G* to obtain the amount of glucose present in the aliquot taken for analysis. Thus, when a 10-ml. aliquot has been taken from 50 ml. of a sugar solution representing 0.8 gram of wet silage material, the total sugar expressed as glucose may be calculated on the dry matter (D.M.) basis as follows:

$$\text{Sugar, \% of dry matter} = \frac{(B - T)G \times 100}{10/50 \times 0.8 \times 10^3 \times \text{D.M.}(\%)/100}$$

$$= \frac{(B - T)G \times 62.5}{\text{D.M. (\%)}}$$

Dry matter determinations on silage

Table II. Sugar Analyses on Silage Extracts

Sample	Sugar, % Oct. 9	Oct. 28
246	1.85	1.85
248	1.36	1.32
249	1.64	1.69
251	1.47	1.44
267	1.74	1.83
268	2.57	2.54
270	2.11	2.03
272	1.38	1.26
273	1.60	1.60
275	2.12	2.08
278	1.79	1.71
288	2.58	2.55
292	2.20	2.16
295	1.67	1.64
300	1.53	1.54
303	2.71	2.66
304	1.78	1.93
305	2.51	2.45
326	1.97	1.94

Table III. Recovery of Clarified and Inverted Sucrose

(Theoretical recovery, 1.500 mg. inverted = 1.579 mg. "glucose found")

Sample	Glucose Found, Mg.	Theory, %
1	1.572	99.56
2	1.567	99.24
3	1.573	99.62
4	1.563	98.99
5	1.566	99.18
6	1.562	98.92
7	1.573	99.62
Av.	1.568	99.30

may be conveniently made by toluene distillation in a Bidwell-Sterling apparatus.

Results

Recovery experiments with standard glucose from the National Bureau of Standards were made to test the effect of the Darco G-60 and Celite 535 and resin treatments. In Table I, satisfactory recoveries are evident.

Satisfactory reproducibility of analyses is shown in Table II. Comparisons of analyses made 19 days apart on silage extracts are listed. Somogyi's reagent of 1945 was used to obtain the data shown in Tables I and II. Dowex 50 and Duolite A-4 were used in the clarification work.

In Table III, recoveries are listed for sucrose samples subjected to clarification, dealcoholization, and inversion. Seven 250-ml. aliquots of sucrose taken from a stock solution containing 3 grams of sucrose in 2 liters of 80% ethyl alcohol were filtered through Darco-Celite. The combined filtrate and washings were made up to 750 ml. in each case. From this volume, 30-ml. aliquots—0.0150 gram of sucrose—were resin-treated, dealcoholized, inverted, neutralized, and made up to 100-ml. volumes. The modified Somogyi reagent giving equal response to glucose and fructose was used to obtain the data in Table III. A Dowex 50-W-X8 and Dowex 3 mixture was used for the resin treatment. The inversion was carried out for 24 hours in the following manner. The acidified sugar solutions were placed in a desiccator vessel which was rapidly evacuated and refilled with nitrogen three times in succession. The temperature of inversion was maintained at 25° C. by placing the vessel in a temperature-controlled water bath.

Discussion

In this laboratory, reducing substances have been encountered in preliminary water extracts of ion exchange resins. The use of resins therefore necessitates adequate water washing to minimize the blanks, as well as the inclusion of resins in blank determinations.

The filtration of the Waring Blendor contents through the Darco-Celite bed yields an essentially colorless filtrate. The deionization by resins is also conducted on the 80% alcoholic filtrate, in contrast to the usual resin treatment of aqueous solutions. These two clarification steps remove ions and organic matter before the sugar solutions are concentrated on the steam bath to remove the alcohol. The solutions, when nearly evaporated to dryness, are free of gummy residues and require no filtration. Because a further increase in volume from the clarification steps is eliminated, a wider choice of final volume is permitted.

Attention is invited to the advantages offered by magnetic stirrings during titrations of the solutions in the 25-mm. diameter test tubes as used in the Somogyi sugar method. Although magnetic stirring bars, 1.25 inches long, stand on one end at an angle in test tubes of this diameter, the bars will rotate to give efficient mixing when held over a magnetic stirring apparatus. Under these conditions, titrations are performed rapidly without repeated removal of the tube to mix the contents by shaking. In addition, larger aliquots can be smoothly handled without danger of spillage and poor mixing. Of greater importance, however, is the need to ensure that the cuprous oxide precipitate formed in the reduction is stirred sufficiently well to react completely with the iodine formed upon addition of potassium iodide and sulfuric acid. Experience in this laboratory has shown that 1 minute of rapid stirring by the magnetic apparatus leads to consistent titration values; without this precaution results often gave considerable variation.

Preparations of the copper reagent at different alkalinities were tested against these sugars to obtain equal response to glucose and fructose. In each of these trials the disodium phosphate concentration prescribed by Somogyi was doubled to increase its buffer action. The intersection of the copper reduction curves of glucose and fructose came at a pH of approximately 9.6. The sodium hydroxide addition, possibly more precisely expressed than the pH, was 82.5 meq. per liter. The two sugars were further tested for equivalence at this alkalinity of the copper reagent. The relative reducing power of 1.5-mg. quantities of glucose and fructose was compared from triplicate determinations on each sugar in each of six trials. The fructose-glucose reducing ratios were 1.005, 1.006, 1.002, 1.005, 1.005, and 0.994.

Because the modified reagent reacts equally to glucose and fructose, the reducing action of inverted sucrose is conveniently twice that of glucose on a molecular basis. In Table III, inverted sucrose would be calculated to yield $(2 \times 180)/342$ times the reduction of glucose. Thus, 1.5 mg. of sucrose when inverted would be equivalent to 1.579 mg. of glucose (Table III). With glucose test solutions containing 0.5, 1.0, and 1.5 mg. per ml., the response of the modified reagent, within experimental error, was found to be linear.

When solutions of sucrose—National Bureau of Standards, sample 17—were inverted in air, consistent losses of approximately 3 to 4% were obtained, which could be tolerated in routine determinations. However, if the extent of aerial oxidation is a function of the concentration of fructose, the exclusion of air should be of more concern in the inversion of solutions relatively rich in fructose.

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Symposium on Soil Testing

The eight papers which follow comprise the Symposium on Soil Testing presented on September 15, 1959, at the 136th National Meeting of the American Chemical Society at Atlantic City, N. J., by the Society's Division of Fertilizer and Soil Chemistry.

The symposium was designed to:

outline the history and bring up to date the known technology of soil testing;

describe current research aimed at improving the accuracy and usefulness of soil-testing techniques;

point the way for future progress, in order that soil testing may be made increasingly effective as a diagnostic tool, assuring more efficient use of lime and fertilizers, and more profitable production of crops and livestock.

The division was fortunate in securing participation of a distinguished panel of experts. Each member of this group of able scientists has specialized in some aspect of soil testing and interpretation, and offers specific knowledge in his selected field.

The symposium as a whole, therefore, covers soil testing both intensively and extensively; it provides useful information at all levels from basic research to practical application.

M. D. SANDERS, Symposium Chairman
Swift and Co., Chicago, Ill.

History and Development of Soil Testing

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The history and development of soil testing are considered in three approximate periods: 1845 to 1906, 1906 to 1925, and 1925 to 1952. Phosphorus and potassium are emphasized, but other elements are considered. Data of the second period provide background information for interpretation of soil tests. More recently many new methods have been developed, some for universal application and others for specific problems.

For a little more than a century soil and plant scientists have sought chemical and biological methods for determining the fertility of a soil. These procedures have often in recent years been related to recommendations issued by governmental agencies for fertilizer practice. The composition of crops has also had an important place in diagnosing the capacity of a soil to deliver nutrients to plants, particularly to crops to be grown subsequently in a field.

The present discussion divides history and development into three imperfectly defined periods. The first is from 1845 to 1906. Review here deals essentially with the work of four authors: Daubeny, Liebig, Hilgard, and Dyer. During the second period, roughly 1906 to 1925, emphasis was placed on the fundamental chemical composition of soils as related to crop production. A third period includes essentially 1925 to 1952, with ramifications extending to the present. This was a period of struggle for methods that could be universally applicable, and of efforts to adapt chemical procedures to specific soil conditions.

Early Work

One of the first records of quick soil tests for an assay of soil fertility is that of Daubeny in England in 1845 (*6*). He suggested the terms "active" and "dormant" to express the more and less soluble nutrient constituents of a soil, respectively. The solvent used for extracting the active portion was carbonated water. Analytical methods in use more than a century ago imposed difficulties in carrying out the suggestions of Daubeny; and the subject of soil testing appears to have been essentially laid aside until revived by Liebig about a quarter century later (*14*).

The procedure used by Liebig included extraction of soil with dilute solutions of hydrochloric, nitric, and acetic acids. Varying quantities of phosphorus and potassium compounds were dissolved by the different acids and the quantities dissolved also varied with the manurial treatments received by the soils. The soils used by Liebig were from the Rothamsted Experiment Station, where fertilizer histories had been known for nearly 30 years.

In 1906 when Hilgard wrote the first edition of his textbook (*11*, p. 353), he

stated that "the soils of Mississippi containing less than about 0.1% of total phosphoric oxide (P_2O_5) usually respond well to applications of phosphatic fertilizers, while those containing more than this amount are not likely to respond profitably to such treatment."

This was more than 50 years ago. Farmers now have better seed for various kinds of crops; they know more about the use of fertilizers and expect higher average yields. It is well, however, to consider the importance of this approximate total value, 0.1% P_2O_5.

This figure provides a yardstick with which to judge a few values in the next items of literature to be examined. In 1894 the first really modern approach to soil testing as a guide for fertilizer practice was made by Dyer (*8*), who worked with soils from the famous Broadbalk and Hoos Fields of the Rothamsted Experiment Station. The plots had then been under observation nearly 40 years.

Dyer has long been known partly on account of his study of the acidity and nutrient composition of root sap as a guide for the selection of a solvent for active or available nutrients. Analytical methods in use at that time were presumably not suitable for a detailed study of acidity essential for carrying out his thesis. He was, however, led to the selection of 1% citric acid as a solvent. This extractant has been used to some extent for several decades.

The data of Dyer show that the quantity of phosphorus dissolved by citric acid is more closely related to crop production than is the total quantity of phosphorus present. The data show much more pronounced phosphorus deficiency than need for potassium. Dyer's paper contains extensive data of interest to anyone investigating soil tests (Table I).

Fundamental Chemical Data

Turning now to the second period, 1906 to 1925, fundamental chemical data are emphasized. This is background information and never was proposed as a substitute for quick tests of soil in individual fields. In some of the states the value of chemical composition as background information is recognized; in other states little attention is paid to these older data.

There is a great volume of data from Iowa showing the phosphorus contents of soils of that state by counties, published in 1914 (*5*). Certain soils of a glacial till area in the northeastern part of the state were shown often to contain about 0.1% P_2O_5 in the surface soil and less in the second foot. Soils of the loess area in western Iowa often contain approximately double the amount of P_2O_5 found in the glacial till area of the northeast. These data are confirmed by analyses published in 1940 (*23*). The Iowa station has properly placed its substation for the evaluation of phosphate rock *vs.* superphosphate in an area of the Carrington-Clyde soil association of northeastern Iowa. These soils frequently have a P_2O_5 content of about 0.1% in the surface soil and less in a layer beneath plow depth.

Arkansas has soils of tremendous contrast in phosphorus content and consequently of recommended use of phosphatic fertilizers. A splendid publication of that state gives data of interest in characterizing the phosphorus status of a number of its soil types (*21*). Table II shows the total phosphoric oxide content of surface soils and subsoils of several series found in Arkansas. The low values indicate where phosphate rock can frequently be used economically for building up the inventory of total phosphorus in the soil.

In Kentucky, soils of the Maury series in the Inner Blue Grass Region usually have P_2O_5 contents of 0.75 to 2.5%. Phosphatic fertilizers are not needed. Less than 100 miles distant is the Knob Country, where soils of medium texture often have P_2O_5 contents of less than 0.1% (*13*). In these areas small grain production is sometimes hopeless without phosphatic fertilizers.

The three states mentioned provide excellent chemical information regarding their soils; each also has a very effective soil-testing service. The background data aid in the interpretation of results obtained from tests of soils sent in by farmers.

Sometimes background data help considerably when the highly desirable tests for more readily available constituents are not obtainable. Kaddah (*12*) has shown that the phosphoric oxide content of suspended matter of the lower Nile River ranges from 0.21 to 0.35%. This type of mud has provided the soil material where the grain grown 6000 years ago presumably fed a vast number of people who built the pyramids.

We have a Biblical record of some 2500 years ago when the Hebrews sent a small committee to Egypt to buy grain during a famine in Palestine. About the same time Pericles, the great Greek statesman, showed some embarrassment over the political necessity of receiving a gift of "corn" from the Egyptians. The Nile Valley always produced good small grain crops because the water supply was assured, and the muds deposited had left a soil with at least 0.2% of phosphoric oxide (*9*, *12*). On the other hand, many soils of Greece have less than 0.1% P_2O_5 (*2*).

With respect to phosphorus-bearing minerals, apatite is of common occurrence in soils, but it is very insoluble in water and the P_2O_5 content is very slowly available. Other forms of calcium phosphate containing some fluorine are also found in soil. When phosphatic fertilizers are applied to land, extensive residues of varying composition accumulate. It is not surprising that the quantities of phosphorus extracted by different solvents at various soil-solution ratios show wide variations.

The potash situation is very different from that of phosphorus with respect to response to testing solutions. Most of the methods are dependent upon base exchange principles set forth by Way (*29*) more than a century ago. The concentration and pH of an extracting solution, as well as soil-solution ratio, usually have only moderate influence on the amount of potash extracted. Furthermore, the amount extracted is greatly influenced by the general composition of the soil.

In many cases the total potash content of a soil bears very little relation to available potash status. Climatic conditions which affect natural leaching appear to be of great importance. Many soils of the Piedmont area extending from New England into Georgia are well supplied with K_2O; values as high as 2 to 2.5% occur in soils of medium to heavy texture (*4*). On these soils potassium fertilizers are frequently economically used.

Table I. Barley Yield and Phosphoric Oxide Contents of Surface Soils Differently Treated, Hoos Field, England

Average of 4 plots (8)

Soil Treatment	Barley Grain Yield, 38th Year (1890) Bushels	Phosphoric Oxide Contents of Soils, % Total	Phosphoric Oxide Contents of Soils, % Soluble in 1% citric acid solution
No minerals	24.7	0.10	0.006
N, K_2O, and MgO, no phosphates	24.4	0.11	0.009
N, P_2O_5, K_2O, and MgO	38.6	0.18	0.050
N and P_2O_5, but no K_2O or MgO	36	0.18	0.043

Table II. Total Phosphoric Oxide Content of Surface Soils and Subsoils of Arkansas (21)

Soil Series	P_2O_5, % Surface soil	P_2O_5, % Subsoil
Baxter	0.06	0.04
Lufkin	0.03	0.02
Atkins	0.09	0.06
Leaf	0.03	0.02
Portland	0.17	0.14
Sharkey	0.18	0.15
Miller	0.18	0.13
Hanseville	0.07	0.06

Table III. Readily Available Phosphorus Obtained from Soils of Fertility Plots, Aroostook Farm, Maine (24)

Description of Soils	Readily Soluble Phosphorus, Lb. P_2O_5/Acre					Truog Values Divided by Values as Indicated			
	Truog	Modified Morgan (15–18)	2.5% acetic acid	Hester (10)	Neubauer	Modified Morgan	2.5% acetic acid	Hester	Neubauer
Virgin soil	62	9.5	19	1.3	...	6.5	3.3	47.7	...
No treatment, 3-yr. rotation	73	5.8	21	1.5	...	12.6	3.5	48.7	...
4–8–8, 2000 lb. in 3-yr. rotation	200	13	87	2.0	100	15.4	2.3	100.0	2.0
4–12–8, 2000 lb. in 3-yr. rotation	300	17	150	2.6	130	17.6	2.0	115.4	2.2
4–8–8, 2000 lb. continuous potatoes	400	24	220	3.2	200	16.7	1.8	125.0	2.0

Fertilizer potash added for one crop may be partly taken up by the crop and partly leached out during a year. A large part may also be fixed by contact with minerals such as micas; these losses may be partly compensated by the decomposition of potash-bearing minerals and organic matter.

Recent Work

In 1951 a report issued by a National Soil Testing Committee (*19*) summarized methods used in various state and other institutional laboratories. At that time 28 laboratories used the same type of extracting solution for available soil phosphorus as for potassium. Strong mineral acids were used in concentrations varying from 0.02*N* to 0.3*N*. Varying kinds and concentrations of buffering salts were used in a few cases with the acid.

Any of these extractants may be expected to produce conventional base exchange reactions when an adequate quantity of liquid is used. The action on phosphorus-carrying compounds is no doubt rather drastic when the more concentrated solutions of mineral acids are used.

The Bray (*3*) or Illinois test for phosphorus employs ammonium fluoride with hydrochloric acid as an extractant. It may be assumed that the fluoride ion tends somewhat to separate the phosphate and silicate radicals that are related chemically.

Water as a solvent was proposed by Spurway of Michigan (*26*), and later used in certain other areas, particularly for soils not strongly leached. It would seem that carbonated water, a slightly more effective solvent than pure water, has met with a little more favor, though not extensively used.

A buffered solution, such as sodium acetate with acetic acid at a pH of approximately 5, has been widely used as an extractant for potassium and less extensively for phosphorus.

Soil-solution ratio is one of the extremely important features for estimating results in terms of pounds of available phosphorus present per acre. These ratios vary from 1 to 2 for several methods to 1 to 40 in Oklahoma when a particular extractant is used for both phosphorus and potassium (*19*). The soil-solution ratio is usually the same for each nutrient.

Some confusion has arisen as to which of the Truog methods for extracting phosphorus from soils should bear his name. Apparently, the one published in 1930 (*27*) was the one he first suggested, as it has frequently been designated as the Truog method (*24*). The distinctive feature of this procedure is that it employs 0.002*N* sulfuric acid neutralized to pH 3.0 after addition of 3 grams of ammonium sulfate per liter. The soil-solution ratio is 1 to 200. Advantages of a wide soil-water ratio are discussed by Truog (*28*).

Many kinds of solutions for extracting available phosphorus from soils have been proposed. Several of these were compared by Rubins and Dean in 1946 (*24*). References to some original publications are given in Table III. The data given here are taken from Rubins and Dean or calculated from their data. A few conclusions are apparent:

The highest phosphorus values are obtained with the Truog procedure. Two factors may be assumed to be important: the low pH of the extracting solution, and the wide soil-solution ratio employed.

The Hester procedure leads to the smallest amount of phosphorus extracted, but the Morgan method (*18*) also gives relatively low values. The main difference between these two procedures lies in the slightly higher pH of the Hester solution and a soil-solution ratio of 1 to 2 instead of 1 to 4 as in the Morgan procedure.

The 2.5% acetic acid solution gave results nearest to the absorption of phosphorus by rye plants (Neubauer method). This does not mean, however, that some other plant would follow a like pattern.

It may be assumed that any of these sets of values may be helpful in arriving at the phosphorus status of a soil. Additional useful information might often be obtainable if two or more kinds of tests were made on a particular soil.

One of the more recently described methods for available phosphorus involves extraction by a solution of sodium bicarbonate (*22*). This type of solution was first used for alkaline western soils, but is applicable to acid soils as well. Miller and Axley extracted phosphorus from 17 soils of Maryland, using four different extracting solutions. The sodium bicarbonate procedure usually gave lowest values (*16*).

Soil-test data, gradually accumulated for a little more than a quarter of a century, have steadily become more useful in interpreting probable fertilizer needs. Relations of test results to soil types and to kinds of crops grown are of particular importance.

In 1932 Scarseth conducted a series of experiments in Alabama (*25*) that are of great value in showing the effects of soil type and time elapsing after fertilizer application in rendering soluble phosphates less available to plants. The experiment was not designed as an aid to interpreting quick test data for local recommendations, but such data can be used in this way as background information.

Another series of experiments valuable as background is that of De Turk (*7*) and others in Illinois. Sweet clover made more effective use of the phosphate rock than did wheat. It has not yet been shown, however, which of the soil test methods thus far proposed is most suitable for use when a field has been treated with a heavy application of phosphate rock. It is possible to use a factor smaller than 1 to diminish values by the Truog or other strong acid method. Or a factor greater than 1 might be used against values obtained with a nearly neutral sodium acetate solution at a narrow soil-solution ratio.

Even if the original thesis of Dyer, developed more than a half century ago, could be fully realized, there is little reason to believe that an extracting solution of conductivity and pH similar to that of the sap of roots and plants could be effectively used. The big question of soil-solution ratio would still be unsolved. Furthermore, the composition of plant sap no doubt varies widely with kind of plant grown. Other complicating factors are also operative here.

It has long been known that soil test data furnish only a part of the information necessary for proper interpretation of results in terms of the most profitable fertilizer practice (*1*, *20*). Such data increase in value as supplemental information is at hand. The necessity for such supplemental information has led to a practice in many laboratories of securing a record of field practice.

Questions such as these are frequently sent to the farmer with a soil container:

What sequence of crops was grown during the past three years?

If commercial fertilizer, farm manure, or lime was added, what was the rate of application of each and the grade of fertilizer?

What was the approximate yield of each crop?

Were crop residues left on the soil in so far as practicable?

A soil map is usually in the office of a county agricultural agent. Furthermore, he travels over the county extensively and observes farm practice and crop yields. In many counties a number of farms are supervised by farm managers who visit fields at frequent intervals. Records of their experience are often available for inclusion with the soil data and other information gathered from varied sources.

The emphasis of this presentation has been upon phosphorus, partly because of the wide differences in values obtained using different tests. Potash and nitrogen should not be neglected, however. The various base exchange processes used should lead to results less variable with potash than with phosphorus.

The question to be decided is not so much whether or not phosphorus, potash, or nitrogen is needed, as what level will prove most profitable for a particular crop. A grower of cotton in any one of several of the southeastern states knows that he usually needs some potash for growing his crop most profitably. Should he apply 40 or 60 pounds of K_2O, for instance? The laboratory test to be an effective aid in answering this question must be supplemented by extensive local data. Time and observation aid in the accumulation of helpful information.

Modern features of soil testing are international in scope. Prominent among those working in this field in different countries are Egner of Sweden, Ferrari of the Netherlands, and Turin of the Soviet Union.

Instrumentation, using such equipment as the flame photometer and the spectrograph, is under way and seems destined for expansion. Minor element studies have been expanded by instrumentation and deserve further consideration in the field of soil tests.

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Research in Maryland on Chemical Methods for Determining Available Phosphorus and Potassium in Soils

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FARMERS in recent years have shown an active interest in soil testing. A survey by the Federal Extension Service showed that nearly a half million more soil samples were tested by state and county soil testing laboratories in 1957 than in 1955. Also a nationwide survey based on personal interviews revealed that soil testing was rated by farmers as the most important single external influence in their fertilization practices (*19*).

About 2,600,000 tons of phosphate (P_2O_5) and 1,900,000 tons of potash (K_2O) are processed in the United States each year (*16*). A more intelligent use of these two fertilizer elements could result through the development of improved soil tests. Although much progress has been made in the development of soil-test methods, many of the tests need to be correlated more extensively with crop response data. In many cases the soil-test correlation data have been obtained from experiments designed for other purposes and there has been a lack of control of the many factors that determine plant growth. As a result the correlation data and calibration scales that have been obtained can in many cases be improved upon.

A number of chemical extraction methods for determining available phosphorus and potassium in soils have been used by soil-testing laboratories in this country. To determine under Maryland conditions the correlation of several soil-test methods with crop response data, a pot culture experiment and several field experiments were conducted. This research has been helpful in evaluating some methods used by soil-testing laboratories, but a great deal more soil-test correlation work is needed for crops and soil conditions that exist in Maryland.

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Research in Maryland on Chemical Methods for Determining Available Phosphorus and Potassium in Soils

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FARMERS in recent years have shown an active interest in soil testing. A survey by the Federal Extension Service showed that nearly a half million more soil samples were tested by state and county soil testing laboratories in 1957 than in 1955. Also a nationwide survey based on personal interviews revealed that soil testing was rated by farmers as the most important single external influence in their fertilization practices (*19*).

About 2,600,000 tons of phosphate (P_2O_5) and 1,900,000 tons of potash (K_2O) are processed in the United States each year (*16*). A more intelligent use of these two fertilizer elements could result through the development of improved soil tests. Although much progress has been made in the development of soil-test methods, many of the tests need to be correlated more extensively with crop response data. In many cases the soil-test correlation data have been obtained from experiments designed for other purposes and there has been a lack of control of the many factors that determine plant growth. As a result the correlation data and calibration scales that have been obtained can in many cases be improved upon.

A number of chemical extraction methods for determining available phosphorus and potassium in soils have been used by soil-testing laboratories in this country. To determine under Maryland conditions the correlation of several soil-test methods with crop response data, a pot culture experiment and several field experiments were conducted. This research has been helpful in evaluating some methods used by soil-testing laboratories, but a great deal more soil-test correlation work is needed for crops and soil conditions that exist in Maryland.

A number of chemical extraction methods have been used in this country for determining available phosphorus and potassium in soils. A pot culture experiment and several field experiments were conducted in Maryland to determine the correlation of several methods with crop response data. In many instances there was little difference between the correlation coefficients for chemical methods used to determine available phosphorus. When all the soils in the investigation were considered, a method employing sulfuric acid and ammonium fluoride as an extractant showed the closest relationship with crop response. The soil series determined to a large extent the correlation between chemically available phosphorus and alfalfa yields. The thermal method proposed by Kolterman and Truog showed promise for determining the relative capacity of soil to supply nonexchangeable potassium to plants.

Materials and Methods

Chemical Extraction Methods. Soluble phosphorus was determined in the soils by the modified Truog method (*13*), the Bray and Kurtz No. 1 method (*2*), the sodium bicarbonate method of Olsen *et al.* (*10*), the Wrenshall and McKibbin method (*20*), the North Carolina method (*4*), and the proposed method of Miller and Axley (*9*). Potassium was determined by the North Carolina method and the proposed method of Kolterman and Truog (*5*).

Field Pot Culture Experiment. A pot culture experiment which consisted of 17 Maryland soils each treated with 20% superphosphate to obtain five levels of soluble phosphorus was conducted at the University of Maryland Plant Research Farm. This experiment was designed as a randomized complete block with five treatments replicated three times on each soil and was used to determine the correlation of phosphorus soil tests with crop response. More specific information on the experimental procedures used in the pot culture experiment, is given by Miller and Axley (*9*).

Field Experiments. The response of crops to phosphate applications was available from field experiments on a Beltsville silt loam, a Monmouth fine sandy loam, and a Penn loam. The response of tobacco to potassium was also available on the Monmouth fine sandy loam. In the field experiments the soil samples were taken from the surface 6 inches of the plots. The fertilizer treatments and the chemical methods employed to determine phosphorus and potassium are shown in Tables V, VII, IX, and X.

Results and Discussion

The phosphorus extracted from the soils in the pot culture experiment by the various chemical methods is shown in Table I. In general, the greatest quantity of phosphorus was extracted by the proposed method of Miller and Axley, which employs 0.03*N* sulfuric acid and 0.03*N* ammonium fluoride as an extractant. The sodium bicarbonate method removed the smallest quantity of phosphorus from the soils, while the modified Truog and Bray and Kurtz No. 1 methods were intermediate and on the average removed about the same amounts of phosphorus. The phosphorus removed from the Chester soil in this experiment by three chemical methods is shown in Figure 1. Similar curves were obtained for several soils. A number of workers (*1*, *7*, *12*, *15*) have reported that the various chemical methods for available phosphorus extract widely different amounts of phosphorus from the same soil. However, this does not present a serious problem as long as the phosphorus extracted shows a good relationship to crop response. For example, the phosphorus extracted by the proposed method averaged about 30% greater than that extracted by the modified Truog method; yet the correlation coefficients obtained for the phosphorus extracted and the crop response data for the pot culture experiment were very similar for the two methods.

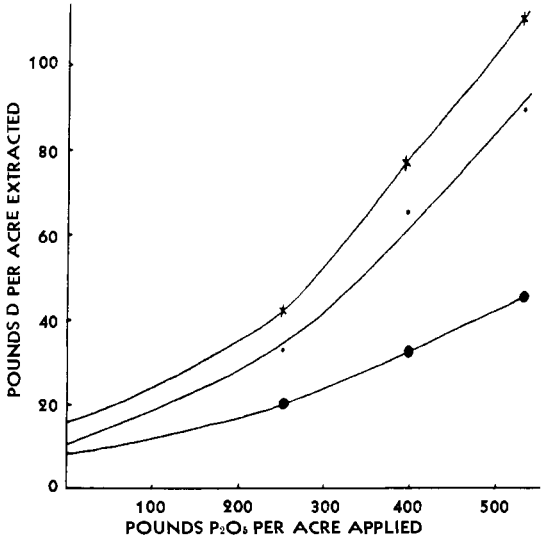

Figure 1. Relation between phosphorus extracted from Chester loam by three chemical methods and phosphate applied to soil

X Proposed method
• Modified Truog
● Sodium bicarbonate

The Galestown, Hagerstown, and Sassafras soils gave no increase in the yields of wheat in the pot culture experiment due to phosphate applications. When alfalfa was the test crop, the same three soils and the Westphalia did not show any response to phosphate applications. The soils that showed no response to phosphorus usually contained a higher level of extractable phosphorus (Table I). The levels of phosphorus in the soils at which phosphate applications often failed to increase the yields of alfalfa and wheat were: 55 pounds per acre, modified Truog; 55, Bray and Kurtz No. 1; 55, North Carolina; 35, sodium bicarbonate; and 70, proposed method.

The correlation coefficients obtained

for all the variables associated in Table II were significant at the 1% level. However, the r^2 values show that much of the variance in yield was related to factors other than soluble phosphorus as determined by the chemical methods. The phosphorus removed by the alfalfa showed a closer relationship than the yield of alfalfa with the phosphorus extracted by the different chemical methods, because at the higher levels of soil phosphorus the plant continued to absorb phosphorus even after there was no further increase in yield. In previous work conducted at this station the "sufficiency value" for phosphorus in alfalfa was estimated to be 0.20% (*8*). On a number of soils at the higher soil phosphorus levels the alfalfa contained considerably more than this quantity of phosphorus.

The correlation coefficients for the phosphorus extracted *vs.* the yields of alfalfa on each of the soils in the pot culture experiment are shown in Table III. Because a plot of the data from this experiment showed that the relationship between extractable phosphorus and yield appeared to be logarithmic rather than linear, the number of pounds of phosphorus extracted per acre was expressed as a logarithm. A comparison of the over-all correlation coefficients for soil phosphorus *vs.* alfalfa yields in Tables II and III bears out this relationship. In many instances there was little difference between the correlation coefficients for the various methods on the same soil. In this investigation the soil series apparently determined to a large extent the relationship between soluble phosphorus and yields of alfalfa. For example, the correlation coefficients calculated for the Bray and Kurtz No. 1 method ranged from 0.120 to 0.885, depending upon the soil type being studied. In a number of cases the soils which gave a poor correlation were high in extractable phosphorus and showed little or no response to phosphate applications. The over-all correlation coefficients for the 17 soils, which included 255 observations, ranged from 0.318 for the Bray and Kurtz No. 1 method to 0.461 for the Truog method. The low values for the over-all correlation coefficients can be explained on the basis that the yield and yield response were not the same for all the soils. The situation was somewhat improved when correlation coefficients were determined for the relationship between soluble phosphorus and yield within soils. The correlation coefficients then ranged from 0.585 to 0.602, which means that the variation in extractable phosphorus accounted for 34 to 36% of the variation in yield within a soil. It is evident that the soil series determined to a large extent the correlation between soluble phosphorus and yields of alfalfa and this apparently must be taken into consideration for more reliable fertilizer recommendations. Data of Olson, Dreier, and Sorensen (*11*) clearly indicate the importance of both soil series classification and soil testing for the most reliable recommendations possible.

The approximate amounts of superphosphate needed to establish similar levels of chemically available phosphorus in the soils in the pot culture experiment varied considerably (Table IV). Toth and Bear (*18*) also showed that there were wide differences in the phosphorus-absorbing capacities for soils in New Jersey. Properties such as texture and type of clay mineral influence markedly the amount of phosphate that must be applied to a soil to establish a certain level of soluble phosphorus. In this experiment the Belts-

Table I. Phosphorus Extracted by Chemical Methods from Soils in Pot Culture Experiment without Added Superphosphate (9)

Soil Type	Phosphorus Extracted by 4 Methods, Lb./Acre: Modified Truog	Bray and Kurtz No. 1	Sodium bicarbonate	Proposed method
Atkins loam	14	23	14	25
Beltsville loam	7	13	7	13
Calvin loam	14	15	13	20
Chester loam	10	14	8	15
Christiana clay loam	7	10	7	13
Collington sandy loam	24	48	22	53
Conowingo loam	9	9	8	11
Galestown sand	81	94	29	116
Golts silt loam	26	73	29	93
Hagerstown loam	74	39	30	50
Lehew sandy clay loam	36	26	17	34
Manor sandy loam	9	14	11	19
Montalto clay loam	7	11	11	13
Myersville loam	18	11	11	16
Penn sandy loam	18	16	10	23
Sassafras loamy sand	81	75	28	101
Westphalia sandy loam	33	55	20	66

Table II. Correlation of Soluble Phosphorus, Determined by Chemical Methods, with Yield, and Phosphorus Removed by Alfalfa in Pot Culture Experiment (9)

Crop Variable	Correlation Coefficients[a] for 4 Methods: Modified Truog	Bray and Kurtz No. 1	Sodium bicarbonate	Proposed method
Total yield of alfalfa for two cuttings	0.295	0.212	0.195	0.251
Phosphorus removed by alfalfa	0.522	0.495	0.421	0.516

[a] Calculated from 255 observations which included soils from 17 soil series; calculations significant at 1% level.

Table III. Correlation between Logarithm of Pounds of Soluble Phosphorus per Acre Measured by Chemical Methods and Total Yield of Alfalfa for Two Cuttings in Pot Culture Experiment (9)

Soil Series	Correlation Coefficients[a] for 5 Methods: Modified Truog	Bray and Kurtz No. 1	Sodium bicarbonate	N. C. method	Proposed method
Atkins	0.675[b]	0.602[c]	0.591[c]	...	0.636[c]
Beltsville	0.842[b]	0.834[b]	0.833[b]	0.829[b]	0.835[b]
Calvin	0.742[b]	0.751[b]	0.727[b]	...	0.749[b]
Chester	0.545[c]	0.531[c]	0.545[c]	0.536[c]	0.538[c]
Christiana	0.794[b]	0.799[b]	0.773[b]	...	0.774[b]
Collington	0.740[b]	0.739[b]	0.725[b]	0.720[b]	0.712[b]
Conowingo	0.827[b]	0.816[b]	0.804[b]	...	0.843[b]
Galestown	0.242	0.241	0.265	...	0.263
Golts	0.609[c]	0.584[c]	0.580[c]	...	0.578[c]
Hagerstown	0.134	0.120	0.108	...	0.104
Lehew	0.526[c]	0.525[c]	0.485	...	0.518[c]
Manor	0.895[b]	0.885[b]	0.876[b]	...	0.884[b]
Montalto	0.705[b]	0.692[b]	0.705[b]	0.706[b]	0.700[b]
Myersville	0.707[b]	0.676[b]	0.724[b]	0.703[b]	0.706[b]
Penn	0.347	0.388	0.359	0.381	0.348
Sassafras	0.268	0.191	0.194	...	0.179
Westphalia	0.253	0.268	0.238	...	0.156
Total for 17 soils	0.461[b]	0.318[b]	0.344[b]	...	0.359[b]
Within 17 soils	0.601[b]	0.601[b]	0.585[b]	...	0.602[b]

[a] Calculated for each soil series from 15 observations; total for 17 soils and within 17 soils calculated from 255 observations.
[b] Significant at 1% level.
[c] Significant at 5% level.

ville loam and Montalto clay loam contained the same levels of Truog phosphorus (Table I), yet the superphosphate required to establish 60 pounds of soluble phosphorus in these two soils by the modified Truog method varied by more than 1500 pounds per acre. Cole and Olsen (*3*) have also shown with calcareous soils that the soil texture affects phosphorus solubility.

The yields of alfalfa and phosphorus extracted from a Penn loam by several chemical methods are given in Table V. The application of 200 pounds of P_2O_5 increased the yield of alfalfa by almost 2 tons per acre. The phosphorus extracted by the modified Truog method and proposed method from the plots receiving this treatment was about 14 pounds per acre greater than that for the no-phosphate plots.

The influence of different phosphate carriers on the yields of corn, wheat, and red clover grown on a Beltsville silt loam is shown in Table VI. Application of equal quantities of P_2O_5 supplied by superphosphate and rock phosphate showed the superphosphate to give larger yields of corn, wheat, and red clover hay. The modified Truog, Wrenshall and McKibbin, and North Carolina methods extracted more phosphorus from the soils treated with rock phosphate (Table VII) than was readily available to the plants, as indicated by the yields of corn, wheat, and red clover. For example, the modified Truog method extracted three times more phosphorus from the rock phosphate–treated plots than from the superphosphate-treated plots which received the same quantity of P_2O_5; yet the yields of all the crops grown were less when rock phosphate was used. Other workers (*14*, *17*) have also noted this difficulty with acid extractants when rock phosphate has been used on soils. It is evident that one cannot make a reliable phosphate recommendation when using these chemical methods unless the past treatment of a soil is known. The correlation coefficients for phosphorus extracted from the Beltsville silt loam by several chemical methods and the yields of corn for 1953 and 1954 are given in Table VIII.

The phosphorus and potassium extracted from a Monmouth fine sandy loam and the yields and value of tobacco are shown in Tables IX and X. The correlation coefficients obtained for the phosphorus and potassium extracted from the soils by the North Carolina method and the yields of tobacco were significant at the 1% level. The r^2 values show that with the phosphorus soil test, 90% of the variation in the yield of tobacco was accounted for and with the potassium test 71%.

Legg and Axley (*6*) have studied the thermal method proposed by Kolterman and Truog for determining fixed potassium in soils. The data obtained by these workers suggest the possibility of using the thermal method for determining the relative capacity of soils to supply nonexchangeable potassium to plants (Table XI). A single heating and extraction treatment was as effective as a series of successive treatments in this regard.

Table IV. Approximate Quantities of Phosphate Needed to Establish Several Levels of Soluble Phosphorus in Soils by Modified Truog Method

	Truog Phosphorus Level and P_2O_5 Applied,[a] Lb./Acre			
Soil Type	40	60	80	100
Penn sandy loam	60	130	210	260
Collington sandy loam	120	240	320	400
Beltsville loam	210	340	440	520
Myersville loam	220	360	480	600
Chester loam	280	390	480	570
Montalto clay loam	410	650	800	960

[a] 20% superphosphate used to supply phosphorus.

Table V. Yields of Alfalfa and Phosphorus Extracted from Penn Loam by Chemical Methods (9)

Soil Treatment,[a] Lb./Acre		Alfalfa Yield,[c] Tons/Acre	Phosphorus Extracted by 4 Methods,[b] Lb./Acre			
P_2O_5	K_2O		Modified Truog	Bray and Kurtz No. 1	Sodium bicarbonate	Proposed method
0	600	2.06	8	6	6	12
80	600	3.51	14	14	10	18
200	600	3.93	21	13	14	26
400	600	3.66	52	38	29	59
L.S.D. at 0.05 level		0.45	..	..	..	..
L.S.D. at 0.01 level		0.59	..	..	..	..

[a] 20% superphosphate and 60% KCl used to supply P and K.
[b] Average of 4 replicates.
[c] Average annual yield from 4 replicates at 14% moisture for 1951 and 1952.

Table VI. Yields of Corn, Wheat, and Red Clover Hay on Beltsville Silt Loam as Influenced by Phosphate Carriers (9)

Soil Treatment[a]		Crop Yields[b]			
		1953		1954	
Source	P_2O_5 applied, lb./acre	Center section corn, bu./acre	South section hay, tons/acre	Center section wheat, bu./acre	South section corn, bu./acre
Superphosphate	75	38.0	3.41	30.0	86.7
Rock phosphate	75	34.0	3.05	24.1	75.0
	300	41.5	3.24	27.0	85.3

[a] All plots received annual application from 1952 to 1954 of 100 pounds of K_2O per acre and 45 pounds of N for wheat and 100 pounds for corn. Plots that received annual application of 75 pounds of P_2O_5 per acre from 1952 to 1954 received 50 pounds annually from 1941 to 1951, and plots that received 300 pounds of P_2O_5 annually from 1952 to 1954 received 200 pounds annually from 1941 to 1951.
[b] Each value represents average for superphosphate of 4 replicates and for rock phosphate of 3 replicates.

Conclusions

There was often little difference between the correlation coefficients obtained for the various chemical methods used to determine phosphorus in the soils. However, when all the soils in the investigation (those treated with rock phosphate or superphosphate) were considered, a proposed method employing sulfuric acid and ammonium fluoride as an extractant apparently showed the closest relationship with crop response. When the soils receiving rock phosphate were not considered, the modified Truog method gave the highest correlation with crop response data of all soluble phosphorus methods studied. However, on rock phosphate–treated soils, the Truog and other methods employing acid extractants removed more phosphorus from the soils than was readily available to the plants as indicated by the yields.

In the pot culture experiment the soil series determined to a large extent the correlation between phosphorus extracted by the different methods and the yields of alfalfa. The quantities of 20% superphosphate required to establish similar levels of chemically available phosphorus in these soils varied in some cases by a ton or more.

The levels of extractable phosphorus in the soils for the different chemical

Table VII. Phosphorus Extracted by Chemical Methods from Beltsville Silt Loam Treated with Superphosphate and Rock Phosphate (9)

Soil Treatment[a]	Phosphorus Extracted by 6 Methods, Lb./Acre											
	Sodium Bicarbonate		Bray and Kurtz No. 1		Proposed Method		Wenshall and McKibbin		Modified Truog		N. C. Method	
	C[b]	S[b]	C	S	C	S	C	S	C	S	C	S
Superphosphate,[c] 75 lb. P_2O_5/acre	15	21	22	29	37	42	25	36	25	33	36	45
Rock phosphate[d]												
75 lb. P_2O_5/acre	3	8	5	10	12	18	48	48	86	88	128	125
300 lb. P_2O_5/acre	9	13	14	16	27	29	149	152	260	276	472	506

[a] See footnotes[a], Table VI, for treatment history.
[b] C center section; S south section.
[c] Each phosphorus value represents average of 4 replicates.
[d] Each phosphorus value represents average of 3 replicates.

Table VIII. Correlation of Soluble Phosphorus Determined by Chemical Methods with Yield of Corn for 1953 and 1954 on Beltsville Silt Loam Treated with Superphosphate and Rock Phosphate (9)

Chemical Method	Correlation Coefficient[a]
Modified Truog	0.123
North Carolina method	0.165
Wrenshall and McKibbin	0.233
Bray and Kurtz No. 1	0.284
Sodium bicarbonate	0.394
Proposed method	0.436

[a] Correlation coefficients calculated from 20 observations. At 5% level $r = 0.444$.

Table IX. Yields and Value of Tobacco and Phosphorus Extracted from Monmouth Fine Sandy Loam by North Carolina Method

P_2O_5 Applied, Lb./Acre	Tobacco		Phosphorus Extracted Lb./Acre
	Yield,[a] lb./acre	Value, $/acre	
0	803	259	13
30	876	420	24
90	942	429	46
180	1000	482	88

[a] Average annual yield for 6 years from 4 replicates. Phosphorus extracted *vs.* yield of tobacco, $r = 0.949$. Significant at 1% level. Cooperative project between University of Maryland and U. S. Department of Agriculture.

Table X. Yields and Value of Tobacco and Potassium Extracted from Monmouth Fine Sandy Loam by North Carolina Method

K_2O Applied, Lb./Acre	Tobacco		K Extracted, Lb./Acre
	Yield[a] lb./acre	Value, $/acre	
0	750	350	151
60	846	465	205
120	892	508	311
240	928	603	490
300	917	637	638

[a] Average annual yield for 6 years from 4 replicates. K extracted *vs.* yield of tobacco, $r = 0.840$. Significant at 1% level. Cooperative project between University of Maryland and U. S. Department of Agriculture.

Table XI. Nonexchangeable Potassium Removed by Cropping and by Single Heating (500° C.) and Extraction Treatment (6)

Soil	By First Heating and Extroction Treatment, P.P.M.	By Prolonged Cropping, P.P.M.
Sable	610	613
Herrick	345	332
Wooster	242	217
Hagerstown	240	128
Decatur	207	122

methods at which phosphate applications often failed to increase the yields of alfalfa in the pot culture experiment were: 55 pounds per acre, modified Truog; 55, Bray and Kurtz No. 1; 55, North Carolina; 35, sodium bicarbonate; and 70, the proposed method.

The correlation coefficient for the relation between potassium extracted from a Monmouth soil by the North Carolina method and the yields of tobacco in a field experiment for a 6-year period was significant at the 1% level. In this same experiment the phosphorus extracted also showed a close relationship with tobacco yields.

The thermal method proposed by Kolterman and Truog showed some promise for determining the relative potassium-supplying power of soils.

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Paper A803, Contribution 3074, Maryland Agricultural Experiment Station (Department of Agronomy).

Correction

The Toxicology of Butoxypolypropylene Glycol 800 (Crag Fly Repellent)

In this article by C. P. Carpenter, C. S. Weil, P. E. Palm, M. D. Woodside, and H. F. Smyth, Jr. [J. AGR. FOOD CHEM. **7,** 763 (1959)], on page 763, the sentence beginning in the eighth line of the abstract should read, "It is not stored in the bodies of animals, and 50% or more of a single dose may be found in the feces unchanged."

Table VII. Phosphorus Extracted by Chemical Methods from Beltsville Silt Loam Treated with Superphosphate and Rock Phosphate (9)

Soil Treatment[a]	Phosphorus Extracted by 6 Methods, Lb./Acre											
	Sodium Bicarbonate		Bray and Kurtz No. 1		Proposed Method		Wenshall and McKibbin		Modified Truog		N. C. Method	
	C[b]	S[b]	C	S	C	S	C	S	C	S	C	S
Superphosphate,[c] 75 lb. P_2O_5/acre	15	21	22	29	37	42	25	36	25	33	36	45
Rock phosphate[d]												
75 lb. P_2O_5/acre	3	8	5	10	12	18	48	48	86	88	128	125
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[a] Correlation coefficients calculated from 20 observations. At 5% level $r = 0.444$.

Table IX. Yields and Value of Tobacco and Phosphorus Extracted from Monmouth Fine Sandy Loam by North Carolina Method

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0	803	259	13
30	876	420	24
90	942	429	46
180	1000	482	88

[a] Average annual yield for 6 years from 4 replicates. Phosphorus extracted *vs.* yield of tobacco, $r = 0.949$. Significant at 1% level. Cooperative project between University of Maryland and U. S. Department of Agriculture.

Table X. Yields and Value of Tobacco and Potassium Extracted from Monmouth Fine Sandy Loam by North Carolina Method

K_2O Applied, Lb./Acre	Tobacco		K Extracted, Lb./Acre
	Yield[a] lb./acre	Value, $/acre	
0	750	350	151
60	846	465	205
120	892	508	311
240	928	603	490
300	917	637	638

[a] Average annual yield for 6 years from 4 replicates. K extracted *vs.* yield of tobacco, $r = 0.840$. Significant at 1% level. Cooperative project between University of Maryland and U. S. Department of Agriculture.

Table XI. Nonexchangeable Potassium Removed by Cropping and by Single Heating (500° C.) and Extraction Treatment (6)

Soil	By First Heating and Extroction Treatment, P.P.M.	By Prolonged Cropping, P.P.M.
Sable	610	613
Herrick	345	332
Wooster	242	217
Hagerstown	240	128
Decatur	207	122

methods at which phosphate applications often failed to increase the yields of alfalfa in the pot culture experiment were: 55 pounds per acre, modified Truog; 55, Bray and Kurtz No. 1; 55, North Carolina; 35, sodium bicarbonate; and 70, the proposed method.

The correlation coefficient for the relation between potassium extracted from a Monmouth soil by the North Carolina method and the yields of tobacco in a field experiment for a 6-year period was significant at the 1% level. In this same experiment the phosphorus extracted also showed a close relationship with tobacco yields.

The thermal method proposed by Kolterman and Truog showed some promise for determining the relative potassium-supplying power of soils.

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Paper A803, Contribution 3074, Maryland Agricultural Experiment Station (Department of Agronomy).

Correction

The Toxicology of Butoxypolypropylene Glycol 800 (Crag Fly Repellent)

In this article by C. P. Carpenter, C. S. Weil, P. E. Palm, M. D. Woodside, and H. F. Smyth, Jr. [J. AGR. FOOD CHEM. **7,** 763 (1959)], on page 763, the sentence beginning in the eighth line of the abstract should read, "It is not stored in the bodies of animals, and 50% or more of a single dose may be found in the feces unchanged."

Current New Jersey Research in Chemical Soil Testing

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Correlation studies are being made between nutrient extraction by electrodialysis and plant response to variable soil fertility levels in greenhouse and field. This is a progress report.

SINCE 1933 a chemical soil-testing service of New Jersey farmers and gardeners has been conducted by the College of Agriculture of Rutgers University. A variety of salt and acid solutions have been used as extractants for the removal of the so-called "available" nutrients from the soil. Among the first solutions was that of Morgan (*13*). Another but more dilute sodium acetate solution employed for a number of years was developed by Hester, Blume, and Shelton in Virginia (*10*). For the past 11 years the method of nutrient extraction from soil samples has been electrodialysis, initiated by Purvis and Hanna (*15*). The testing procedures now in use are described in a bulletin (*8*).

Electrodialysis has been used for the extraction of exchangeable cations from soils since the early work of Bradfield (*2*), Mattson (*12*), and Humfield and Alben (*11*). Several investigators, including Harper (*9*) and Dean (*3*), have also studied the release of phosphorus from soils by electrodialysis. Dean found a higher correlation between crop response to applied phosphorus and soil measured by electrodialysis than to soil phosphorus measured by any of several other extractive means.

Tests have been under way in New Jersey for the past 12 years to compare electrodialysis with other means of nutrient extraction as well as with plant response to and uptake of nutrients in the soil. Results from a laboratory and greenhouse test conducted in 1950 (*7*) are shown in Table I. Correlation of phosphorus extracted by electrodialysis with growth and phosphorus uptake by snap beans from two soils supplied with phosphorus from eight sources compares favorably with similar correlations of phosphorus extracted by four other methods. Yield of beans and phosphorus uptake were based on the whole above-ground portion of plants. The eight sources of phosphorus were normal superphosphate, aluminum, tricalcium, iron, magnesium, and manganese phosphates, ground phosphate rock, and residual soil phosphorus. Electrodialysis and the strong acid extractant of Bray dissolved relatively more phosphorus from the soils treated with rock phosphate and tricalcium phosphate than was available to the bean plants. Other tests of this type, including *A* value determinations with phosphorus-32 have been and are being conducted.

Two types of field tests for soil-test correlation are being conducted in various soil areas of the state. The first is the usual measure of crop response to applied fertilizer. A series of tests at different sites on the same or different soil types is run with a single crop. The tests are then repeated for more than one year with the same crop but on different sites. Tests have been designed to measure the optimum amounts of phosphorus and potassium that should be applied for various soil-test levels. The plot layouts have been according to a central composite design similar to that used by Baird and Fitts (*1*) and Hader *et al.* (*6*).

In the second type of test, various crops are grown on the same site for more than one year. Rather than crop response to applied fertilizer, crop growth is measured at variable residual nutrient levels in the same soil. Field plot designs are simple complete factorials with three levels of phosphorus and potassium. Two levels of nitrogen are superimposed, but only the phosphorus and potassium soil tests are under calibration. Locations of these tests, the crops grown in 1959, and the soils at the three sites are:

Beemerville, Sussex Co., upper Piedmont. Alfalfa, corn, oats (Duchess silt loam).

New Brunswick, Middlesex Co., upper coastal plain. Peppers, potatoes, snap beans, barley (Nixon loam).

Glassboro, Gloucester Co., lower coastal plain. Asparagus, peppers (Downer sandy loam).

Tomato yields from the Glassboro plots in 1958 are summarized in Table II. High yields from low fertility treatments are due to residual phosphorus and potassium from fertilizer applications made prior to start of test. No general conclusions may be drawn from these data, as they are limited to one soil and one year. Examination of results from several such tests is necessary to formulate changes in fertilizer

Table I. Correlation of Extracted Phosphorus with Snap Bean Yield and Uptake

	Norton Silt Loam		Sassafras Sandy Loam	
Extractant	Yield	P uptake	Yield	P uptake
Electrodialysis	0.882	0.901	0.934	0.943
Na acetate, 1	0.841	0.906	0.818	0.855
Na acetate, 2	0.918	0.953	0.902	0.877
Acetic acid	0.881	0.935	0.878	0.883
Bray No. 1	0.899	0.861	0.913	0.867

Table II. Tomato Yields, Glassboro, 1958

Fertility, P	Treatment, K	Yield, Tons/Acre 1	Yield, Tons/Acre Total
Low	Low	19.7	20.6
	Medium	18.1	19.2
	High	22.3	23.3
Medium	Low	18.4	19.2
	Medium	22.6	24.0
	High	23.7	24.8
High	Low	20.3	21.4
	Medium	25.1	26.2
	High	25.0	26.3
Low	...	20.0	21.0
Medium	...	21.6	22.7
High	...	23.8	24.6
...	Low	19.5	20.4
...	Medium	21.9	23.1
...	High	23.7	24.8

recommendations. It is interesting to note a yield response to additional potassium and phosphorus, even when the residual soil level of these nutrients is sufficiently high to produce more than 20 tons of tomatoes an acre. Although information on the fertilizer requirements of a number of crops, for a variety of New Jersey soils, in relation to soil-test results has been accumulated over the past 12 years, a great deal more work remains to be done.

Results of calibration tests to date are best summarized by listing present interpretations of soil test results for various crops. Interpretations of phosphorus tests are given in Table III. Five crop groupings have been made on the basis of present information. In a general way, the crops with the lower phosphorus requirements are the perennials and the long-season grain and forage crops. Perhaps some of these crops obtain an adequate supply of phosphorus for their needs from soils with lower levels of phosphorus as measured by the soil tests. Crops that have shown growth response to added phosphorus at higher soil levels are the short-season, rapid-growing vegetables.

Potassium test interpretations are listed in Table III. Only four crop groups have been established for potassium test levels. Grains, grasses, and perennial fruits show less yield response to added potassium than do most of the vegetables grown in New Jersey. The legumes for hay or pasture respond in increased growth to added potassium at relatively high soil potassium levels.

Combining the phosphorus and potassium test interpretations has given some 26 groups of crops for recommending grade and amount of fertilizer according to soil-test results. An example of the use of these groupings is the fertilizer recommendations (Table IV) for alfalfa, clovers, and pastures at time of seeding. The fertilizer recommendation for a soil in which both the phosphorus and potassium tests are medium is the general recommendation for this or other groups of crops (*16*). Recommendations for soils testing other than medium in phosphorus or potassium are made by adjustment of the phosphorus or potassium to be added by selection of the proper grade of fertilizer from the approved list of ratios or from straight phosphorus and potassium goods.

While the most important use of soil-test results is that of obtaining information to aid in determining the fertilizer needs of a particular crop on the particular field from which the sample for testing was taken, these results are useful for a variety of purposes. Summaries

Table III. Soil-Test Interpretation

(Pounds per acre)

Crop Group	Very Low	Low	Medium	High	Very High
		PHOSPHORUS			
Blueberries, cane fruit, soybeans	0–5	5–10	10–20	20–30	30
Alfalfa, clover, and grass hay and/or pastures, corn, small grains, apples, grapes, turf	0–5	5–10	10–20	20–40	40
Peaches	0–10	10–20	20–35	35–50	50
Asparagus, beans, beets, cucurbits, crucifers, peppers, white and sweet potatoes, strawberries, young fruit trees	0–10	10–20	20–40	40–60	60
Celery, eggplants, onions, peas, sweet corn, and leafy vegetables such as lettuce, spinach, etc.	0–15	15–30	30–60	60–90	90
		POTASSIUM			
Corn, small grains, grass pasture and hay, all fruit except strawberries, soybeans, turf	0–40	40–80	80–120	120–160	160
Asparagus, beans, beets, cucurbits, crucifers, peppers, tomatoes	0–50	50–100	100–150	150–200	200
Celery, eggplants, onions, peas, sweet corn	0–75	75–125	125–200	200–300	300
Alfalfa, clover pasture, and hay	0–100	100–200	200–300	300–400	400

Table IV. Fertilizer Recommendations for Alfalfa, Clovers, and Pastures at Seeding

(Pounds per acre)

Available P in Soil[a]		Available Potassium in Soil: 0 Very Low		75 Low		150 Medium		225 High		300 Very High	
		Fertilizer Recommendations									
0	Very low	160 160	P_2O_5 K_2O	160 100	P_2O_5 K_2O	160 80	P_2O_5 K_2O	160 60	P_2O_5 K_2O	160 30	P_2O_5 K_2O
		400 800	0–20–20 5–10–10	350 900	Super 5–10–10	400 800	Super 5–10–10	500 700	Super 8–8–8	550 600	Super 8–8–8
5	Low	100 160	P_2O_5 K_2O	100 100	P_2O_5 K_2O	100 80	P_2O_5 K_2O	100 60	P_2O_5 K_2O	100 30	P_2O_5 K_2O
		300 700	0–20–20 7–7–14	1000	5–10–10	900	5–10–10	1100	5–10–5	300 600	Super 8–8–8
10	Medium	80 160	P_2O_{52} K_2O	80 100	P_2O_5 K_2O	80 80	P_2O_5 K_2O	80 60	P_2O_5 K_2O	80 30	P_2O_5 K_2O
20		300 700	0–10–20 7–7–14	900	5–10–10	800	5–10–10	800	5–10–10	800	5–10–10
	High	60 160	P_2O_5 K_2O	60 100	P_2O_5 K_2O	60 80	P_2O_5 K_2O	60 60	P_2O_5 K_2O	60 30	P_2O_5 K_2O
40		200 800	Muriate 5–10–5	800	7–7–14	700	7–7–14	700	8–8–8	700	5–10–5
	Very high	30 160	P_2O_5 K_2O	30 100	P_2O_5 K_2O	30 80	P_2O_5 K_2O	30 60	P_2O_5 K_2O	30 30	P_2O_5 K_2O
		200 500	Muriate 8–8–8	200 500	Muriate 8–8–8	600	7–7–14	500	7–7–14	400	8–8–8

[a] As determined by electrodialysis at New Jersey Agricultural Experiment Station.

Table V. Soil Test Results for Tomatoes, 1958

Phosphorus	Potassium			
	Low	Medium	High	Total
	Per Cent of Samples			
Low	7	1	3	11
Medium	5	19	4	28
High	13	14	34	61
Total	25	34	41	

Table VI. Soil Test Results in Warren County

(Per cent each soil)

Land Type Areas	Phosphorus			Potassium		
	Low	Medium	High	Low	Medium	High
Early drift						
Shale uplands	75.8	24.0	0.2	65.6	34.4	0
Limestone valleys	68.5	23.1	8.4	46.4	46.1	7.3
Gneiss highlands	86.4	10.4	3.2	59.2	32.0	8.8
Later drift						
Shale uplands	73.0	21.9	5.1	70.9	24.1	5.0
Limestone valleys	49.4	37.1	13.5	71.2	24.3	4.5
Gneiss highlands	72.7	22.1	5.2	59.7	39.6	1.3
The Moraine	61.1	25.0	13.9	48.1	43.6	8.3
Muck	6.1	21.7	72.2	3.5	23.5	73.0

from a large number of samples from different soils and crops will show areas of nutrient deficiencies, fertilizer use patterns, and probable percentage of samples, in a given area and for a given crop, requiring various grades of fertilizer. The use of summaries of soil-test results to show probable fertilizer needs for a particular crop is demonstrated in Table V. Soils used for tomatoes in New Jersey tend to be low in potassium and high in phosphorus. These data indicate that a 1–2–2 or 1–1–1 fertilizer would be recommended for about 61% of the soils used for tomatoes; 1–1–2 for some 32% of the samples; and 1–2–1 for only 7% of the soils. Fitts, Welch, and Nelson (*4*) have used this same type of data to predict fertilizer ratios needed for a particular crop in various areas of North Carolina. Only a limited number of such crop summaries have been made in New Jersey, because soil test data will be completely transferred to punch cards only as funds permit. Rather complete summaries have been made (*5, 14*) for three counties—Warren, Monmouth, and Gloucester—where recent soil surveys have been completed.

One type of summary of soil-test data that has been made is illustrated in Table VI. Phosphorus and potassium tests for Warren County have been sorted according to age of soil and type of parent material. In this county there is a rather sharp boundary between areas covered during the glacial periods. The shale soils show little difference in phosphorus or potassium levels between the two time periods. Soils of the limestone valleys have become more acid with time and as a result soils on the earlier drift are more acid, as indicated by pH tests, and have lower available phosphorus. On the other hand, these limestone soils have higher potassium in the area of the earlier drift, where a longer time of weathering and soil formation has resulted in more acid soils. These data point to the difference in the amount of weathering with time between the shale and gneiss parent materials. Highly fertilized vegetables are grown on the muck.

All soil-test calibration studies are being expanded as funds permit. Fertilizer recommendations are modified according to results of laboratory, greenhouse, and field experiments as needed to give New Jersey growers the best possible information on the fertilizer requirements of their crops and soils.

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Interpretation of Soil Tests and Application as Charted by Current Research

E. J. KAMPRATH and J. W. FITTS

Department of Soils, North Carolina Agricultural Experiment Station, Raleigh, N. C.

Soil tests should provide information about the soil which will serve as a guide to liming and fertilization. Since the time of Liebig, the goal of agronomists has been evaluation of soil fertility and prediction of crop yield from soil tests. However, the yield of a crop (both quantity and quality) is a function of several factors, which can be expressed by the equation:

$$\text{Yield} = f(\text{crop} + \text{soil} + \text{climate} + \text{management})$$

If an index or value can be obtained for each variable, the yield can be calculated. Soil testing furnishes information about the soil variable in the equation in respect to fertility or special soil conditions. Other sources of information are needed for the other variables.

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$$\text{Yield} = f(\text{crop} + \text{soil} + \text{climate} + \text{management})$$

If an index or value can be obtained for each variable, the yield can be calculated. Soil testing furnishes information about the soil variable in the equation in respect to fertility or special soil conditions. Other sources of information are needed for the other variables.

Soil tests furnish information to guide in liming and fertilization of soils. In the future soil tests will be very important as tools to assess the condition of the soil and provide information about building and maintaining soil fertility. Basic research is necessary to provide information about the chemical properties of the soils.

Fitts and Nelson (*3*) point out that there are four major phases of soil testing: calibration of the test with crop response, securing of representative samples, chemical testing procedures, and interpretation and recommendation. This paper deals largely with interpretation of results.

Early Attempts at Soil Testing

Soil testing has been viewed in many ways—from a psychological tool to a single-value soil management cure-all. One of the first problems encountered was interpretation of the results obtained from various procedures. Crop rotations, with and without application of manure, were the principal field studies under way in the early days. Unsuccessful efforts were made to calibrate soil tests by taking soil samples from the rotation plots and explaining crop yields on the basis of the soil-test results.

Soil Testing Today

Information gained from soil testing is used in many ways. Some of the more important objectives (*3*) include:

To group soils into classes for suggesting fertilizer and lime practices.
To predict the probability of getting a profitable response to the application of plant nutrients.
To help evaluate soil productivity.
To determine specific soil conditions which may be improved by addition of soil amendments or cultural practices.

Soil tests are calibrated in several ways. One procedure is the correlation of soil test values with per cent yield of plant material or with total uptake of the nutrient element being studied from treated and untreated soils. In this technique, yield responses to fertilization at a given soil level may vary considerably because of differences in properties of the soil other than fertility or other variables in the yield equation.

Another method of predicting lime and fertilizer responses from soil tests values is the "probability" approach suggested by Fitts (*2*). The probability of obtaining a profitable increase in yield for a given fertilizer treatment is plotted against the soil-test results. The index of probability used is the percentage of fields within a given range of soil-test values that do or do not respond to application of the soil amendment. The chances of getting a profitable response to fertilization are much greater on a soil that tests low in a given nutrient than on one that tests high. This concept does not rule out the possibility of a profitable response from fertilizer application at a high level of fertility, if yield factors other than fertility are optimum. Likewise, a profitable response on soils of low fertility is not assured when other factors such as climate and management are poor.

Trends in Soil Testing

A big problem in soil-test interpretation and recommendations is how much fertilizer should be applied. Although crops vary in their nutrient requirements, the addition to the soil of amounts equivalent to those absorbed by the plants is not valid, because only a portion of the applied fertilizer will be utilized. The remainder may be lost by leaching or made immobile by fixation. The release of nutrients from the soil must also be taken into consideration.

The addition of fertilizers and lime to the soil changes the immediate environment of the plant roots. Although a large increase may be obtained from application of a fertilizer to a soil low in a given element, the yield obtained is not likely to approach that obtained on a "fertile" soil.

The importance of the fertility level in the yield of crops is shown by experiments on a Norfolk soil (*5*).

P_2O_5 Added, Lb./Acre	Soil Phosphorus Level	
	Low	High
	Yield of Seed Cotton, Lb./Acre	
None	834	2112
50	1403	2287

The highest yields of seed cotton were obtained on the soil with a high phosphorus level. Addition of phosphorus to the soil deficient in this element increased yield but did not bring it up to that obtained at the high soil level. When all the other factors affecting yield are satisfactory, a profitable response can be obtained to fertilization even at a high soil level, as shown by the increase in the yield of seed cotton when 50 pounds of P_2O_5 were applied to the soil high in phosphorus.

Interpretation of soil-test results and recommendations become a question of how to improve the fertility status of the soil. How much fertilizer will be needed to change the soil from low to medium or high in that element? What soil factors will influence the amounts of fertilizer required? What will be the most economical level at which to maintain the nutrient status of the soil? Many factors will influence the possibility of building and maintaining high nutrient levels. An important one is the capacity of the soil to fix phosphorus in insoluble forms.

In Table I are shown the amounts of phosphorus that had to be applied to bring three soils to the same phosphorus level. On the Norfolk considerably less phosphorus is required to raise the soil phosphorus level than on the Cecil or Rabun soils. The relative phosphorus-fixing capacities of soils can be determined by laboratory methods.

Table I. Phosphorus Additions Required to Increase Soil Phosphorus Level (6)

Soil	Initial P Soil Level, P.P.M.	P_2O_5 Added, Lb./Acre	Final P Soil Level, P.P.M.
Norfolk	13	200	28
Cecil	6	400	28
Rabun	7	800	29

Table II. Relation of Corn Yields to Rate of Potassium Fertilization on a Portsmouth Soil (7)

K_2O Added, Lb./Acre	Initial Soil K Level, Meq./100 G.	
	0.08	0.29
	Average Corn Yield, Bushels/Acre	
0	82	103
20	97	105
80	96	103

Table III. Influence of Rate of Potassium Fertilization on Soil K Level of Portsmouth Soil (7)

K_2O Added, Lb./Acre	Initial Soil K Level, Meq./100 G.	
	0.08	0.29
	Soil K Level, Meq./100 G.	
0	0.08	0.22
20	0.11	0.26
80	0.15	0.28

Soil	P-Fixing Capacity, Meq./100 G.
Norfolk (*4*)	2.0
Cecil (*7*)	6.8
Rabun (*7*)	14.4

Once this information is available, the soils of an area can be grouped according to their relative phosphorus-fixing capacities, and recommendations for phosphorus fertilization can be made.

The available potassium content is another important factor in evaluating the fertility level of a soil. The availability of potassium in a given soil for annual crops is related in large part to the amount of exchangeable potassium, which is the form generally measured by a soil test. The ability of a soil to supply potassium over a long time, however, depends upon the amount of nonexchangeable potassium present. Therefore, in evaluating the potassium status of a soil the level of both exchangeable and nonexchangeable potassium must be considered.

The importance of a high level of soil potassium is shown by Table II. The highest average yields of corn for a 3-year period were obtained at the high soil potassium level. The yield response data indicate that at the low soil level 20 pounds of K_2O per acre was sufficient, while at the high soil level none was needed. However, a fertilization program should take into account not only the effect on the yield but also the effect on the soil. The soil-test data show that to increase the potassium level of the soil appreciably at least 80 pounds of K_2O per acre must be applied; to maintain the high level at least 20 pounds of K_2O must be applied (Table III). In making recommendations based on soil tests it is important to consider what is happening to the fertility level of the soil as well as the nutrient requirements of the crop.

The level to which potassium can be built in a soil depends upon the cation exchange capacity and the cations present on the exchange complex. Generally, the higher the cation exchange capacity, the larger is the reservoir in which potassium can be stored. Therefore, it is usually possible to build the available potassium content of soils with a high cation exchange capacity to a higher level than those of a low capacity.

Soil	% of Applied K Retained	
	Acid	Limed
Norfolk (low cation exchange capacity)	22	49
Cecil (medium cation exchange capacity)	60	89

The amount of potassium held was greatly increased when the soils were limed, which resulted in the replacement of exchangeable hydrogen and aluminum by calcium and magnesium (*8*). If the potassium level of acid soils is to be increased by fertilization, it is important that the soils first be limed.

Information about the lime status of a soil also is obtained from a soil test. Generally, the pH of the soil is used as the criterion for determining whether or not a response will be obtained to the addition of lime. Soil pH alone, however, is not a good index for determining the lime needs of a soil. Coleman, Kamprath, and Weed (*7*) pointed out that the nature of the ion exchange material is important in determining at what pH level a response to lime will be obtained. The amount and kind of clay and the amount and characteristics of the organic matter determine the ion exchange properties of the soil. Plants differ as to the optimum pH and lime level necessary for good growth. Therefore, it is necessary to consider the kind of plant to be grown as well as the chemical properties of the soil when making lime recommendations for acid soils.

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Submitted with the approval of the director as Paper 1084 of the Journal Series.

Commercial Laboratory and Advisory Procedures in Subtropical Agriculture

BENJAMIN WOLF

Dr. Wolf's Agricultural Laboratories, Hollywood, Fla.

Advisory systems based on soil and plant analysis are of great value in subtropical agriculture. Considerable attention must be paid to trace elements because of light soils and extreme leaching. The combination of soil and climate necessitates sampling on a 2-week schedule soils producing vegetables, gladioli, chrysanthemums, and other plants having limited food storage capacity. A much less frequent schedule (three to four times per year) is needed for citrus, cane, pasture, sod, and other perennials.

THE importance of advisory procedures based on adequate analysis of soil and plant has increased considerably in the last decade. Today it has come of age, although much remains to be done to increase its accuracy and usefulness.

Procedures and calibrations were established in the Middle Atlantic states, primarily in New Jersey. The methods used are based on Morgan's extracting solution, carefully calibrated with crop response at Seabrook Farms and later at laboratories at Bridgeton, N. J., and Hollywood, Fla. The requirements in terms of soil nutrient levels have been established for a number of crops, but correlations are still going on to define nutrient levels and amounts of fertilizer necessary to change one level to another.

About 10% of our work deals with determination of soluble nutrients and total nutrients in plants. Plant tests check on soil tests and also point out possible deficiencies. Soil tests point out deficiencies or excess of nutrients in time to make corrections without seriously lowering crop production. Often by the time such deficiencies or excesses are noted in plant analysis,

Once this information is available, the soils of an area can be grouped according to their relative phosphorus-fixing capacities, and recommendations for phosphorus fertilization can be made.

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serious crop losses have resulted.

Many calibrations for vegetables in New Jersey held well for Florida, but several new problems presented themselves.

Trace Elements

The first major change involved certain trace elements. In New Jersey we had run tests for pH, organic matter, available nitrogen, phosphorus, potassium, calcium, and magnesium, and occasionally boron and manganese. Under Florida conditions it was also necessary to determine copper, iron, and zinc. Now the complete list is run as a matter of routine.

The need for checking trace elements is especially acute under Florida conditions, where light soils originally contained small amounts of these elements and high annual rainfall has leached them from the soil. It is highly advantageous to test for all these elements in New Jersey, Pennsylvania, Delaware, Maryland, Virginia, Georgia, and the Caribbean Islands. Seldom are as many trace elements in short supply as in Florida, but routine testing has helped uncover both shortages and excesses in other areas.

Chemostatic Control

The light soils and high annual rainfall make control of all elements a more serious problem than on soils of high exchange capacity and in a more equitable climate.

While Florida has a number of soils with a relatively good exchange capacity, most of the soils we are dealing with have an exchange capacity of less than 2 meq. per 100 grams. Many are sand with a little organic matter. This influences the frequency of testing for many elements as well as the need for determining trace elements. Soils of low exchange capacity are definitely limited as to amounts of nutrients they will hold under leaching conditions. Nearly everything must be supplied from the fertilizer bag, yet not too much can be applied at one time, lest we "burn" the crop.

This has prompted large use of organic sources of nitrogen. Unfortunately when extra nutrients are needed because of heavy leaching, the organics often fail to do the job. Cold weather following rains, or the lowered oxygen content of the soil, slows down decomposition of the organic nitrogen to a point where it is unsatisfactory. Fortunately, periodic and frequent testing can evaluate such a situation and a grower can usually apply available nitrogen quickly enough to save his crop.

To evaluate the changes brought about by variable weather and on soil of limited holding capacity has required frequent testing. For most annuals which store little plant food, it has been necessary to test areas every 2 weeks, and occasionally extra samples are taken.

The frequency of testing is much less for perennial crops that have much greater storage capacity, and therefore are less dependent on daily or weekly fluctuations in soil nutrients. For pastures and citrus, good results can be obtained by making tests three or four times a year.

Such frequent testing of the soil may be called "chemostatic" control of plant nutrition. Much as a thermostat controls the operation of a furnace to provide a uniform temperature, chemostatic control can provide a chemical climate or a range of nutrients favorable for crop production. Although not automatic, chemostatic control can provide much more favorable growth than any hit-or-miss system.

In practice we try to establish a schedule of fertilization which provides a basis for crop nutrition. Then periodic testing determines whether applications should be reduced, maintained, or increased.

Nitrogen tests are most useful. We determine all three forms of available nitrogen in one test. Nitrate and nitrite forms in the Morgan's soil extract are reduced with titanous chloride solution to the ammonium form. This ammonium nitrogen, plus what was originally available as ammonium, is determined with a modified Graves reagent, and as in other tests, is measured photometrically.

Fertility Levels

Additional problems are presented with new crops, or with familiar crops grown under vastly different conditions. Experience with avocado and mango is still rather meager. Generally, nutrient levels are kept under conditions similar to those for citrus.

In most cases pH is adjusted to 6.0 to 6.5 once a year by use of dolomite.

Citrus. Citrus is marked by rather high requirements for nitrogen and calcium, but low for phosphorus and potassium. An examination of the leaf often shows 5% or more of dry weight as calcium, with less than 1% of potassium. Such trees produce a normal crop. Under certain conditions these potassium levels may be allowed to fall too low, with resultant poor growth (Table I). Nevertheless, frost resistance to a certain degree is inversely related to potassium fertilization and within certain ranges is directly related to nitrogen fertilization. Excellent growing conditions can be maintained by adjusting nutrient levels (pounds per acre) three or four times a year, based on an over-all treatment.

N	75–100	B	2–4
P	15–25	Mn	2.5–10
K	100–150	Cu	1–4
Ca	1000+	Fe	2.5–5
Mg	150+	Zn	2.5–20

A major problem on old groves is accumulation of copper in the soils to toxic levels, which interfere seriously with root development and iron uptake. Generally, this can be corrected by raising the pH to 6.5 to 7.0 with dolomite, increasing the available phosphorus content to 50 to 75 pounds per acre, including as much organic matter as possible through cover crops and weeds, and increasing the iron content. Where

Table I. Analysis[a] of Pineapple Orange Leaves

	Good Tree, %	Poor Tree, %	Dying Tree %,
Nitrogen	2.00	2.42	2.40
Phosphorus	0.14	0.14	0.14
Potassium	0.46	0.45	0.36
Calcium	6.0	6.1	6.4
Magnesium	0.17	0.17	0.14
Boron	0.0038	0.0042	0.0040
Manganese	0.0017	0.0015	0.0015
Copper	0.0020	0.0020	0.0030
Iron	0.0070	0.0065	0.0100
Zinc	0.0050	0.0080	0.0034

[a] Dry weight basis.

Table II. Influence of Soil Treatment on Leaf Analysis and Yield of Sugar Cane

Treatment[a]		Leaf Analysis, %[b]			Yield		
P_2O_5, lb.	K_2O, lb.	N	P	K	Cane, tons/acre	%	Sugar, tons/acre
0	150	1.92	0.18	1.08	40.03	10.02	4.01
25	150	2.22	0.23	1.04	42.87	9.84	4.22
50	150	2.32	0.26	1.04	41.62	9.77	4.10
100	150	2.40	0.28	0.96	42.75	9.67	4.13
200	150	2.30	0.33	0.85	43.75	9.21	4.02
0	300	1.92	0.21	1.18	42.50	9.98	4.24
25	300	2.40	0.24	1.18	45.73	9.81	4.45
50	300	2.32	0.28	1.08	43.84	9.63	4.22
100	300	2.60	0.27	1.00	43.45	9.40	4.08
200	300	2.60	0.30	1.00	42.08	9.44	3.97

[a] Pounds per acre applied as 20% superphosphate and 60% KCl.
[b] Dry weight basis.

Table III. Analysis[a] of Good and Poor Cane

Element	Leaf Analysis, P.P.M.[b] Good cane	Poor cane
Aluminum	25–75	25–75
Cobalt	0.1–1.0	0.1–1.0
Copper	25–75	25–75
Iron	4	2.4
Manganese	250	60
Molybdenum	25–75	2–6
Titanium	25–75	25–75
Vanadium	25–75	25–75
Zinc	18	0.6

[a] Mature leaves of 10-month-old cane. [b] Dry weight basis. Spectrographic analysis except for iron, manganese, and zinc which were done colorimetrically.

injury is severe, recovery is greatly hastened on acid soils by addition of chelated iron at the rate of 4 to 8 ounces per tree.

Sugar Cane. Most of the sugar cane in Florida is grown on the muck soils close to Lake Okeechobee. However, about 14,000 acres are grown in the vicinity of Fellsmere, which may be on muck, sandy muck, or sand. The muck soils require little or no nitrogen; evidently liberation of available nitrogen from the muck is able to keep up with the normal rate of growth. Relatively small amounts of phosphorus have been used, with emphasis on potassium and secondaries of manganese and copper.

The practice has been to supply about 600 pounds of an 0–8–30 with 3% MnO, 1.5% CuO, 0.15% B_2O_3, and 0.45% ZnO, with 8 pounds of chelated iron per ton to newly set cane; the ratoons receive 150 pounds of K_2O per acre with minors as indicated, or K_2O alone. Long cropping of the muck without further additions of phosphate has resulted in a condition giving response to added phosphates. When several levels of phosphorus were applied to the soil with two applications of potassium, there was a response to 25 pounds of phosphorus per acre after a check receiving no phosphorus, but indication that higher phosphorus levels may depress yields (Table II). Some of this depression may be a result of trace element deficiencies (Table III).

Applying about 18 pounds of P_2O_5 per acre to ratoon crops in fields testing less than 25 pounds of available phosphorus has substantially increased yield. Response to sulfur, or the effect of sulfur in lowering pH with resultant increase in availability of trace elements, has given improved yields (Table IV).

We are trying to establish a 15- to 25-pound level of available phosphorus and 250-pound level of available potassium per acre of muck soil at time of planting or shortly after harvesting. Experiments are under way to determine whether higher levels of secondaries may be used, particularly in conjunction with larger amounts of phosphorus and potassium.

On sands and sandy mucks, several tests are under way to determine the feasibility of side dressing with extra fertilizer during the growing season. At present, one application is made to bring available nitrogen in the 15- to 25-pound range and available potassium in the 100- to 150-pound range, and 50 pounds of nitrogen per acre are applied about 6 weeks later. Current tests are made to maintain the levels of nitrogen, phosphorus, and potassium as at first fertilization by applications 6 and 10 weeks after first application.

Table IV. Influence of Treatment on Yields[a] of Sugar Cane

(Ratoon crops)

Treatment[b]	No. of Fields	Yield, Tons/Acre
Minus phosphorus	24	36.1
Plus phosphorus	18	44.1
Minus sulfur	23	37.9
Plus sulfur	19	43.8
Minus phosphorus and sulfur	13	35.6
Plus phosphorus and sulfur	10	47.5

[a] Weight of commercially harvested cane. [b] All fields received approximately 250 pounds KCl, 50 pounds $MnSO_4$, 8.5 pounds CuO, 5 pounds $ZnSO_4$, and 2 pounds borax per acre. Phosphate treatments consisted of 18 pounds of P_2O_5 per acre from triple super. Sulfur applied at 200 pounds per acre.

Pastures

Florida is the largest beef-producing state east of the Mississippi and ranks with a number of western states. In recent years it has built up a large population of dairy cattle. Some horse breeding is also done.

There is a great need for adequate pastures. The poor soils on which most of the pasture is grown require large amounts of fertilizer. In many areas it takes 15 acres to support an animal in poor style. By proper use of improved pasture grasses, and adequate irrigation, drainage, and fertility practices, it is possible to supply good support for three to four animals per acre the year around. Most operators have made some changes, but there is vast room for improvement.

If the grower has improved pastures with desirable grasses such as Pangola, bahia, fescue, St. Augustine, and Carib, and a good drainage and irrigation system, the level (pounds per acre) is adjusted three or four times a year to:

	Sandy Soils	Muck Soils High in Ca and Mg
N	75–150	25–50
P	15–25	15–25
K	100	150–200
Ca	1000+	...
Mg	100+	...
Mn	5–20	...
B	1–2	1–5
Cu	1–2	1–2
Fe	1–5	...
Zn	2.5–20	2.5–20

pH is adjusted to less than 6.0 once a year with sulfur, if necessary to increase availability of trace elements.

We try to maintain different fertility levels for pasture legumes such as white Dutch clover. Nitrogen is kept at a low range of about 25 pounds per acre, phosphorus at 25, potassium at 150 to 250, calcium at 1000+, magnesium at 150+, manganese at 10 to 20, boron at 2 to 4, copper at 1 to 4, and zinc at 10 to 20.

Pangola does better on a high nitrogen level of 75 to 150 pounds. Bahia gives satisfactory growth at levels of about 50 pounds per acre.

Foliage Plants

Florida has come into its own as a large producer of foliage plants, such as philodendron, diffenbachia, and dracaena. Little is known about satisfactory nutrient levels.

We have been getting good growth by maintaining nitrogen levels at 50 to 150 pounds, and phosphorus levels at 15 to 25 pounds. On soils in Dade County considerable oölitic limestone may be close to the surface, so that calcium levels are already in the 5000- to 8000-pound range. With such high calcium levels, growth is better when potassium is maintained at about 250 rather than 100 to 150 pounds per acre. However, a constant check must be maintained on magnesia, as some limestone may be low in this element, and potassium application may cause magnesium deficiency.

Boron has been maintained at 2 to 4, manganese at 2.5 to 10, copper at 1 to 2, and zinc at 2.5 to 10 pounds per acre. Satisfactory growth has been obtained with such levels, although further calibration may show need for revamping these ranges.

Flower and Vegetable Crops

The flower and vegetable crops have presented somewhat different problems in subtropical agriculture.

Chrysanthemums. A relatively mild climate has permitted growing chrysanthemums in the open or under saran. Schedules of lighting, planting, and spraying have been worked out so that the industry has prospered, with huge increase of acreage.

Cost of installation and culture runs

rather high. Because of close cropping (plants approximately 7 × 5 inches apart) and controlled water conditions, it is desirable to maintain higher nutrient levels than with many other crops. We try to maintain nitrogen at 150 to 250, phosphorus at 50 to 75, potassium at 200 to 300, calcium at 1000, magnesium at 150+, boron at 2 to 4, manganese at 2.5 to 10, copper at 1 to 2, iron at 2.5 to 5, and zinc at 2.5 to 10 pounds per acre. The nutrients are checked at 2-week intervals and adjusted if necessary.

Gladioli. About 10,000 acres of gladioli are grown annually in the open in Florida. Original concepts of feeding with low nitrogen levels to reduce bulb rots have had to be markedly reversed under Florida conditions. Because gladioli are grown mostly on sandy soils, lack of nitrogen is often a problem. Better dipping procedures with new fungicides have greatly lessened the bulb losses to Fusarium, permitting much higher nitrogen levels.

At present we try to maintain an available nitrogen level of 50 to 75, phosphorus of 15 to 25, potassium of 100 to 150, calcium of 1000+, magnesium of 100+, boron of 2 to 4, manganese of 2.5 to 10, iron of 1 to 5, and zinc of 2.5 to 20 pounds per acre until time of spiking. These levels are adjusted on a 2-week interval analysis, if scheduled applications have not maintained desired amounts. At time of spiking the potassium level is raised into the 150- to 250-pound range and maintained until shortly before the spike is cut.

On light soils calcium deficiency often causes bud malformation. Part of the difficulty is brought about because of the high potassium level necessary to produce good quality bulbs and the antagonism between the two elements. To correct this situation, several sprays of calcium chloride or nitrate at the rate of 4 pounds per 100 gallons are applied to plants beginning shortly before spiking has started.

Vegetables. Practically all the vegetables common to other areas, except such perennials as asparagus, rhubarb, and dill, can be grown in Florida. Special varieties adapted to the area have improved yields and quality; skill in proper planting, insect and disease control, and fertilization have made this production feasible.

Generally we maintain nitrogen at 50 to 150, phosphorus at 12 to 25, potassium at 150 to 250, calcium at 1000+, magnesium at 100+, boron at 1 to 2, manganese at 2.5 to 10, copper at 1 to 2, and zinc at 2.5 to 20 pounds per acre.

Nearly all vegetable crops are checked often, key samples usually being taken every 2 weeks.

What Is Required for a Commercial Consulting and Soil-Testing Service

JACKSON B. HESTER

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In conducting a commercial laboratory, factors to be considered are: accuracy of soil analyses, type of extractant and interpretation of results, nutritional elements involved, the specific soil problems of concern, and achievements expected. With these data available and a broad knowledge of soil, plant, and animal mineral nutritional requirements, the laboratory is well on its way toward success.

In 1934 only a few scientists were interested in soil testing, and many were opposed because of lack of information. The first publication of the author (*7*) in 1934 culminated several years of chemical research aimed to assist the grower in crop production.

Within the next few years scientists in every state and in many parts of the world began to take seriously this approach to a solution of the grower's problems. Publications began to appear in the scientific literature, practically all in favor of soil testing. Different methods were developed, some adapted to specific locations and conditions.

A method of soil testing which is chemically accurate and gives basic information about a soil is desirable, and interpretation of the data accumulated from the use of this system, in the light of the crop concerned, is the most scientific approach to the solution of the problem. Any organization that

Table I. Variations in Soil Conditions on One Farm

(Soil type RBSiL)

Field	pH	% Organic Matter	Pounds per Acre: Nitrogen NO_3	Nitrogen NH_3	P_2O_5	K_2O	Ca	Mg	Fe	Mn	B	Cu	Zn	Toxic Al	Mo, Parts per 2 Billion
3	5.8	3.8	0.9	12	32.5	126	2225	106	0.4	0.65	0.34	0.5	0.27	0.60	50
4	6.0	3.6	5	8	50	191	2225	98	0.3	1.30	0.28	1.0	0.19	1.48	50
8	5.4	3.0	7	4	32.5	126	921	98	0.3	1.69	0.36	1.0	Trace	0.60	20
9	5.8	3.1	1	5	50	126	1150	130	0.4	1.56	0.38	0.5	1.5	0.12	10
10A	5.8	3.4	11	7	42	99	1880	126	0.3	1.65	0.25	1.6	2.3	0.18	50
12	4.8	3.4	8	10	35	140	921	50	0.4	4.80	0.42	3.3	2.5	0.72	20
25	5.9	3.5	11	12	31	82	1650	130	0.4	1.69	0.38	1.3	Trace	0.60	20
26	4.7	3.1	3	5	44	112	1185	32	0.3	5.25	0.37	4.3	2.5	0.48	50
Mean	5.53	3.36	5.86	7.88	39.63	125.25	1519.63	96.25	0.35	2.32	0.35	1.69	1.54	0.60	33.75
Mean dev.	0.42	0.22	3.39	2.63	6.88	20.69	475.38	27.63	0.05	1.48	0.04	1.06	0.89	0.25	16.44

rather high. Because of close cropping (plants approximately 7 × 5 inches apart) and controlled water conditions, it is desirable to maintain higher nutrient levels than with many other crops. We try to maintain nitrogen at 150 to 250, phosphorus at 50 to 75, potassium at 200 to 300, calcium at 1000, magnesium at 150+, boron at 2 to 4, manganese at 2.5 to 10, copper at 1 to 2, iron at 2.5 to 5, and zinc at 2.5 to 10 pounds per acre. The nutrients are checked at 2-week intervals and adjusted if necessary.

Gladioli. About 10,000 acres of gladioli are grown annually in the open in Florida. Original concepts of feeding with low nitrogen levels to reduce bulb rots have had to be markedly reversed under Florida conditions. Because gladioli are grown mostly on sandy soils, lack of nitrogen is often a problem. Better dipping procedures with new fungicides have greatly lessened the bulb losses to Fusarium, permitting much higher nitrogen levels.

At present we try to maintain an available nitrogen level of 50 to 75, phosphorus of 15 to 25, potassium of 100 to 150, calcium of 1000+, magnesium of 100+, boron of 2 to 4, manganese of 2.5 to 10, iron of 1 to 5, and zinc of 2.5 to 20 pounds per acre until time of spiking. These levels are adjusted on a 2-week interval analysis, if scheduled applications have not maintained desired amounts. At time of spiking the potassium level is raised into the 150- to 250-pound range and maintained until shortly before the spike is cut.

On light soils calcium deficiency often causes bud malformation. Part of the difficulty is brought about because of the high potassium level necessary to produce good quality bulbs and the antagonism between the two elements. To correct this situation, several sprays of calcium chloride or nitrate at the rate of 4 pounds per 100 gallons are applied to plants beginning shortly before spiking has started.

Vegetables. Practically all the vegetables common to other areas, except such perennials as asparagus, rhubarb, and dill, can be grown in Florida. Special varieties adapted to the area have improved yields and quality; skill in proper planting, insect and disease control, and fertilization have made this production feasible.

Generally we maintain nitrogen at 50 to 150, phosphorus at 12 to 25, potassium at 150 to 250, calcium at 1000+, magnesium at 100+, boron at 1 to 2, manganese at 2.5 to 10, copper at 1 to 2, and zinc at 2.5 to 20 pounds per acre.

Nearly all vegetable crops are checked often, key samples usually being taken every 2 weeks.

What Is Required for a Commercial Consulting and Soil-Testing Service

JACKSON B. HESTER

Jackson B. Hester Agricultural Research Laboratory, Elkton, Md.

In conducting a commercial laboratory, factors to be considered are: accuracy of soil analyses, type of extractant and interpretation of results, nutritional elements involved, the specific soil problems of concern, and achievements expected. With these data available and a broad knowledge of soil, plant, and animal mineral nutritional requirements, the laboratory is well on its way toward success.

In 1934 only a few scientists were interested in soil testing, and many were opposed because of lack of information. The first publication of the author (*7*) in 1934 culminated several years of chemical research aimed to assist the grower in crop production.

Within the next few years scientists in every state and in many parts of the world began to take seriously this approach to a solution of the grower's problems. Publications began to appear in the scientific literature, practically all in favor of soil testing. Different methods were developed, some adapted to specific locations and conditions.

A method of soil testing which is chemically accurate and gives basic information about a soil is desirable, and interpretation of the data accumulated from the use of this system, in the light of the crop concerned, is the most scientific approach to the solution of the problem. Any organization that

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establishes a commercial laboratory with a conscientious approach must have basically a very broad knowledge of the history of soil testing and the requirements of the crops concerned.

Table I illustrates in what great detail a grower must consider his problems to be a successful producer. These data are from one farm, but the average of these tests is virtually worthless. There is such a great variation in the analyses that each field presents a separate fertilizer and soil amendment problem.

Probably no soil in the world has the ideal concentrations of all the elements essential to the proper growth of plants and animals. The fertilizer industry has striven energetically to supply these essential elements to the grower in the proportions needed. It has recognized the very great differences in the requirements of plants. Two very different crops are blueberries and alfalfa. Blueberries thrive on an extremely acid soil with high magnesium and potash content and other essential elements. Alfalfa requires a high pH, which controls the availability of molybdenum, essential to nodule formation.

The methods of soil analysis developed by this laboratory are based on extraction of the soil with sodium acetate at pH 5.0. The sodium, a very active element, replaces most of the readily available cations in the soil. The acetate anion has the specific ability to replace available anionic plant nutrients such as phosphates. This salt complex is desirable from the standpoint of analytical procedures, because it does not involve essential plant nutrients.

Commercial laboratories have a unique position, because they are not hampered by limitation of field activities and concentration on any one crop. They correlate data and information accumulated for all crops into a rounded picture of soil conditions and plant nutrient requirements. This laboratory has analyzed soil from 24 states and many South and Central American countries. Correlation of the information from all these sections adds to the value of information ascertained from a single analysis of a specific crop.

Progressive plant and animal breeders recognize that there are superior genes, but they have conceded that the essential nutrition of plants and animals helps them to achieve maximum efficiency. This point is illustrated by the fact that this laboratory has been called upon periodically to analyze the soil on which is produced feed for the race horses of a number of the leading breeders and racers.

Literature Cited

(1) Hester, J. B., Va. Truck Expt. Sta., Bull. *82* (January 1934).

Operation of a Soil-Testing and Recommendation Service in a Plant Food Sales Program

LEONARD L. SCHRADER

Standard Oil Co., 910 South Michigan Ave., Chicago 80, Ill.

New laboratory facilities provide farmers in the Corn Belt with a means of getting their soil tested by the newest equipment and the latest analytical procedures.

MANY new, modern soil-testing laboratories employing the very latest methods and procedures have been established in recent years throughout the United States. Farmers are benefiting from this service, through which they have been able to keep their total production costs at a minimum while increasing their yields and profits. The farmer feels that the ideal soil fertility program is the one that makes the most money for him, considering his time and investment. He is, therefore, depending more and more on the interpretation of soil tests to aid him in making profits.

Many farmers do not know the proper procedure for collecting soil samples—how to take them, how many to take, or how to interpret the recommendations of the soil-testing laboratory. However, in most instances, they know they can depend on local agricultural authorities for assistance. They are "getting the message" from all directions: The efficient way to raise any crop is to farm intensively with the heaviest practical fertilizer applications on fewer acres. They are being told again and again that proper fertilizer usage takes a big share of the "gamble" out of farming, that during periods of drought and in areas of consistently low rainfall crops grown on fertilized acres make more efficient use of rainfall and soil moisture, and that crops grown on properly fertilized land are better able to withstand the effects of diseases, insects, and other natural enemies.

In any fertilizer soil-testing program, the basic and primary requisite is to provide recommendations on properly feeding the soil, so that the soil, in turn, can properly feed the crop being grown. Recommendations should be aimed at building up good soil tilth, providing adequate quantities of plant food, and supplying an abundance of organic matter throughout the surface of the soil where all of the roots—as well as the important soil bacteria—can be nourished to full capacity at all times. Recommendations for supplemental plant food, however, cannot be used as a formula that will give an exact answer in terms of crop yields. A great deal depends on the kind of season experienced, and how efficiently a soil management program is followed. The soil test serves only as a guide to good fertilizer and management practices to produce the desired yield.

A soil testing service must consider five general phases of soil testing:

Obtaining the sample.
Analyzing the sample.
Calibrating.
Interpreting and recommending.
Evaluating the soil test.

Consolidated Laboratories, Congerville, Ill., is an example of the workings of a modern soil-testing laboratory. This laboratory was opened September 10, 1958, to serve the needs of farmers in the Corn Belt area. It uses the newest equipment and the very latest analytical procedures. Facilities were installed following a thorough study of laboratory techniques as practiced in several state universities in the Midwest. The laboratory technicians were sent to the various colleges and universities to learn firsthand the latest techniques and procedures of soil testing employed in each state. Their resulting Soil Master Series specializes in the following tests:

Measuring the exchange capacity (the ability of the soil colloids to hold plant food), and determining what needs to be done to utilize the capacity of the colloids.

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Measuring soil pH, to determine percentage or degree of acidity or alkalinity of the soil.

Determining hydrogen—this being a by-product of plant root activity, it denotes the soil's degree of activity and reserve acidity.

Determining magnesium.
Determining calcium.
Determining nitrogen.
Determining phosphorus.
Determining potassium.
Interpreting results with regard to the soil type tested.

Also made available are the Illinois, Missouri, Iowa, Wisconsin, and Indiana series of soil testing, plus tests for manganese and boron. Equipment in the laboratory includes the Spectronic 20 used for interpretation, a custom-built pH meter, a distillation apparatus for determining available nitrogen, a soil-grinding and screening unit, automatic pipet equipment for metering reagents, a soil dryer, an exhaust system, a glass dryer, special shakers, and an air conditioner for maintaining constant temperatures. Plans are also in the making to add a flame photometer to aid in the speed and accuracy of testing.

The laboratory's facilities are geared to handle 2000 soil samples per day. The final results and the interpretation are mailed back to the fertilizer dealer—or salesman—who is trained to explain the results of the test to the farmer and to assist him in procuring the needed fertilizer materials. A copy of the test results and the laboratory's recommendations is retained by the laboratory and filed alphabetically by area or state. Samples are retained only briefly. When a retest is needed, new samples are collected from the farmer's field.

Consolidated Laboratories was established for a number of reasons—primarily to give faster service and more complete tests than were readily available to farmers in the sales territory service by the laboratory. Simplicity in analyzing the report was a must to enable the farmer to understand the fertilizer requirements of his soil. Another reason was to strengthen fertilizer sales programs, for it has been proved that after a dealer or salesman collects the soil samples and helps the farmer to interpret the results, it is easier to break down sales resistance and to promote his buying the right kinds of fertilizer materials.

One of the biggest problems confronting salesmen and dealers is getting farmers to take accurate samples. The farmers themselves are aware of this failing, and consequently, a high percentage will wait for the dealer or salesman to take the samples for them. In many cases it is even difficult to get the farmer to accompany the dealer or salesman while his fields are being sampled. He is content to allow the dealer or salesman to collect the samples and then wait to review the final report as to what is recommended to bring his farm up to the desired level of fertility.

Value of Soil Testing

For years articles in farm magazines, farm newspapers, and college bulletins and pamphlets have stressed the value of soil testing. Yet, in spite of all this publicity, including specific instructions on obtaining accurate soil samples, many farmers still do not take the time to have their soil tested. Most farmers do not use enough fertilizer. Others may purchase quantities of various costly elements not required by their soil. In some instances they purchase fertilizer materials on the basis of what their neighbor buys, even though they are not farming exactly as he is, and may not have the same type of soil. With a soil test—and perhaps by spending a few more dollars per acre—profits can be materially increased.

A soil test report is vital to the farmer. He should keep it in a safe place, but available for easy reference. Consolidated Laboratories emphasizes the importance of the soil test by forwarding the laboratory report in an attractive, durable folder, suitable for placing in a file or bookcase. Handled in this manner it has less tendency to become mixed with other papers and misplaced.

In the Midwest, and in the center of the Corn Belt, there is an extremely wide variation in soil types and classifications. Soils range from practically pure sand to highly organic to almost pure clay—with all the various combinations in between. Some soils are highly calcareous, others podzolic, others well drained, still others subject to severe erosion. The midwest area has been farmed a shorter length of time than most other areas in the United States, and only in recent years have recommendations called for fairly high rates of supplemental plant food. Climatic factors generally favor high yields of corn, soybean, and hay crops.

Soil testing has definite value—to the dealer and the customer. However, experience has shown also that the report on the soil test must be expressed in simple terms so the farmer can understand its meaning. By having the report sent to the dealer or salesman, a twofold purpose is accomplished. After discussing the farmer's soil requirements, both know what fertilizer materials are needed to raise the desired bushels per acre. And after following recommendations, the farmer knows the soil-testing program really works.

Looking Ahead in Soil Testing

WERNER L. NELSON, J. FIELDING REED, and R. D. MUNSON

American Potash Institute, Inc.

It is essential to think ahead and consider what will be important 10 years from now. The prime objective is to bring about the most efficient and profitable use of plant nutrients by the agricultural producer.

WHILE soil testing is only one of many tools to assist the farmer in doing a precision job, it is an important link that demands top-flight programs. Thus we need to think ahead and consider what the agricultural producer might want in the future—say 10 years hence. We must keep in mind that he will have a high investment business operation and will be interested in maximum profits. He will want to eliminate as many limiting factors as possible. Through adequate soil testing one controllable factor, plant nutrient supply, will be taken care of. The prime objective is the most efficient and profitable use of lime and fertilizer by agricultural producers.

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All business operations take a periodic inventory of their resources. Each farmer knows how many cattle, hogs, and machines he owns. Today, however, very few have an inventory of their soil resources. But before long those who obtain all their income from the farm will be forced to have an inventory of their soils to stay in business. Soil testing will thus become a necessary operation in farming.

Some soil scientists have had reservations as to the value of soil testing. These reservations are gradually being dispelled and soil testing is enjoying greater acceptance from this group. The ultimate aim of soils research is to assist in solving crop production problems. Soil testing is a very important link between this research and the farmer. Admittedly much is yet to be learned in the field of soil and plant chemistry, but much progress has been made in the last few years.

Sampling

Who Will Do the Sampling? One might predict that as soil testing and fertilizer usage mature, proper amounts of plant nutrients will be supplied more on a service basis. This would mean that someone other than the farmer would take over much of the sampling. Sampling could be a part of the complete service rendered by a laboratory or the fertilizer industry. A sampling service on a charge basis has been initiated in several Michigan counties as a part of the extension program.

Number of Samples. From 1955 to 1957 the number of samples increased 35% in the United States. In some states numbers of samples might double in the next ten years. Certainly the trend will be upward, as soil testing becomes a cog in the farmer's business operation.

Frequency of Sampling. Sampling every three to five years should be adequate for most purposes. With heavy corrective applications of plant nutrients the soil would probably not reach a stage of adequate mixing for sampling in much less than three years. With the gradual nutrient build-up type of recommendation, the fertility level of the soil or the needs of the crops would not change rapidly enough to warrant resampling in much less than five years. On the basis of the one sample more effort should be made to provide recommendations for the next three or four crops in the rotation.

Method of Sampling. As fertilizer use increases, it becomes more difficult to take a representative sample. One core of soil which contains a high concentration of phosphorus or potassium may distort the reading for the whole sample. There may be a trend to fewer cores per composite sample and more samples. Illinois has been using a system of five cores per composite and 11 composite samples per 40 acres.

Who Is Reached in a Soil-Testing Program?

It is important to know what level of farmer is reached in a soil-testing program. Where soil samples are taken and sent in outside of governmental aid programs, the better farmer is no doubt sending in the samples. Where the samples are taken through ASC programs, or where a county makes soil tests mandatory in order to receive payment for certain practices, the entire range of farmers will be represented.

The following estimates come from a survey conducted in the Midwest by a NPFI Research and Education Committee.

	No. of States	State Average, 1953–57	Yield of Farmers Sending in Soil Samples	Goals in Soil Test Program
Corn, bushels	9	44.6	61.2	82.4
Wheat, bushels	9	20.3	27.2	36.0
Alfalfa, tons	8	1.9	2.8	3.8
Soybeans, bushels	7	18.6	24.0	31.1

The obvious conclusion is that the higher yields are due to more effective use of fertilizer and lime. However, the fact that these farmers are able to employ high rates to advantage indicates that they probably possess above-average management ability. The very fact that the farmer uses the soil-testing service is a favorable reflection on his management ability.

Economists have predicted that in the not too distant future there will be only 1,000,000 farmers in the United States. What will these farmers want in the way of soil-testing service? It seems of increasing importance that we know with whom we are working and their objectives. A summary of certain data on the soil-test information sheet, such as previous yields, yield desired, size of farm, and number of acres sampled in any one year might assist in formulating future soil-testing programs.

Level of Recommendations

One of the best ways to encourage the use or sale of a product is to have one that performs much better than the one the consumer already possesses. If a farmer is averaging 80 bushels of corn per acre, are our recommendations based on soil tests enough better really to sell the soil testing program? Are the top 5 to 10% of the farmers already ahead of our educational programs and information?

If the recommendation is aimed merely at achieving average yields, the value of soil testing is greatly reduced. It may do more harm than good, as it creates doubt in the minds of top farmers. In the next few years, as the farmers' usage of fertilizer increases, recommended rates may be considerably less than the amounts used by the leaders.

Two or More Levels. In some soil-testing programs recommendations are made at two or more levels. Iowa uses three levels. An example from Minnesota is shown below:

Such an approach seems to have merit. It provides something for the highest quartile of farmers, who really are making a significant contribution. It provides for the lower level farmers, some of whom need slower education.

In ten years a more positive approach might be to give the top level recommendations and management practices as the first choice in all reports. Then qualification might be made that if the farmer is not planning to do his best in stand, pest control, variety, and management, he can use the lower recommendation. The penalty he is imposing on himself might well be spelled out, however.

Soil Test	N,[a] Lb./Acre	P_2O_5, Lb./Acre	K_2O, Lb./Acre
75 bushels of corn			
High	60	20	0
Medium	80	40	60
Low	100	40[b]	120
100 to 140 bushels of corn			
High	60	20	20
Medium	100	60	80
Low	140	80	160

[a] After nonlegumes.
[b] Very low P test, apply additional 20 to 30 pounds.

Build-Up Approach. In this type of recommendation enough nutrients are supplied for good crop yields and also for nutrient build-up on soils testing low and medium. In Ohio, the suggested average annual application of P_2O_5 and K_2O in the rotation is 80, 60, and 40 pounds per acre at the low, medium, and high soil levels, respectively. Ni-

trogen is suggested according to crop and rotation.

These recommendations are designed to supply nutrients for 100 to 125 bushels of corn, 40 to 50 bushels of wheat, 80 to 100 bushels of oats, 35 to 40 bushels of soybeans, and 4 to 5 tons of hay per acre.

A well balanced fertility program involves building fertility reserve to a reasonably high level, maintaining fertility once a high level is reached. Much emphasis will be placed on this approach in the next few years, with the idea of keeping the sights high.

Detail of Recommendations

There is a trend to report a fewer number of levels. Only five are being used in some states, with some reporting just three—low, medium, and high.

Perhaps the soil test should be used merely to indicate levels in the soil. Recommendations may then indicate the amounts necessary to raise the nutrient to the optimum level. Below is an example from the Purdue testing program:

Test Value	P_2O_5 to Raise P Test Level to 180 Lb./Acre
0- 25	400
26- 50	300
51- 75	225
76-100	150
101-125	90
126-150	50

Possibly too much emphasis has been placed on the "recommendation" as such. Actually, the soil test does not tell the kind of fertilizer to use. It assists in evaluating the fertility level of the soil. With this information, one can better estimate the fertilizer needs.

If a soil is acid or low in a particular plant nutrient, a farmer can take this into account and plan to correct the condition. How this is done is bound to vary with the farmer. Over the years, records can be kept of changes in fertility levels and corrective treatments.

Uniformity of Reporting

The variation in reporting from one state to another is confusing to the person attempting to use soil testing. Farmers operating near a state line or fertilizer producers operating in several states have difficulty in seeing why a river or a state line should materially affect recommendations.

Sometimes it is said that uniformity would not be desirable, because it would kill initiative. There is ample opportunity for initiative in research and development of soil testing. When it comes to actual use, however, there may not be too much reason for widely divergent methods of reporting results and making recommendations. With the various regional soil-test committees at work, some of these problems are gradually being brought out and approached.

Amount of Service Demanded

As the farmer operates more land, he will want more specialized information. When a program passes from the research to the service stage, the question arises as to who should provide the service.

The following are possibilities:

More from Extension? While Extension at present is not set up to provide much more specialized information, it is using soil testing as an educational tool.

Service from industry agronomists.

Service from a commercial laboratory with advice on a number of management phases.

A specialist employed by a group of farmers.

A full-time specialist employed by one farmer.

It appears that the farmer will demand more and more service, depending on the size of his operation. He will be willing to pay for it. If the extension service cannot meet this demand, commercial agencies will step in and do the job.

Who Will Be Doing the Testing and Recommending?

Official Laboratories (Central or County). They will be doing much of the testing, particularly in the newer testing areas, and where the official leaders insist on it. In the central lab system there is a trend to have more and more of the recommendations made by county agents or by district agronomists. This follows the need for more intelligent recommendations based on individual needs.

This testing service might be used by the specialist the farmer employs. The specialist would make the recommendation based on response curves obtained by the college.

Private Laboratories and Consulting Firms. A package program of testing and management service should gain rapidly in popularity. Service might be an all-inclusive soil, crop, and livestock management program. The colleges might develop standard procedures for use by such laboratories.

Fertilizer Companies. The industry can do much to foster getting the samples taken and many feel this to be its most important role in soil testing. Its expansion in the area of actual testing probably will be limited.

Evaluation of Residual Fertility

A major problem in many fertilizer recommendations is inadequate attention to residual effects. While immediate return on the fertilizer investment is important, the better farmers are interested in top returns over the years.

The need to place a price tag on residual fertility will be a must for the progressive grower 10 years from now. Detailed economic evaluation is needed. The calibration of our soil tests to evaluate residual effects and the establishment of response tables will be an important task.

Research

Many of our field calibration studies are already out of date, because of limiting factors such as stand, insect damage, inadequate variety, or improper placement. It is difficult to predict what management practices farmers will be using 10 years from now, but it would seem essential to conduct field calibration studies with the best practices we now know. This will ensure being ready with the answers when the grower demands them.

Research on soil and plant chemistry for both major and minor elements must continue if diagnostic techniques are to continue to improve. Up to now the land grant colleges and USDA have been doing the research. Who will be doing the research for the private laboratories and consulting firms in the future? If the research is to be continued by official agencies, more help should be given by industry either by direct grants or by aid in obtaining appropriations. Will the agricultural producer be sufficiently convinced of the value of the service to pay not only for the technological performance of the test and its interpretation, but also for the research behind the soil test?

Plant Tests

This diagnostic tool appears to have great potential for use along with soil tests. Actually plant composition should better reflect availability of nutrients to the plant than soil tests can. Perhaps the greatest use is in annual crops for predicting needs in subsequent years.

Personnel

An important problem in soil testing is setting the sights high in quality of the leaders and then keeping this

personnel. In some states the soil-testing laboratory is used as the training ground for graduate students. As soon as one completes his graduate work another moves in. Too, it may be difficult to keep well trained personnel. In one state testing laboratory there were six directors in 10 years, all Ph.D's. Fortunately, more emphasis on the key nature and the importance of the soil testing program is now being given by the administration in most states.

Marked changes in certain aspects of soil testing appear to be in store for the near future. It will take top-flight personnel to stay on top of the problems and to adjust with changing times.

This is a condensed version of the meeting paper. The complete article appeared in the October 1959 issue of Commercial Fertilizer and Plant Food Industry.

END OF SYMPOSIUM ON SOIL TESTING

PLANT GROWTH REGULATORS

Synthesis and Preliminary Evaluation of Amino Acid Derivatives of 2-(2,4,5-Trichlorophenoxy)propionic Acid

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A series of D-, L-, and DL-amino acids involving 2-(2,4,5-trichlorophenoxy)propionic acid has been prepared and evaluated. A wide variety of notable differences in behavior pattern in this new series is in sharp contrast to generalities reported for the halogen-substituted phenoxyacetic acid series. The derivatives of L- and DL-amino acids reported here proved generally to be active plant-growth regulators with high selectivity during the test period on the assay plants used; an exception was the derivative of L-tryptophan, which was completely inactive. The D-amino acid derivatives were almost completely lacking in growth-regulating properties with the exceptions of the D-alanine and D-tryptophan derivatives which showed some degree of activity. Most of these derivatives are easily prepared and purified and those with sharp melting points may be useful in characterization of amino acids.

Aryloxyalkyl carboxylic acids form an important group of synthetic growth-regulating substances now available; of these DL-2-(2,4,5-trichlorophenoxy)propionic acid, designated 2,4,5-TP, has achieved merit. Recently the optical forms of the amino acids derivatized with 2-(2,4-DP) (*4*) have shown a specificity in their mode of action on plant growth, indicating a varying relationship between the optical configuration of the amino acid and the biological activity. The need for further studies using an extended series of these amino acids was evident. Hence the reason for preparing this series of D-, L-, and DL-amino acid derivatives of 2,4,5-TP. This work with 33 new compounds represents an elaboration of previous studies (*4–6*) in which only 18 compounds were prepared for each series.

Many of the compounds in this and in series previously reported (*3–8*) have now been submitted to various agencies for evaluation as anticancer agents, estrogenic substances, fungicides, herbicides, insecticides, and nematocides.

[1] Present address, Courtaulds, Inc., Mobile, Ala.

Experimental

The general procedure used in the preparation of the amino acid derivatives of 2,4,5-TP was that previously described (*5*) utilizing Schotten-Baumann techniques. The 2,4,5-TP used was of technical grade and the amino acids were the best obtainable from commercial sources.

2-(2,4,5-Trichlorophenoxy)propionyl Chloride. A 73% yield of this compound was obtained by the reaction of 2,4,5-TP (1 mole) with thionyl chloride (1.7 moles) essentially as described (*7*). The acid chloride boiled at 111–17° C. and 0.3 mm. of mercury, with a constant index of refraction (n_D^{26} = 1.5601).

An abbreviated description illustrates the general procedure employed (*5*) in the preparation of the amino acid derivatives of 2,4,5-TP.

***N*-[DL-2-(2,4,5-Trichlorophenoxy)-propionyl]-L-methionine.** In the cases involving the preparation of L-methionine, L-leucine, and L-phenylalanine derivatives of 2,4,5-TP, 0.03 to 0.04 molar ratios of amino acid to acid chloride were used. For cystine derivatives ratios were 2 to 1; all other derivatives were synthesized with 1 to 1 proportions. For synthesis of the L-methionine derivative 4.48 grams of the amino acid were used with 11.5 grams of 2-(2,4,5-trichlorophenoxy)-propionyl chloride; this produced a crude yield of 9.80 grams (81.5%) with a melting range of 120° to 128° C. The crude product was dissolved in boiling ethyl acetate and precipitated with petroleum ether (boiling point 63° to 70° C.). The final yield was 7.93 grams (66.0%) melting at 127° to 130.5° C. (Table I).

The most difficult amino acid derivatives to prepare were those of DL-aspartic acid, DL-cystine, and D-, L-, and DL-leucine. These separated as oils and

personnel. In some states the soil-testing laboratory is used as the training ground for graduate students. As soon as one completes his graduate work another moves in. Too, it may be difficult to keep well trained personnel. In one state testing laboratory there were six directors in 10 years, all Ph.D's. Fortunately, more emphasis on the key nature and the importance of the soil testing program is now being given by the administration in most states.

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The most difficult amino acid derivatives to prepare were those of DL-aspartic acid, DL-cystine, and D-, L-, and DL-leucine. These separated as oils and

Table I. Yields, Physical Properties, and Analyses of Amino Acid Derivatives of DL-2-(2,4,5-Trichlorophenoxy) propionic Acid

N-[DL-2-(2,4,5-Trichlorophenoxy) propionyl]-	M.P., °C.[b] (Corr.)	Yield, % Crude	Yield, % Refined	Formula	Analyses[a] Chlorine, % Calcd.	Chlorine, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Optical Rotations	$[\alpha]_D^{25}$	C.g./100 ml., in pyridine
L-Alanine	161.5–163.5	48.3	18.0	$C_{12}H_{12}Cl_3NO_4$	31.23	31.36	4.11	4.09	Levo	13.2 ± 0.6	2.01
D-Alanine	157 –160	61.7	47.5		31.23	31.11	4.11	4.15	Dextro	13.31 ± 0.6	2.02
DL-Alanine	173 –176	54.9	43.8		31.23	31.07	4.11	4.15			
D-Asparagine	155 –157.5[c]	56.3	13.3	$C_{13}H_{13}Cl_3N_2O_5$	27.73	28.19	7.30	6.95	Levo	13.45 ± 0.6	2.00
L-Asparagine	152 –154[c]	33.0	16.9		27.73	27.70	7.30	7.16	Dextro	11.12 ± 0.6	2.55
DL-Asparagine	173 –178[d]	35.6	5.7		27.73	28.44	7.30	6.55			
DL-Aspartic acid[e]	178 –181[f]	24.3	11.4	$C_{13}H_{12}Cl_3NO_6$	27.66	27.63	3.64	3.65			
L-Hydroxyproline	202 –204[f]	32.2	16.7	$C_{14}H_{14}Cl_3NO_5$	27.80	27.32	3.66	3.73	Dextro	9.36 ± 0.4	2.00
L-Isoleucine	113 –116[g]	90.6	62.7	$C_{15}H_{18}Cl_3NO_4$	27.80	27.64	3.66	3.61	Levo	6.97 ± 0.5	2.01
D-Isoleucine	106 –113[g]	34.0	18.6		27.80	27.34	3.66	3.74	Dextro	6.66 ± 0.5	2.00
DL-Isoleucine	100 –104[f]	94.1	33.9		27.80	27.72	3.66	3.64			
L-Leucine	52 – 58[h]	88.0	38.7	$C_{15}H_{18}Cl_3NO_4$	27.80	27.73	3.66	3.65	Levo	17.02 ± 0.5	2.00
D-Leucine	44 – 49[i]		19.3		27.80	27.39	3.66	3.62	Dextro	15.27 ± 0.6	2.00
DL-Leucine	145 –148	76.7	35.1		27.80	27.56	3.66	3.65			
L-Methionine	127 –130.5	81.5	66.0	$C_{14}H_{16}Cl_3NO_4S$	26.55	26.36	3.50	3.47	Levo	7.80 ± 0.5	2.00
D-Methionine	129 –131	46.6	30.1		26.55	26.56	3.50	3.56	Dextro	7.40 ± 0.5	2.00
DL-Methionine	130 –132	57.5	35.1		26.55	26.46	3.50	3.51			
L-Phenylalanine	145 –150[f]	85.6	28.4	$C_{18}H_{16}Cl_3NO_4$	25.53	25.47	3.36	3.36	Dextro	5.87 ± 0.5	2.00
D-Phenylalanine	149 –152	25.2	13.0		25.53	25.48	3.36	3.37	Levo	0.27 ± 0.4	2.01
DL-Phenylalanine	145 –148	51.9	40.3		25.53	25.47	3.36	3.36			
DL-Serine	137 –140	95.3	53.3	$C_{12}H_{12}Cl_3NO_5$	29.83	29.27	3.93	3.86			
L-Threonine	156 –161[f]	85.4	8.4	$C_{13}H_{14}Cl_3NO_5$	28.70	29.03	3.78	3.56	Levo	9.26 ± 0.6	2.00
D-Threonine	153 –155	55.8	12.2		28.70	28.41	3.78	3.73	Dextro	9.82 ± 0.5	2.01
DL-Threonine	133 –135	41.7	32.6		28.70	28.56	3.78	3.81			
L-Tryptophan	171 –173[f]	73.2	17.4	$C_{20}H_{17}Cl_3N_2O_4$	23.34	23.29	6.15	6.20	Levo	4.75 ± 0.6	2.01
D-Tryptophan	169.5–170.5	69.3	32.5		23.34	23.09	6.15	6.08	Dextro	4.38 ± 0.6	2.05
DL-Tryptophan	203 –205	49.9	15.7		23.34	23.23	6.15	6.17			
D-Valine	121 –123[f]	93.2	69.4	$C_{14}H_{16}Cl_3NO_4$	28.85	28.89	3.80	3.79	Dextro	15.16 ± 0.5	2.01
DL-Valine	140 –145[f]	99.5	56.0		28.85	28.57	3.80	3.80			
N,N'-Bis[DL-2-(2,4,5-trichlorophenoxy) propionyl]-											
L-Cystine	121 –123	68.6	40.5	$C_{24}H_{22}Cl_6N_2O_8S_2$	28.62	28.28	3.77	3.65	Levo	92.97 ± 0.4	3.00
D-Cystine	109 –119	86.5	18.2		28.62	27.85[k]	3.77		Dextro	92.6 ± 1.0	3.40
DL-Cystine	136 –138[f]	28.2	17.9		28.62	28.08	3.77	3.76			
DL-Lysine	109 –116	73.7	44.4	$C_{24}H_{24}Cl_6N_2O_6$	32.77	32.25	4.32	4.27			

[a] Analyses by J. S. Ard.
[b] Recrystallized one or more times from ethyl acetate–petroleum ether unless otherwise indicated.
[c] Recrystallized from ethyl alcohol-water previous to ethyl acetate–petroleum ether.
[d] Recrystallized from ethyl alcohol-water and from methanol-water.
[e] Repeated attempts to prepare L- and D-aspartic acid derivatives were unsuccessful.
[f] And from ethyl alcohol–water.
[g] Not recrystallized, but washed with petroleum ether.
[h] And derivatized with cyclohexylamine (*2*).
[i] And from ethyl alcohol–water and from methylene chloride–petroleum ether.
[k] Value before final recrystallization.

hardened either after refrigeration or following evacuation treatment. To achieve chemical and optical purity of these compounds additional recrystallizations were necessary as indicated in the footnotes of Table I.

Preliminary Evaluation as Plant Regulators

Twenty-two of the 33 amino acid derivatives of 2,4,5-TP prepared (Table I) were evaluated for plant growth regulating properties on Pinto bean, large seeded sunflower, and Arlington White Spine cucumber. The lanolin assay technique previously described (*6*) was employed. The compounds evaluated were: the parent acid 2,4,5-TP, the DL-aspartic acid, and D-, L-, and DL-forms of the amino acids alanine, cystine, leucine, methionine, phenylalanine, threonine, and tryptophan derivatives of 2,4,5-TP.

Observations were made 7 to 9 days after treatment and the degree of growth modification induced by the various compounds was estimated and scored as 0, none; 1, slight; 2, moderate; and 3, marked. The responses studied were: stem curvature, growth inhibition, epinasty, formative effects, and induced cell proliferation (gall formation).

Results of Plant Tests

With all the derivatives of 2,4,5-TP except those of tryptophan, the L- and DL- forms were active, although less active, quantitatively, than the parent acid (Table II). In general the D-amino acid derivatives were only slightly active or inactive. Growth inhibition formative effects, and cell proliferation of the hypocotyls of plants treated with the derivatives indicated that translocation of the amino compounds or their biologically active metabolites was similar to that of the parent acid. In contrast, the L-tryptophan amino acid derivatives induced no apparent growth regulation,

Table II. Plant-Growth Modifying Activity of Amino Acid Derivatives of DL-2-(2,4,5-Trichlorophenoxy)propionic Acid on Pinto Bean (*B*), Sunflower (*S*), and Cucumber (*C*)[a]

N- DL-*2-(2,4,5-Trichloro-phenoxy)-propionyl* -	*Stem Curvature*			*Growth Inhibition*			*Epinasty*			*Formative Effects*			*Cell Proliferation*								
													First Internode			*Hypocotyl*			*Treated Area*		
	B	*S*	*C*	*B*	*S*	*C*	*B*	*S*	*C*	*B*	*S*	*C*	*B*	*S*	*C*	*B*	*S*	*C*	*B*	*S*	*C*
L-Alanine	3	1	3[b]	3	3	3	0	3	3	0[b]	3	3[b]	3	0[b]	0	3	2[b]	0	3	2[b]	0
D-Alanine	3	0	0	2	2	3	0	1	0	3	1	0[b]	2	0	0[b]	1	1	1	3	1	1
DL-Alanine	3	0[b]	3[b]	3	3	3	0	0[b]	3[b]	0	1[b]	3[b]	3	0[b]	0[b]	2	2[b]	2[b]	3	2[b]	2[b]
DL-Aspartic acid	3	1	1[b]	1	3	3	0	1	1[b]	0	3	0[b]	2	0	0[b]	2	0	1[b]	3	0	3[b]
L-Cystine[c]	3	3	2[b]	3	3	3	0	2	0[b]	0	3	0[b]	2	0[b]	0[b]	2	0[b]	2[b]	3	3	3[b]
D-Cystine[c]	0	0	2	2	0	2	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0
DL-Cystine[c]	1	1	0[b]	1	2	3	0	1	0[b]	0	2	0[b]	1	1	0[b]	0	0	2[b]	2	0	2[b]
L-Leucine	3	3	3[b]	3	3	3	0	3	3[b]	0[b]	3	3[b]	3	3	0[b]	3	3	1	3	3[b]	0
D-Leucine	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
DL-Leucine	3	3	3[b]	3	3	3	0	3	3[b]	0	3	3[b]	2	2	0[b]	3	1	2[b]	3	2	3[b]
L-Methionine	3	3	1	3	3	3	0	1	1	0[b]	1	3[b]	3	3	0	3	2	0	3	3[b]	0
D-Methionine	0	0	0	2	1	3	0	1	0	0	2	0	1	0	0	0	0	0	2	0	0
DL-Methionine	3	3	2[b]	3	3	3	0[b]	1	1[b]	0[b]	3	0[b]	3	3	0[b]	3	0	2[b]	3	3	2[b]
L-Phenylalanine	2	1	2	2	2	3[b]	0	0	3	0[b]	3	3[b]	3	0	0	3	0	0	3	1	0
D-Phenylalanine	0	0	1	0	0	2	0	0	0	0	1	0	0	0	0[b]	0	0	1	0	0	2
DL-Phenylalanine	2	0	2[b]	1	0	3	0	1	0[b]	0	1	2[b]	0	0	0[b]	1	0	1[b]	3	0	1[b]
L-Threonine	2[b]	2	3[b]	3	3	3	0[b]	2	3[b]	0	1[b]	3[b]	0	0[b]	0[b]	2[b]	1	2[b]	3[b]	2	3[b]
D-Threonine	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DL-Threonine	3	3	2[b]	2	3	3	0	2	1[b]	0	0	3[b]	3	3	0[b]	3	0	1[b]	3	1	2[b]
L-Tryptophan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Tryptophan	0	2	2[b]	1	2	3	0	0	1[b]	0	1	3[b]	0	0	0[b]	0	0	1[b]	1	1	2[b]
DL-Tryptophan	1	1	2[b]	3	3	3	0	2	1[b]	3	3	2[b]	1	0	1[b]	0	0	1[b]	3	0	2[b]
Parent acid	2[b]	1[b]	3[b]	2[b]	2[b]	3[b]	1[b]	0[b]	3[b]	0[b]	2[b]	3[b]	0[b]	1[b]	0[b]	2[b]	2[b]	2[b]	3[b]	2[b]	3[b]

[a] Evaluations made 7 to 9 days after treatment. 0, no effect; 1, slight effect; 2, moderate effect; 3, marked effect.
[b] Response obtained with a mixture containing 1% of compound was so marked that evaluation of responses listed could not be made and it was necessary to retest the compound at 0.1% concentration. Figures, therefore, represent responses obtained with 0.1% concentration.
[c] *N,N'*-Bis[DL-2-(2,4,5-trichlorophenoxy)propionyl]-derivative.

while the D- and DL- forms were active.

Comparison of the tryptophan and threonine derivatives illustrates the wide difference of growth responses caused by variation of optical configuration of the amino acid derivatives.

Discussion

Growth-modifying properties of the amino acid derivatives of 2,4,5-TP demonstrate that there is, in general, lower activity for the majority of D-, L-, and DL- compounds of this series than for those reported for the halogenated phenoxyacetyl compounds such as 4-chloro- (*5*), 2,4-dichloro- (*8*), and 2-methyl-4-chlorophenoxyacetyl derivatives (*6*). The behavior pattern of the 2,4,5-TP compounds follows somewhat that of the amino acid series of the 2-(2,4-dichlorophenoxy)propionic acid, 2-(2,4-DP) (*4*), in that plants for the most part showed little or no response to D-amino acid derivatives of either series. However, the selective and widely variable behavior of the L- and the relatively inexpensive DL-amino acid forms makes these 2,4,5-TP compounds, like those of the 2-(2,4-DP) series, exceptionally attractive from the standpoint of further study.

In a previous paper (*4*) it was pointed out that considerable weight had been given to the idea that plant-growth response in the case of the L- and DL-amino acid derivatives of halogen-substituted phenoxyacetic acids (*5*, *6*, *8*) depends upon the presence of cellular hydrolytic enzymes which are capable of splitting the amide linkage to free the carboxyl group (considered essential for activity) of the substituted phenoxy acid. Also, it was mentioned that the assay plants may be partially or completely incapable of splitting the D-amide linkage. Although the results obtained with the amino acid derivatives of 2-(2,4-DP) (*3*, *4*) and 2,4,5-TP to some extent support these ideas, the data obtained with the phenoxypropionic acid series seem to lend some support tc another concept concerning mode of action—that is, activity may be due to effect per se; it may depend upon the translocation and specificity of the whole intact molecule. The almost complete inactivity of the D-amino acid isomers of the phenoxypropionic acid series evaluated, and the lowered, but selective, activity of the L- and DL-isomers are the contributory factors to this concept.

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MEASUREMENT OF INSECTICIDE EXPOSURE

Ether-Extractable Urinary Phosphates in Man and Rats Derived from Malathion and Similar Compounds

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The urinary excretion of malathion-derived materials was studied to develop a chemical method which could be used as a measure of exposure. Following exposure to malathion, ether-extractable phosphatic compounds appear in the urine of man and rats. Quantitative determination was accomplished by a colorimetric method for phosphates. The amounts of these materials were proportional to dosage. Preliminary studies with other similar compounds were made. This method is useful in detecting and evaluating the degree of exposure to malathion. It is far more sensitive than measurement of cholinesterase inhibition.

MALATHION, *S*-[1,2-bis(ethoxycarbonyl)ethyl] *O*,*O*-dimethyl phosphorodithioate, is being used more widely as time goes on, especially in agriculture. A chemical method for analysis of body fluids or tissues which would give a measure of exposure to malathion would be very useful in studying exposure of workers. The excretion of malathion in urine was studied to find a method of analysis which might be correlated with the amount absorbed or ingested.

Reports have appeared concerning the excretion of malathion metabolites in the urine of various animals (*4*, *5*). Studies on mice using phosphorus-32–labeled malathion have shown that large amounts of the malathion-derived material are excreted in the urine, largely as compounds not extractable by chloroform (*5*). However, as the metabolism is complex, solvents other than chloroform or carbon tetrachloride might extract a portion of the metabolites. This presents a method for the extraction and colorimetric analysis of a portion of the malathion-derived materials excreted in the urine of man and rats, and application of this method to some studies of the excretion of malathion.

Urinary Excretion by Rats

Normal urine contains large amounts of inorganic phosphates with relatively small amounts of organic phosphorus compounds. Preliminary results using a colorimetric method showed that when technical malathion was given intraperitoneally to rats there was a definite increase in the amount of organic phosphorus compounds excreted in the urine. However, the increase in relation to the inorganic phosphate was not great enough to enable very accurate determinations to be made. Therefore it was necessary to study the separation of at least a portion of these organic phosphorus compounds before the malathion-derived material could be determined colorimetrically. To facilitate this study radioactive malathion labeled with phosphorus-32 was synthesized. This compound was synthesized as described by March *et al.* (*5*). The first step of the synthesis as described by these authors was eliminated by starting with phosphorus-32–labeled phosphorus pentasulfide. This material was obtained by irradiating phosphorus pentasulfide (technical grade, recrystallized twice from carbon disulfide) in the neutron pile at Oak Ridge for 4 weeks. Radioassay was done in solutions using a dipping Geiger tube. The initial activity of the malathion was 36 counts per second per γ per ml.

In the first experiment four rats were used. One male and one female were given 100 mg. per kg. of malathion intraperitoneally in peanut oil daily for 5 consecutive days, while one male and one female were given peanut oil only. Urine and feces were collected for each 24-hour period. A device was used to separate the feces from the urine during collection, but some cross contamination between feces and urine may have occurred. The urine was first assayed for the total amount of radioactive materials present (Table I). The per cent of the dosage so recovered for each 24-hour period ranged from 73 to 20 (average 46%) for the 5-day period of daily dosage of the animals. No great efforts were

Table I. Excretion of Malathion–Phosphorus-32 Derived Materials in Rat Urine Following Intraperitoneal Injection

Sample No.	Sex	Time Since Dosed, Hours	Urine Volume, Ml.	Total[a] Found by Radio-assay, Mg.	Daily Dose, %		Urinary Material Ether-Extractable, %
					Total in urine	Ether-extractable	
100 Mg. per Kg. per Day for 5 Consecutive Days							
1	M	24	6.9	14.5	73	43	59
	F	24	3.7	10.6	51	40	78
2	M	24	7.0	13.0	65	43	66
	F	24	4.0	8.5	45	33	73
3	M	24	5.5	6.7	34	16	47
	F	24	4.5	9.5	51	36	70
4	M	24	3.2	3.9	20	13	65
	F	24	3.2	5.4	29	20	69
5	M	24	7.5	10.0	50	32	66
	F	24	...	Lost	...	...	..
6	M	48	...	0.180	0.90	0.31	35
	F	48	...	Lost	...	...	..
7	M	72	9.0	0.110	0.6	...	..
	F	72	4.5	0.220	1.2	...	..
8	M	96	6.0	0.050	0.25	0.045	18
	F	96	5.0	0.100	0.53	0.14	26
25 Mg. per Kg. per Day for 5 Consecutive Days							
1	M	24	6.0	2.34	47	24	51
	F	24	4.5	1.65	37	14	38
2	M	24	5.5	2.13	43	27	63
	F	24	4.0	1.67	37	28	76
3	M	24	6.0	2.28	46	23	50
	F	24	6.0	1.79	40	30	75
4 & 5	M[b]	...	...	...	...	...	..
	F	...	...	...	...	...	..
6	M	48	5.0	0.028	0.6	0.1	17
	F	48	4.5	0.110	2.5	1.3	52
7	M	72	5.0	0.019	0.4	0.04	10
	F	72	5.0	0.050	1.1	0.4	36
8	M	96	5.0	0.015	0.3	...	..
	F	96	4.5	0.042	0.9	...	..
9	M	120	6.0	0.010	0.2	...	..
10	F	120	5.0	0.026	0.6	...	..

[a] Calculated as malathion equivalent.
[b] Daily injections continued for 5 days. Samples saved for other uses.

made to obtain quantitative recovery of all the urine; hence some of the variation in percentage recovery may have been due to losses in collection of the urine. However, the assay for total malathion metabolites established the presence of large amounts of these materials in the rat urine. Samples 6 (48 hours), 7 (72 hours), and 8 (96 hours) represent succeeding 24-hour periods of collection after cessation of treatment. The excretion falls off rapidly but was measurable after 96 hours.

Extraction of the urine using various solvents was then tried. Several samples of male and female urine were acidified and extracted with carbon tetrachloride. Any unchanged malathion would have been extracted by this solvent. The amount of such material found represented less than 1% of the dosage. As the amount was so small, the chances of detecting any unchanged malathion when smaller dosages were given would be remote. Therefore, most of the samples were not extracted with carbon tetrachloride. Ether, on the other hand, extracted large amounts of radioactive materials (Table I). The percentages of the daily dosage recovered by ether extraction of the acidified urine ranged from 43 to 13 for the treatment period. This ether-extractable material represented from 47 to 78% (average 66%) of the total malathion-derived material excreted in the urine. The samples collected during 24 to 48 hours (sample 6) and 72 to 96 hours (sample 8) show that ether-soluble malathion-derived materials are excreted for a considerable time after the last treatment.

Having shown that relatively large amounts of malathion-derived ether-extractable phosphatic materials were excreted in rat urine following a dosage at the 100 mg. per kg. level, the dosage level was lowered to 25 mg. per kg. The results with the lower level (Table I) are in general agreement with those found for the higher dosage. The percentage of total intake excreted in the urine was slightly lower but more consistent (ranging from 37 to 47% and averaging 42%). The samples collected for daily intervals starting 24 hours after treatment (samples 6, 7, 8, and 9; 48 to 120 hours) showed excretion of malathion-derived material in amounts detectable by radioassay. The proportion of ether-extractable materials (ranging from 38 to 75% and averaging 69%) for the period of daily treatment was in the same range as found for the higher level of intake. Thus the amount of ether-extractable phosphates would seem to be almost directly proportional to dosage at these two levels of dosage. The degradation of the malathion to materials not extractable by ether such as orthophosphate did not proceed to a markedly greater degree at the lower dosage level. The urine samples collected 48 and 72 hours after the last treatment showed some ether-extractable materials detectable by radioassay. However, by the colorimetric method used they approached or fell below the limits of detectability.

Orally administered malathion was excreted in the same way as the intraperitoneally injected malathion. Male and female rats given 100 mg. per kg. of phosphorus-32 malathion excreted a total of 24 and 48%, respectively, in the urine, of which 31 and 36%, respectively, was ether soluble.

Colorimetric determination of phosphates in the ether-soluble materials gave good correlation with the radioassay. Of greatest importance was the fact that ether extracts of urine from the control animals showed no detectable amounts of phosphate. This indicated that analysis for urinary ether-extractable phosphatic materials could be used as an index of malathion ingestion. Therefore methods for extraction and colorimetric determinations were developed and standardized.

Method of Extraction and Analysis

General Scheme for Extraction and Analysis. The urine samples were acidified and extracted with ether. Anhydrous sodium sulfate was used to remove traces of urine and water containing inorganic phosphates. The ether solution of extracted metabolites was treated with alkali to prevent losses during evaporation of the ether and then the solvent was removed. Water was added to the residue and aliquots were taken for oxidation by perchloric acid (*7*). Colorimetric determinations were usually made by the method of Fiske and Subbarow (*3*). Where greater sensitivity was desired the method of Ammon and Hinsberg (*1*) as modified by Chen, Toribara, and Warner (*2*) was used.

Reagents. Aminonaphtholsulfonic acid reagent, 0.2% of 1,2,4-aminonaphthol sulfonic acid, 12% sodium bisulfite, and 2.4% sodium sulfite.

Ascorbic acid solution (10%), Mallinckrodt U.S.P. ascorbic acid (store in refrigerator).

Ascorbic acid reagent. One volume of 6*N* sulfuric acid, 2 volumes of distilled water, and 1 volume of 2.5% ammonium molybdate are mixed; 1 volume of 10% ascorbic acid is added and mixed well.

This solution must be made freshly each day.

Extraction and Evaporation of Solvent. In this laboratory, an automatic extraction apparatus is used, which limited the maximum urine sample volume to 40 ml. When smaller volumes of urine are used, enough water is added to bring the total volume of urine and water to 40 ml. Two of these automatic extractors are used, enabling ten samples of urine to be extracted simultaneously. The urine is acidified with concentrated hydrochloric acid to pH 2.0 or slightly less. A volume of 40 ml. of ether (analytical grade) is added and the extraction is carried out. Some emulsions are formed, but ordinarily the two phases separated adequately. Any emulsions are carried along with the ether phase. Rapid agitation seems to give less emulsion than does the gentler shaking of the funnels. After the first extraction, the urine is drained off into a beaker. The ether phase is put into a 500-ml. glass-stoppered bottle. A $^{24}/_{40}$ ⧩ joint is placed in the mouth of the bottle. The urine sample is returned to the separatory funnel and extracted again with 40 ml. of ether. The urine phase is then discarded and the second ether solution is combined with the first ether solution.

This solution is dried by adding sufficient solid anhydrous sodium sulfate to break any emulsion present and to take up the water. Careful but vigorous shaking at this point is required to break emulsions and to remove traces of water. The use of the ⧩ joints allows more vigorous shaking than glass stoppers. The sodium sulfate is removed by filtering the ether through a glass wool plug inserted in the stem of a short-stemmed funnel. The filtrate is caught in another 500-ml. glass stoppered bottle. The bottle is rinsed with ether and filtered as before. The ether solution is washed once by adding 2 ml. of 0.1*N* hydrochloric acid and shaking the bottles vigorously. Anhydrous sodium sulfate is again used to absorb the water and the bottles are again shaken. The ether is filtered as before into 300-ml. flat-bottomed $^{24}/_{40}$ ⧩ flasks. Ten milliliters of absolute ethyl alcohol and 1 ml. of 0.1*N* potassium hydroxide are added. Several glass beads are added and the mixture in the flask is refluxed for 10 minutes using cold finger condensers. The ether is then boiled off on the steam bath until only a small volume remains in the flask. The remaining solvents are removed by means of a rotating evaporator until only a small amount of nonvolatile residue remains. A measured amount of deionized (phosphate-free) water is added. The amount of water to be added depends on the amount of phosphatic material expected. Usually 3 to 5 ml. of water are added and aliquots of 2 ml. are taken for oxidation.

Table II. Ether-Extractable Phosphates in Human Urine Following Oral Ingestion of Malathion

(Colorimetric analyses)

Sample No.	Urine Volume, Ml.	Accumulative Time after Ingestion, Hours	Accumulative Time after Ingestion, Min.	Ether Extraction: Malathion equivalent per sample, mg.	Ether Extraction: Urine, p.p.m.	Ether Extraction: Mg./hour	Dose, %	Accumulative % of recovery
58 Mg. Technical Malathion (0.84 Mg./Kg.)								
1	250	1	20	1.26	5	0.94	2.2	9
2	180	2	50	4.70	26	3.10	8.1	44
3	210	5	20	5.62	27	2.20	9.7	86
4	64	7	35	1.55	24	0.69	2.7	97
5	205	16	20	0.43	2	0.05	0.7	100
6	165	22	20	<0.08[a]	<0.5	...	...	...
		Total		13.56	...	...	23.4	...
11 Mg. of 99% Pure Malathion (0.16 Mg./Kg.)								
1	370	1	30	0.38	1.0	0.25	3.5	18
2	370	3		1.02	2.8	0.68	9.2	65
3	127	4	30	0.59	4.7	0.39	5.4	94
4	170[b]	6		0.14	0.8	0.09	1.3	100
5	235	7	30	<0.035[b]	<0.15	...	...	...
6	200	8	30	<0.030[b]	<0.15	...	...	...
		Total		2.13	...	...	19.4	...

[a] Not detectable, method of Fiske and Subbarow.
[b] Not detectable, method of Ammon and Hinsberg.

Table III. Fractionation by Solvents of Extractable Phosphatic Malathion-Derived Materials Found in Human Urine

Sample No.	Accumulative Time after Ingestion	% of Total Solvent-Extractable Material: CCl_4	$CHCl_3$	Ether
2	2 hours 50 min.	62.7	22.5	14.7
3	5 hours 20 min.	42.5	17.9	39.6
4	7 hours 35 min.	18.4	23.0	58.6

Oxidation and Colorimetric Determination. The aliquots are placed in test tubes (175 × 22 mm.). Perchloric acid (0.5 ml. of 60%) is added and the tubes are heated at 190° to 210° C. to evaporate the water and oxidize the organic material. Heating is conveniently done in an aluminum block into which holes to receive the test tubes have been drilled to a depth of 4 cm. When the samples have become colorless, the tubes are removed from the block and cooled.

Color development is usually carried out by the method of Fiske and Subbarow. To the oxidized sample 4.0 ml. of water are added. Then 0.2 ml. of the 5% ammonium molybdate reagent is added and mixed. This is followed by 0.2 ml. of the aminonaphtholsulfonic acid reagent. The color is allowed to develop for 15 minutes and then read at 700 mμ in a spectrophotometer using a 1-cm. cell. Determinations of unknowns are made by calculation using an extinction coefficient determined from malathion standards. The urine metabolites are calculated in terms of malathion. By this method an absorbance of about 0.1 will be found for 35 γ of malathion.

When the more sensitive Ammon and Hinsberg method is used according to the modification of Chen *et al.*, 4 ml. of water are added to the oxidized solution, then 4 ml. of the ascorbic acid reagent. The color is developed by incubation at 37° C. for 0.5 to 1 hour. Colorimetric measurements are made at 820 mμ in a spectrophotometer in a 1-cm. cell. A fourfold increase in sensitivity over the Fiske and Subbarow method can readily be obtained by this procedure.

Excretion by Humans. The method described was applied to human urine obtained from a male who ingested 58 mg. of technical malathion (0.84 mg. per kg.). This dose was below that capable of causing any detectable cholinesterase inhibition. Pre-exposure urine samples showed no detectable amounts of ether-extractable phosphates. The amount of ether-extractable phosphates rose quickly after dosage (Table II) and then decreased to the predosage level. A total of 23% of the dose was recovered after $16^1/_3$ hours and 97% of this was excreted in the first $7^1/_2$ hours. The rate in terms of milligrams per hour was greatest in the sample taken $1^1/_3$ to 3 hours after ingestion. A smaller dose of 11 mg. of malathion (0.16 mg. per kg.) gave similar results (Table II). The percentage recovery was similar. However, metabolites could be detected for a shorter time after ingestion, even though the more sensitive method for detection was used.

Dermal application to a human male

of malathion in a talc dust also produced ether-extractable phosphatic materials in the urine. When 100 mg. of malathion as a 1% dust was applied for 14 hours, 1% of the dose was recovered during this period. With higher amounts of malathion, the percentage recovery of metabolites from the urine was of the same order. No cholinesterase inhibition was detectable in any case.

Nature of Excretory Products. The malathion metabolites in rat urine contained only small amounts of carbon tetrachloride–extractable materials. The human urine, however, contained an appreciable amount of phosphatic material which was carbon tetrachloride–extractable. An experiment was done wherein the urine was extracted successively with carbon tetrachloride, chloroform, and ether. The results of the analysis by the colorimetric phosphate method (Table III) show a change in the composition of the metabolites with length of time after dosage. The carbon tetrachloride–extractable material decreases from 63 to 18% of the total solvent extractable compounds during a 5-hour period. This decrease is reflected in an increase in the material extracted by the ether from 15 to 59% of the total extracted.

The Norris, Vail, and Averell method (*8*) for malathion was applied to the carbon tetrachloride extracts. (This method will be referred to as the Norris method as contrasted to the organic phosphate method.) The Norris method gave a positive test with the carbon tetrachloride–extractable materials. Control samples had no interfering materials. This colorimetric method depends on the hydrolysis of malathion to dimethyl phosphorodithioate salt in alkaline solution. Any compounds which can be hydrolyzed to this material will give a positive reaction. Thus, in addition to malathion, either of the half esters, *S*-[(1-carboxy-2-carbethoxy)ethyl] *O,O*-dimethylphosphorodithioate or *S*-[(2-carboxy-1-carbethoxy)ethyl] *O,O*-dimethylphosphorodithioate is converted to the dimethylphosphorodithioate salt and therefore will give a positive test. The extractability of the half esters by carbon tetrachloride under the conditions used here is not known at present. Therefore, the carbon tetrachloride–extractable material could be malathion, one or both of the half esters, or other unknown compounds. Colorimetric organic phosphate determinations carried out on the same carbon tetrachloride extracts agreed with the results by the Norris method. Thus all of the carbon tetrachloride–extractable phosphatic material was detected by the Norris method.

The urine samples collected after the lower dosage (11 mg.) of malathion did not show the same results as at the higher dosage. From 23 to 27% of the ether-extractable material was found to be carbon tetrachloride–extractable in samples collected at 3 and $4^1/_2$ hours after dosage. However, this carbon tetrachloride–extractable material failed to give a positive color with the Norris method. There is apparently a variability in the nature of the excretory products which may be dependent on amount ingested. Thus the extraction by ether and determination of phosphatic materials are preferable to the Norris method, in that it determines a larger amount of materials for a longer period of time after ingestion of malathion and at lower concentrations of malathion ingestion.

Malathion is readily hydrolyzed by acid chiefly to *O,O*-dimethylphosphorothionic acid and by alkali chiefly to *O,O*-dimethylphosphorodithioate (*6*). It was thought that the ether-extractable urinary metabolites could be materials derived from one of the above dimethyl esters. As phosphorodithioate is rather unstable and is converted at least partially to the phosphorothionate, treating a rat with *O,O*-dimethylphosphorodithioic acid should produce ether-extractable phosphatic compounds in the urine if malathion degradation depended wholly on hydrolysis to these compounds. When *O,O*-dimethylphosphorodithioic acid was given intraperitoneally at the level of 100 mg. per kg. as the ammonium salt in water, the amount of ether-extractable material recovered was only 1% of the dose during the first 24 hours after dosage. This recovery, compared to that found for malathion, indicates that hydrolysis of malathion to the secondary dithio or thionophosphates is not responsible for the formation of the major portion of the ether-extractable urinary malathion metabolites.

Other Organic Phosphorus Insecticides. Some preliminary work was done using other organic phosphorus insecticides to determine whether appreciable amounts of ether-extractable phosphatic materials were excreted in the urine of rats following treatment with them. These compounds were given intraperitoneally in peanut oil to female and male rats and the urine was collected for two 24-hour periods.

The compounds tested were as follows:

I. 2,3-*p*-Dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate) (Delnav) (Hercules Powder Co., Inc., Wilmington, Del.), 10 mg. per kg.

II. *O*-(3-Chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate (Chlorthion) (Chemagro Corp., New York, N. Y.), 50 and 100 mg. per kg.

III. *O*-(2-Chloro-4-nitrophenyl)*O,O*-dimethyl phosphorothioate (Dicapthon) (American Cyanamid Co., Stamford, Conn.), 50 mg. per kg.

IV. *O,O*-Diethyl *O*-*p*-nitrophenyl phosphorothioate (Parathion), 2 mg. per kg.

V. *O,O*-Dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate (Dipterex) (Chemagro Corp., New York, N. Y.), 100 mg. per kg.

One of these compounds is a phosphorodithioate (I), three are phosphorothionates (II, III, IV), and one is a phosphonate (V). The phosphonate (V) was chosen because of its low mammalian toxicity. No detectable phosphatic materials were extracted by ether when V was given. Parathion is so toxic that only a small amount could be given to the rats. No urinary metabolites were detected by ether extraction when parathion was given. However, had the parathion been metabolized in a fashion similar to that of malathion the concentrations in the urine of ether-extractable materials should have been detectable. The phosphorodithioate (I) did show some excretion of ether-extractable phosphates in the urine but only to the extent of 3% for the male and 2% for the female. Such small amounts could possibly be due to impurities. The other two phosphorothionates (III and IV) also showed the excretion of some ether-extractable urinary products. For Chlorthion the recovery was 2% for both the male and female rats. The Dicapthon gave the highest results, with a recovery of 7% for the male and 1% for the female. None of these compounds approaches the recoveries found for malathion. The metabolism of malathion apparently differs from that of the other compounds tested. Nevertheless it is possible that excretion in man differs from that in rats with these latter compounds and that determination of urinary ether-extractable phosphates could be of value in the detection of exposure of man to various organic phosphorus compounds other than malathion.

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A Sensitive Procedure for Urinary *p*-Nitrophenol Determination as a Measure of Exposure to Parathion

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A sensitive procedure has been developed for the determination of *p*-nitrophenol in urine. The major variation from earlier published procedures involves extraction with acetonitrile to remove interfering impurities. The present method allows determination of as little as 10 γ of *p*-nitrophenol and permits the use of urine samples as large as 100 ml.

THE ACTIVITY OF THE ORGANIC phosphorus insecticides in inhibiting the enzyme cholinesterase is well known. Measurement of blood cholinesterase level has been widely used as a measure of exposure of workers and others to these agents. However, there has been a need for a more sensitive measure of exposure. In certain instances, particularly in cases of multiple exposure, it would be an advantage to have a test which is specific for a certain compound or for a certain restricted group of compounds. Urine samples are easier and less painful to acquire than blood samples requiring venipuncture, particularly where a number of tests may be made on the same person over the course of a season's work. The determination of urinary *p*-nitrophenol as a measure of exposure to those organic phosphorus compounds, including parathion and EPN, which yield this material on hydrolysis has been investigated.

There are in the literature a number of methods for the determination of *p*-nitrophenol in urine. All involve the reduction of *p*-nitrophenol to *p*-aminophenol, which then is coupled with a phenol to form indophenol blue. They differ as to whether or not hydrolysis is considered to be necessary and as to methods for extraction and purification. All suffered from the limitation that quantities of urine greater than 10 ml. could not be used because of interfering substances. In addition, with some of the methods, recovery from urine of known added amounts of *p*-nitrophenol was erratic.

The present paper points out that hydrolysis is a necessary step in the procedure. An additional cleanup step involving use of acetonitrile brings about much more complete separation of *p*-nitrophenol, from interfering impurities than was possible with earlier methods and thus makes possible the use of large quantities of urine (up to 100 ml.).

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Procedure

A sample of urine (not more than 100 ml. and containing not less than 10 γ of *p*-nitrophenol) is placed in a 500-ml. Erlenmeyer flask fitted with a water-cooled condenser. Twenty milliliters of concentrated hydrochloric acid are added per 100 ml. of urine, and the mixture is boiled gently for 1 hour. The hydrolyzed urine is cooled and extracted with 60 ml. of ethyl ether–benzene (20 to 80) by shaking for 5 minutes. The water phase is aspirated off and sufficient anhydrous sodium sulfate (about 5 to 10 grams) added to congeal any residual emulsified aqueous phase and to dry the ether-benzene.

Fifty milliliters of the organic phase are transferred to a 250-ml. Erlenmeyer flask or glass-stoppered bottle and shaken for 5 minutes with 5 ml. of 20% sodium hydroxide. The organic layer is aspirated off, and the sodium hydroxide is poured into a 15-ml. glass-stoppered centrifuge tube. Any accompanying ether-benzene is aspirated off and residual amounts are finally removed by a gentle air stream. The previous container is rinsed with 5 ml. of acetonitrile, and the rinsings are carefully added to the glass-stoppered centrifuge tube containing the sodium hydroxide.

The centrifuge tube is stoppered and shaken for two approximately 1-minute periods. The tube is centrifuged briefly to effect a clear separation of the layers and the sodium hydroxide phase is aspirated off.

Five milliliters of 3*N* ammonium hydroxide and 1 ml. of *o*-cresol reagent (2% in 0.2*N* sodium hydroxide) are added and mixed. The solution should be observed at this time (prior to reduction) for blue color which would indicate the presence of amino compounds. One milliliter of freshly mixed titanium trichloride reagent (1 ml. of 20% titanium trichloride to 16 ml. of water) is then added. The tube is immediately shaken for about 15 seconds or until the dark precipitate disappears. Thirty minutes is allowed for full development of the indophenol blue color. Once developed, the color is stable for several hours. The tubes are then centrifuged for 2 to 5 minutes or until optically clear. The absorbance of the colored solution is then read against a reagent blank at a wave length of 620 mμ. The *p*-nitrophenol content is determined by calculation using the absorptivity obtained in the usual manner from carrying known quantities of *p*-nitrophenol through the complete procedure (Table I).

Results

The effects of varying certain of the prescribed conditions in the method were measured. Among the items studied were:

Hydrolysis. *p*-Nitrophenol may be present in human urine in either the free form or conjugated with glucuronic, sulfuric, or acetic acid (*5*). The conjugated *p*-nitrophenol must be converted to the free form before it can be successfully carried through the series of extractions and the color development step described above. In almost every instance, acid hydrolysis of urine samples from persons exposed to parathion gave higher *p*-nitrophenol values than corresponding aliquots which were not hydrolyzed. An additional advantage of acid hydrolysis was that troublesome emulsions were less frequently encountered when extractions with organic liquids were carried out following this procedure than when extractions were made from neutral or alkaline media. In a limited series of samples, it appeared that differences between hydrolyzed and nonhydrolyzed samples were smaller for samples of higher *p*-nitrophenol content. This may be an indication that, at higher levels of parathion exposure, the ability of the organism to detoxify *p*-nitrophenol by conjugation is exceeded.

Table I. Absorptivity and Recovery of Known Amounts of p-Nitrophenol[a]

p-Nitrophenol Content	Direct Color Development		Full Procedure		
	Absorbance	Absorptivity[b] × 10^2	Absorbance	Absorptivity[b] × 10^2	Recovery, %
10	0.150	1.50	0.115	1.15	75
20	0.305	1.53	0.230	1.15	75
30	...	...	0.335	1.12	73
40	...	...	0.465	1.16	75
50	0.790	1.58	0.535	1.07	70
100	1.55	1.55	1.10	1.10	71
Mean		1.54		1.13	73.2
Standard error of mean		0.017		0.015	

[a] Determinations reported in columns 4 and 5 were carried through entire procedure described above. Determinations in columns 2 and 3 involved color development only.
[b] Calculated using concentration expressed as micrograms per 12 ml.

The results noted could be due to excretion of free parathion in the urine following high-level exposure. However, no parathion could be detected either by chemical or by enzymatic methods in urine from persons known to have large parathion exposure and who were, at the time of study, excreting high levels of *p*-nitrophenol.

No advantage was noted in extending hydrolysis time up to 4 hours or in increasing acid strength beyond that recommended above. A 1-hour hydrolysis period gave somewhat better results than did shorter times.

In contrast to results with human urine, hydrolysis brought about no increase in recovery of *p*-nitrophenol from the urine of parathion-dosed rats. This variation is thought to be a species difference, as the dosage levels in the two instances were of about the same order of magnitude. The mature male rats had been fed a diet containing 50 p.p.m. of parathion, which determines an oral dosage of about 2 mg. per kg. per day. The daily exposure of orchard spraymen to parathion has been estimated to be about 8.9 mg. per kg. (*2*), chiefly by the dermal route.

Extraction. The use of ion exchange resins, including Amberlite resins IR4-B, IR45, IR50, IR120, IR400, and IR401 (Rohm & Haas Co., Philadelphia, Pa.) to separate *p*-nitrophenol from interfering substances in urine was investigated. A number of resins were found which would absorb *p*-nitrophenol. However, of the resins tested, none would selectively adsorb *p*-nitrophenol without interfering substances and allow quantitative recovery of the adsorbed *p*-nitrophenol. Better purification and better recovery were obtained with solvent extraction than with any resin tested.

All three of the extraction steps involved, including ether-benzene from aqueous hydrochloric acid; aqueous sodium hydroxide from ether-benzene; and acetonitrile from aqueous sodium hydroxide, are almost quantitative and give recoveries of known added amounts of *p*-nitrophenol between 80 and 100%. The latter transfer (from sodium hydroxide to acetonitrile) requires vigorous shaking to mix the phases and it is necessary that the alkali be concentrated (20% or above) so that the acetonitrile will not extract water as well as *p*-nitrophenol.

Recovery. The relationship between *p*-nitrophenol content and absorbance of the indophenol blue product at 620 mμ as measured in a Beckman Model B spectrophotometer is shown in Table I. The agreement of the absorptivity values shows that the reaction follows Beer's law closely between 10 and 100 γ of *p*-nitrophenol. At higher values of *p*-nitrophenol content (above 250 γ), the relationship between *p*-nitrophenol content and absorbance is no longer linear. However, it is possible to obtain fairly accurate estimations of *p*-nitrophenol content of solutions too deeply colored for accurate direct reading (above absorbance of 1.55) by dilution with acetonitrile.

The column in Table I headed Direct Color Development represents values obtained by adding known amounts of *p*-nitrophenol directly to the tubes in which the final color reaction took place so that no recovery of *p*-nitrophenol by extraction was involved. The absorptivities are higher (average 0.0154) than those obtained when the nitrophenol was extracted from the urine (average 0.0113). The average absorptivity, corrected for aliquots taken, of the Full Procedure determination, is only 73% as large as that of solutions in which the color was developed directly. An average loss of 27% of the added *p*-nitrophenol is incurred in carrying out the full procedure. While desirable, it is not necessary that 100% recovery of material be possible in analytical methods of this type. It is, however, essential that a constant percentage be attained. The present method yields a rather constant recovery of from 70 to 75%. No samples of known amounts of conjugated *p*-nitrophenol were available, so no studies were made of the completeness of hydrolysis. Therefore, it must be understood that in actual practice the recovery of total (including both free and conjugated) *p*-nitrophenol from urine may be slightly lower because of possible incomplete hydrolysis. However, the samples referred to in Table I under Full Procedure were carried through the hydrolysis step to allow for any losses incurred during these manipulations.

Specificity. The specificity of the *p*-nitrophenol analysis would be improved if one could determine whether any of the final colored complexes represented *p*-aminophenol. *p*-Aminophenol might occur in the urine as a natural metabolite of parathion following in vivo reduction of parathion (*1*), of *p*-nitrophenol (*3*), or as a biotransformation product of certain common drugs, such as acetophenetidin. Distinction between amino and nitro compounds is not possible by previously published procedures. However, by simply adding the color-producing reagent to the reaction mixture before carrying out the reduction step, it is possible to determine pre-existing amino compounds. (Any blue color formed after addition of *o*-cresol and before addition of titanium chloride could be ascribed to amino compounds.) Tests have shown that the nitro compounds give the same eventual depth of color regardless of whether the *o*-cresol reagent is added before or after reduction. In none of the samples analyzed have the authors noted the presence of amino compounds, following the recommended cleanup procedure.

The authors have found it convenient to express *p*-nitrophenol excretion in terms of parathion equivalent. This conversion can, of course, be made by multiplying *p*-nitrophenol excretion by a factor of 2.09.

Storage. Formalin (2 to 3 drops per 100 ml. of urine) is a good preservative for urine samples collected for *p*-nitrophenol determination. Refrigeration is also recommended where samples are to be kept for several days before analysis.

Unpreserved human urine samples showed losses of *p*-nitrophenol, on standing, ranging from 1 to 24% per day. A more rapid loss of *p*-nitrophenol occurred in those urine samples with stronger odor of ammonia.

Discussion

Preliminary tests indicated that, with minor modifications, this procedure could be made applicable to other organic phosphorus insecticides which yield substituted nitrophenols on hydrolysis. Among the compounds in this category which have been considered are Chlorthion [*O*-(3-chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate] and Dicapthon [*O*-(2-chloro-4-nitrophenyl)*O,O*-dimethyl phosphorothioate]. The former compound would yield 3-chloro-4-nitrophenol and the latter material would give rise to 2-chloro-4-nitrophenol.

A number of common insecticides and drugs have been tested for possible interference in this procedure. Of the materials tested, the only substance which interfered in the analysis was dinitro-*o*-cresol (DNOC). This finding is not surprising in view of the similarity between the dinitro-*o*-cresol molecule (which is really a disubstituted *p*-nitrophenol) and that of *p*-nitrophenol. Dinitro-*o*-cresol produces a yellow color in the reaction which has the effect of a high blank value. The magnitude of this interference is not very great even at low *p*-nitrophenol values and is undetectable at levels above 25 γ.

Studies of exposed workers carried out during the 1958 spray season have shown that, when measured by the present method, urinary *p*-nitrophenol excretion is significantly more sensitive than blood cholinesterase in measuring absorption of parathion. This statement is true even under optimal conditions for usage of blood cholinesterase values—when the individual's pre-exposure enzyme level is known. The results of these studies will be reported in detail elsewhere.

The present method for determination of urinary *p*-nitrophenol content can be carried out in about the same period of time as required for blood cholinesterase determination by the Michel procedure (*4*). It is not proposed that measurement of urinary *p*-nitrophenol be substituted for blood cholinesterase determination in all cases involving exposure to parathion or EPN. In some instances, one or the other analysis may be preferable; in other cases, it may be informative to carry out both tests.

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SOIL FUMIGANT DETERMINATION

Extraction and Determination of Ethylene Dibromide in Soils

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A method utilizing vacuum distillation and catalytic oxidation is described for the determination of ethylene dibromide in soils. Recovery is reported in the range of about 82 to 0.2 mg. of ethylene dibromide per 50 grams of soil. Moisture and organic matter affect recovery.

IN STUDIES INVOLVING penetration and phytotoxic effects of ethylene dibromide (EDB) in soils it is necessary that an analytical technique be developed to determine the low concentrations of the fumigant usually encountered in soil studies.

Previous studies were either lengthy and prone to both oxidizing and reducing side reactions (*1*), or lacking in sensitivity at low concentrations (*4*).

This investigation is an attempt to develop a simple but sensitive technique for the determination of ethylene dibromide in soils. This determination is carried out in three stages: recovery of ethylene dibromide from the soil sample, conversion of ethylene dibromide to inorganic bromide, and determination of inorganic bromide.

Reagents and Apparatus

2,4,5,7-Tetrabromofluoroscein, sodium salt (eosin Y), 0.1% solution.

The catalytic oxidation of ethylene dibromide is based on a modification of the method described by Lubatti and Harrison for the determination of methyl bromide (*3*). The combustion or reaction chamber is shown in Figure 1. Two 15-mm. lengths of 20-gage platinum wire are introduced into the stopper, *B*, by means of capillaries, *C* and *C'*; and sealed in airtight with Apiezon W wax. To the lower ends of the thicker platinum wires are connected 16 mm. of coiled 30-gage platinum wire which serves as the oxidizing surface. During the catalytic oxidation, the leads of *C* and *C'* are connected to an electrical circuit through a variable transformer. In this study, 5 volts were sufficient to bring the filament to a bright red glow. The 14/35 taper attached to *B* is used to connect the reaction chamber to the vacuum manifold for the evaluation of the flask. The vacuum system used maintained a pressure of 1 to 5 microns as measured with a McLeod gage.

Procedure

The purity of ethylene dibromide used in the study was determined by adding, with a micropipet, known amounts of ethylene dibromide to flask *A*, then immediately immersing the flask in a dry ice–acetone bath. To the frozen system were then added 2 ml. of 0.8*N* sodium hydroxide and 1.5 ml. of 30% hydrogen peroxide. Stopper *B* was placed on the flask and the system was completely evacuated while still immersed in the freezing bath. The system was then removed from the vacuum manifold, was thawed, and sufficient voltage was placed across the filament to produce a bright glow. The combustion period consisted of alternate closing and opening the circuit at 1-minute intervals, to prevent overheating, for about 15 minutes. Stopper *B* was then removed and rinsed. The solution in flask *A* was then directly titrated with either 0.01*N* or 0.025*N* silver nitrate using a microburet and following the eosin Y method of Kolthoff and Sandell (*2*).

At two concentration levels of 21.7 and 43.4 mg. of ethylene dibromide added to the reaction flask successive determinations indicated a recovery of 96.3, 96.5, and 96.7% and 95.5, 96.6, and 96.7%, respectively. Hence under

A number of common insecticides and drugs have been tested for possible interference in this procedure. Of the materials tested, the only substance which interfered in the analysis was dinitro-*o*-cresol (DNOC). This finding is not surprising in view of the similarity between the dinitro-*o*-cresol molecule (which is really a disubstituted *p*-nitrophenol) and that of *p*-nitrophenol. Dinitro-*o*-cresol produces a yellow color in the reaction which has the effect of a high blank value. The magnitude of this interference is not very great even at low *p*-nitrophenol values and is undetectable at levels above 25 γ.

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Table I. Recovery of EDB from Air-Dried Mineral Soils

Columbia Fine Sandy Loam				Yolo Silty Clay			
EDB dosage, mg.	Glycerol added, ml.	EDB Recovered Mg.	EDB Recovered %	EDB dosage, mg.	Glycerol added, ml.	EDB Recovered Mg.	EDB Recovered %
82.4	20	81.9	99.4	82.4	20	81.1	98.4
	20	80.4	97.6		20	80.7	97.9
	20	81.7	99.2		20	80.7	97.9
	0	70.6	85.7		0	72.0	87.4
	0	70.7	85.8		0	68.5	83.0
2.14	20	2.09	97.7	2.14	20	2.10	98.1
	20	2.04	95.3		20	2.14	100.
	20	2.08	97.2		20	2.07	96.7
0.256	20	0.235	91.8	0.256	20	0.216	84.4
	20	0.226	88.9		20	0.226	88.3
	20	0.226	88.9		20	0.220	85.0
	0	0.197	77.0		0	0.188	73 4
	0	0.196	76.6		0	0.189	73.8

Table II. Recovery of EDB from Air-Dried Organic Soil

(Staten peaty muck)

EDB Dosage, Mg.	EDB Recovered, Mg.	Recovery, %
82.4	79.4	96.4
	79.5	96.5
2.14	1.76	82.2
	1.76	82.2
0.188	0.132	70.2
	0.140	74.4

Table III. Recovery of EDB at Field Capacity

(Yolo silty clay)

EDB Dosage, Mg.	EBD Recovered, Mg.	Recovery, %
82.4	78.8	95.7
	79.1	96.0
	79.3	96.3
3.36	2.99	89.1
	2.95	87.8
	3.02	89.8
0.217	0.178	82.5
	0.171	78.8
	0.171	78.8

experimental conditions the ethylene dibromide was considered 96.4% pure. The above value also includes the efficiency of the oxidation procedure for the analysis of ethylene dibromide.

The extraction of ethylene dibromide from air-dried soils was as follows. Two milliliters of 0.8*N* sodium hydroxide and 2 ml. of 30% hydrogen peroxide were added to flask *A*. The flask was then immersed in a dry ice–acetone bath, connected to the vacuum distillation manifold, and evacuated. Flask *A* remained in the freezing bath and acted as the cold trap throughout the extraction period. In a similarly modified 125-ml. Erlenmeyer flask a given amount of ethylene dibromide was placed at the bottom and 50 grams of sieved, air-dried soil were added. The flask was immersed in a dry ice bath and 20 ml. of technical grade glycerol were added. The sample flask was then connected to the vacuum distillation manifold through a reflux condenser and evacuated while frozen out. The distillation system was then closed from the pumps and the cold bath was removed and replaced by a sand bath maintained at 150° C. for 2 hours. At 20- to 30-minute intervals, a stopcock was closed to isolate the sample and the connecting system between the flasks was open to the pumps to remove noncondensable gases. Care must be exercised when returning the sample to distillation, after pumping out noncondensable gases, to prevent the blowing of the sample into the vacuum lines. After 2 hours of distillation, flask *A* was removed from the system and stoppered with *B*, and the condensate was oxidized and titrated as previously described.

Field capacity samples were prepared by adding adequate water to 50 grams of air-dried soil and allowing the mixture to equilibrate for 24 hours. The ethylene dibromide was then added to the moist soil, and the flask was stoppered and immersed in a dry ice bath. When the contents were frozen, 20 ml. of glycerol were added and the flask was attached to the vacuum manifold through a reflux condenser and evacuated. Because of the greater amount of vapor distilled over to flask *A*, field capacity soils were heated at 150° C. for 3 hours. The remaining procedure was the same as with air-dried soils.

The ethylene dibromide was added to the soils either by micropipets or as an aqueous solution of the fumigant. The actual amount of ethylene dibromide delivered to the soil by both methods was determined by catalytic combustion analysis.

Results and Discussion

Table I shows the recovery of ethylene dibromide from two air-dried mineral soils and the effect of glycerol on the recovery at high and low rates of application. The beneficial effect of glycerol is ascribed not only to its refluxing action during distillation, but also to its displacement of sorbed ethylene dibromide from the soil surface in the air-dried systems. The recovery of ethylene dibromide from an organic soil, Staten peaty muck (40% organic matter), is shown in Table II. At the lower rates, a definite decrease in recovery is noted. This relative inability to recover ethylene dibromide from this organic soil indicates either a rapid interaction between the organic fraction and the fumigant or that a modification of the procedure is necessary in organic systems. The data from soil blanks did not significantly alter the results of the procedure and hence are not reported.

Of interest is the applicability of this determination in soils supplied with adequate moisture for plant growth. Table III shows the determination of ethylene dibromide in soils at field capacity.

These data indicate that moisture does decrease the apparent recovery of ethylene dibromide when compared to soil analyzed in the air-dried state. This decrease is ascribed mainly to the accumulation of moisture in the cold trap during distillation of the wetted sample. Data obtained in this laboratory indicate that the presence of excess water in the reaction chamber definitely decreases the efficiency of the catalytic oxidation reaction. The magnitude of this decrease is in the order of 10 to 15% in the presence of 10 ml. of excess water. The detrimental effect of moisture is probably due to the hindering action of moisture on the vaporization of ethylene dibromide from its liquid and solution phases.

The data of this study indicate that vacuum distillation coupled with the catalytic oxidation reaction shows promise as a method of determining

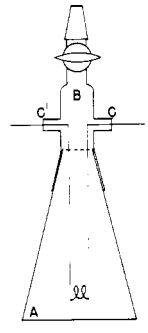

Figure 1. Reaction flask used in catalytic oxidation of ethylene dibromide

A. 125-ml. Erlenmeyer flask with 24/40 taper
B. 4-mm. stopper with 14/35 taper and mounted platinum filament

ethylene dibromide in dry and moist soils at dosages approaching field applications of ethylene dibromide (4 to 6 gallons per acre). At present, this method shows particular usefulness in dry systems where the complicating factor of moisture is minimized.

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INSECTICIDE RESIDUES

Persistence of Dimethoate and Metabolites Following Foliar Application to Plants

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DIMETHOATE [*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate] is active as a systemic insecticide for grub control in cattle (*6*). This same chemical (designated as Rogor in England and Europe) has been used for insect control on plants for several years (*12, 13*). It displays good systemic activity following foliar application (*2*) and as a side dressing in the soil when formulated on granules (*11*). The oxygen analog of dimethoate [*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate] has been demonstrated in bean and cherry plants after root and foliar dimethoate treatment, respectively (*12, 13*). Studies on the mammalian metabolism of dis methoate were reported in a previou paper (*5*).

The present investigation concerns the persistence and metabolites associated with the use of dimethoate as a systemic insecticide for treating plant foliage.

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Methods

Dimethoate Derivatives Utilized. In addition to dimethyl phosphoric, phosphorothioic, and phosphorodithioic acids, the following compounds related to dimethoate were studied: *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate, $(CH_3O)_2P(O)SCH_2C(O)NHCH_3$, the oxygen analog of dimethoate; *O,S*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate, $(CH_3O)(CH_3S)P(O)SCH_2C(O)NHCH_3$, the *S*-methyl isomer of dimethoate; *O,O*-dimethyl *S*-carboxymethyl phosphorodithioate, $(CH_3O)_2P(S)SCH_2C(O)OH$, the thio-carboxy derivative of dimethoate; *O,O*-dimethyl *S*-carboxymethyl phosphorothiolate, $(CH_3O)_2P(O)SCH_2C(O)OH$, the oxy-carboxy derivative of dimethoate; *O*-methyl *O*-hydrogen *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate, $(CH_3O)(HO)P(S)SCH_2C(O)NHCH_3$, the des-methyl derivative of dimethoate; and *O,O*-dimethyl *S*-(*N*-ethylcarbamoylmethyl) phosphorodithioate, $(CH_3O)_2P(S)SCH_2C(O)NHC_2H_5$, the *N*-ethyl analog of dimethoate which is designated as CL 18,706.

Synthesis. Radioactive dimethoate was prepared, purified, and characterized as previously described (*5*) using isotopic exchange to obtain the phosphorus-32 pentasulfide intermediate (*4*). The radioactive product was identical in infrared spectrum with highly purified dimethoate and had a specific activity of about 25 mc. per gram. Radioactive CL 18,706 was prepared in 60% yield by the same procedure as for dimethoate, except that *N*-ethyl α-chloroacetamide replaced *N*-methyl α-chloroacetamide. The radioactive product was identical in chromatographic properties and infrared spectrum with a highly purified sample of known CL 18,706.

Volatilization. The volatilization rates for radioactive dimethoate from cotton leaves and the surfaces of metal, wood, glass, plastic, rubber, Masonite, and Pressboard were determined. Approximately 5 γ of radioactive dimethoate in 0.1 ml. of acetone were applied to the upper leaf surface of each cotton plant in a spot 0.5 inch in diameter, or to comparable areas of the other surfaces. The treatments were replicated six times and the materials were held in a greenhouse at about 28° C. Radioassays (*7*) were made periodically after treatment.

The volatilization rate for radioactive

ethylene dibromide in dry and moist soils at dosages approaching field applications of ethylene dibromide (4 to 6 gallons per acre). At present, this method shows particular usefulness in dry systems where the complicating factor of moisture is minimized.

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INSECTICIDE RESIDUES

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The volatilization rate for radioactive

CL 18,706 from cotton leaves was also determined using the same technique.

Persistence and Absorption. Potted seedlings of corn (variety 1512), cotton (Lankart 55), and potato (Irish cobbler) with above-ground heights of 6, 4, and 6 inches, respectively, were treated by dipping the plants into a solubilized dimethoate solution composed of 30 mg. of phosphorus-32 dimethoate, 3.26 mg. of Alrodyne 315, 3.26 mg. of Aerosol OT, and 8.70 mg. of methyl Cellosolve per 50 ml. of water. This formulation was equivalent to about 0.5 pound of dimethoate per 100 gallons of water. Four plants of each species were periodically removed for analysis. The leaf surfaces were washed with 20 ml. of water followed by two 20-ml. portions of acetone. The acetone wash fractions were combined and stripped of solvent, and the residue was dissolved in the water fraction from the surface wash. Leaves from each individual washed sample were then weighed and 2 to 4 grams were homogenized with 7 ml. of water in a Potter-Elvehjem glass homogenizer. Total radioactivity was determined for each fraction. Four milliliters of the surface wash and the leaf homogenate, respectively, were partitioned against an equal volume of chloroform to determine the per cent chloroform-solubles. The same procedure was used for seedling cotton plants treated with radioactive CL 18,706 and for near mature pea plants (Perfection 15 variety) treated with phosphorus-32 dimethoate.

Chemical Changes in Dimethoate on the Plant Surface. Corn, potato, and cotton plants were fractionated as described above at 2 and 12 days after treatment, while peas were fractionated only at 12 days after treatment. Partition chromatography with silica gel columns (*5*, *14*) was utilized to separate the dimethoate derivatives which partitioned into chloroform from neutral aqueous solutions, and ion exchange chromatography (*8*) was used to separate the water-soluble metabolites from the same extractions. Resolution with the ion exchange chromatograms was greatly improved over a previous study (*5*) by substituting cylinders (*3*) for separatory funnels in the gradient elution apparatus (*8*). To obtain sufficient radioactivity, the surface washes from three plants were combined before being chromatographed.

Stability of Dimethoate on Glass Surfaces. One hundred micrograms of radioactive dimethoate in 2 ml. of acetone were added to a Petri dish and the solvent was evaporated. The same amount of the compound was also added to a Petri dish containing a surface extract of potato leaves obtained by scrubbing the leaves with acetone, chloroform, and benzene and then allowing this combined extract to evaporate in the dish. After 7 days of exposure in the greenhouse at approximately 28° C. the per cent hydrolysis was determined by a chloroform-water partition and the chloroform-solubles were fractionated by partition chromatography.

Metabolism of Dimethoate Absorbed by Leaves. Using the above described techniques, the surface residues at 2 and 12 days after treatment were removed by washing with water and acetone. These leaves which were free of surface residue were then homogenized with 7 ml. of water in a Potter-Elvehjem glass homogenizer and the homogenate was partitioned with an equal volume of chloroform. The chloroform-soluble metabolites were fractionated on a silica gel column and the water-soluble metabolites on an ion exchange column.

Dimethoate Residues in Peas. Flowering pea plants (Perfection 15 variety) were sprayed with radioactive solubilized dimethoate to yield an initial residue of 25 p.p.m. based on the wet weight of the whole plant. Immediately after treatment, four whole plants were fractionated, and 17 days after treatment an additional four plants were separated into pods and nonfruiting tissues which were analyzed separately. Extraction consisted of homogenizing the tissue in a Waring Blendor with 100 ml. each of water and chloroform followed by centrifugation to separate the water, chloroform, and insoluble residue fractions. This residue was re-extracted with 100 ml. of acetone and after the solvent was stripped the acetone-soluble materials were added to the chloroform. Total radioactivity was determined for each fraction, including the residue which was first predigested in nitric acid to allow more efficient counting. Using the fractionation system just described, untreated pea samples to which 1 p.p.m. of dimethoate was added gave recovery rates of 85 and 93% for the pods and plants, respectively.

Other Methods. Other techniques not discussed in detail were the same as in a previous study on dimethoate (*5*).

Results and Discussion

Volatilization. Dimethoate was lost from the surface of treated cotton leaves at the rate indicated in Figure 1. Approximately 58% of the total dimethoate equivalents were lost during the first 10 days with no substantial loss thereafter. The rate of CL 18,706 loss from cotton leaves was similar to that of dimethoate (Figure 1), the major loss occurring during the first 10 days and amounting to 63% of that initially applied. Loss of total dimethoate equivalents from the surface of metal, plastic, rubber, and glass followed first-order kinetics. The number of days until one-half volatilization had occurred was 35, 39, 71, and 130, respectively, for the surfaces. With wood, Masonite, and Pressboard the rate of dimethoate volatilization was too low to be accurately measured.

The major loss recorded by this technique was probably by volatilization. However, with cotton leaves some loss also occurred from penetration into the leaf resulting both in self-absorption of the beta-radiation by the leaf tissue and partial translocation of the material away from the area assayed (*13*). Codistillation may have also been involved in the residue loss from plants.

Persistence and Absorption. Dimethoate residues in and on plant leaves were dissipated at the rate indicated in Table I, this loss resulting both from volatilization and increase in plant weight from growth. The total parts per million of dimethoate equivalents diminished most rapidly with corn.

As shown in Figure 2, solubilized dimethoate rapidly penetrated into the foliage of corn, cotton, and potato. From the data in Table I and Figure 2 it is possible to calculate the actual parts per million in each fraction of the leaf. For example, at 32 days after treatment of potato foliage there were 4.8 p.p.m. total dimethoate equivalents on the surface and 23.2 p.p.m. inside the potato leaf. As for the chloroform-soluble dimethoate derivatives, 0.1 p.p.m. was found on the surface in contrast to 0.46 p.p.m. inside the leaf at this time.

Similarly, on cotton and potato foliage dimethoate penetrated rapidly and the breakdown was much slower on the surface than inside the leaf (Figure 2). It seemed to be metabolized more slowly in cotton than potato, as at 8 days there were 18% as chloroform-solubles inside the cotton leaf but only 7% in potato. CL 18,706 also penetrated rapidly into

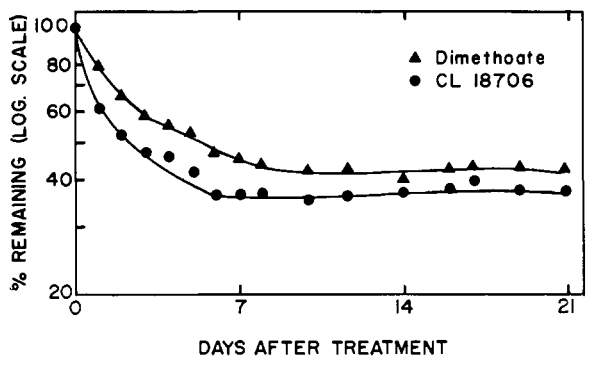

Figure 1. Per cent of total applied dimethoate and CL 18,706 equivalents persisting on and in cotton leaves

cotton foliage with slower degradation on the surface than inside the leaf (Figure 2). Comparing the two compounds at 32 days after treatment of cotton, the amount of chloroform-solubles was similar, but the per cent of the total applied equivalents on the surface was about 20% for dimethoate and 12% for CL 18,706, indicating a somewhat more rapid penetration of CL 18,706.

Of the plant species studied, the rate of penetration of dimethoate into the leaf was the slowest with corn (Figure 2). The rate of metabolism inside the corn leaf was much faster than with potato or cotton as shown by the rate of decrease of the chloroform-soluble materials, but the surface alteration was the slowest.

Chemical Changes in Dimethoate on Plant Surface. For evaluation of residue data it is essential to know the composition as well as the total level of residues. Column chromatography of the chloroform-soluble compounds on the surface 2 and 12 days after treatment showed that in addition to dimethoate a compound eluting with 100% chloroform was present. The first radioactive peak was characterized as dimethoate based on chromatography with known carrier compound and similar rates of hydrolysis of the radioactive component and the carrier in 0.1*M* aqueous sodium carbonate as ascertained by chloroform-water partitioning after various incubation times. The second peak was found to co-chromatograph with the known oxygen analog and not with the *S*-methyl isomer of dimethoate. Paper chromatography with 85 to 15 acetonitrile-water also showed co-chromatography of the second peak from the column with the known oxygen analog, both having an R_f value of 0.74. Therefore oxidation and not isomerization was the principal nonhydrolytic alteration in dimethoate on the surface. The per cent of oxygen analog (Table II) was similar for all the plant species with respect to the surface residues.

The amount of dimethoate, oxygen analog, and water-soluble derivatives on the surface 2 and 12 days after treatment of potato is shown in Table III. With the longer time interval there was a decrease in the total amount of oxygen analog on the surface but an increase in the proportionate amount with respect to dimethoate (Table II). The decrease in total dimethoate equivalents between 2 and 12 days is partially attributable to a loss from the surface by absorption, volatilization, and possibly co-distillation.

The water-soluble derivatives on the surface of corn, cotton, and potato were fractionated by ion exchange chromatography into four materials (Table IV). Dimethyl phosphoric acid, *O,O*-dimethyl phosphorothioic acid, and the des-methyl derivative of dimethoate were identified by co-chromatography with known carrier compounds. The fourth material was probably the oxy-carboxy derivative of dimethoate based on the following evidence: not extracted by chloroform at pH 7 but readily extracted at pH 1.3 suggesting that the material is a weak acid; co-chromatography on an ion exchange column with a minor product (7.6%) from the hydrolysis of the oxygen analog of dimethoate in 1*N* hydrochloric acid for 12 hours at 28° C.; and co-chromatography on an ion exchange column with a product formed in 26% yield by bubbling nitrogen tetroxide (N_2O_4) through a carbon tetrachloride

Table I. Persistence of Dimethoate and CL 18,706 or Their Metabolites on and in Plant Foliage

Days after Treatment	Dimethoate[a] Corn	Peas	Potato	Cotton	CL 18,706[a] Cotton
0	246 (195–298)[b]	129 (112–150)	791 (590–1040)	65 (55–72)	66 (58–75)
4	24 (9–46)	...	428 (300–500)	28 (24–32)	50 (40–65)
12	7.0 (5–10)	34 (32–40)	98 (75–180)	20 (16–22)	25 (19–31)
32	...	...	28 (16–39)	7.0 (5–10)	10 (9–12)

[a] Figures represent total parts per million of dimethoate or of CL 18,706 equivalents.
[b] Numbers in parenthesis give the range (four replicates).

Table II. Per Cent of Chloroform-Soluble Dimethoate Equivalents as Oxygen Analog of Dimethoate

(Remainder as dimethoate)

	Corn	Peas	Potato	Cotton
Surface				
2 days	0.9	...	0.9	0.8
12 days	5.4	4.8	6.3	6.8
Internal				
2 days	1.4	...	3.9	3.4
12 days	11.8	10.5	25.0	8.8

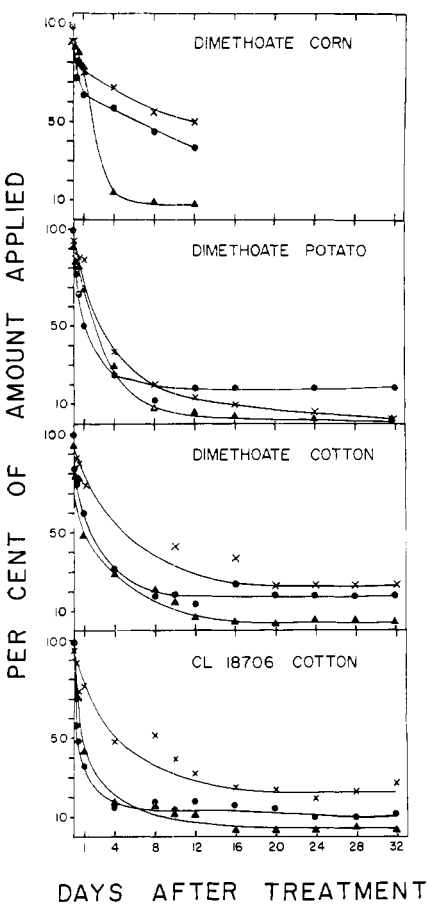

Figure 2. Persistence of dimethoate on or in leaves of cotton, potato, and corn and CL 18,706 on or in cotton foliage

● % total residue on surface
× % surface residue as chloroform-soluble dimethoate equivalents
▲ % absorbed residue as chloroform-soluble dimethoate equivalents

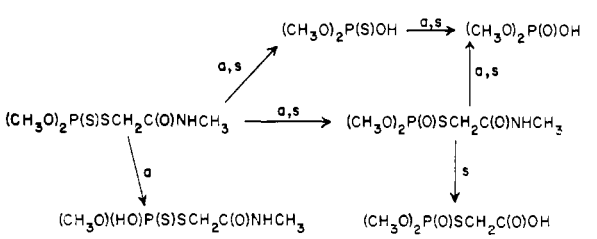

Figure 3. Proposed metabolic pathway for 0,0-dimethyl S-(N-methylcarbamoyl-methyl) phosphorodithioate applied to surface of corn, cotton, and potato

a. Reactions of absorbed residues, presumably enzymatic
s. Reactions of surface residues, presumably nonenzymatic
This decomposition pathway does not incorporate the large amount of phosphoric acid formed in peas

Table III. Nature of Residues from Dimethoate Applied to Potatoes

Days after Treatment	Surface			Internal		
	Dimethoate	Oxygen analog	H_2O sol.	Dimethoate	Oxygen analog	H_2O sol.
2	77.5	0.7	11.1	27.8	1.7	39.7
12	1.3	0.1	6.2	2.0	0.6	50.2

Results expressed as micrograms of total dimethoate equivalents per plant rather than as p.p.m. to minimize changes resulting from increase in plant weight due to growth.

Table IV. Composition of Water-Soluble Metabolites on Plant Surface 2 and 12 Days after Treatment

(Expressed as per cent)

	H_3PO_4	$(MeO)_2P(O)OH$	Oxy-Carboxy Derivative	$(MeO)_2P(S)OH$	Des-methyl Derivative
2 days					
Corn	0.0	3.2	77.4	9.0	10.5
Potato	0.0	0.7	93.8	2.7	2.6
Cotton	0.0	2.4	94.1	2.5	1.1
12 days					
Corn	0.0	6.3	87.5	2.7	3.5
Pea	51.7	7.6	8.8	11.3	20.7
Potato	0.0	4.4	81.0	6.0	8.6
Cotton	0.0	6.2	70.9	4.6	18.3

Table V. Composition of Water-Soluble Metabolites Inside Leaves 12 Days after Treatment

(Expressed as per cent)

	H_3PO_4	$(MeO)_2P(O)OH$	Oxy-Carboxy Derivative	$(MeO)_2P(S)OH$	Des-methyl Derivative
Corn	0.0	13.8	4.1	17.8	64.3
Pea	47.8	12.0	5.7	16.8	17.7
Potato	0.0	25.9	10.4	18.4	45.3
Cotton	0.0	12.8	5.4	12.6	69.2

Results for homogenates of leaves washed to remove surface metabolites.

solution of the thio-carboxy derivative of dimethoate at 0° to 10° C., a procedure which oxidized the thiono group based on infrared evidence. From large amounts of plant foliage 300 γ of this fourth radioactive material were recovered, but attempts to separate the compound from 2 mg. of nonradioactive impurities in order to obtain an interpretable infrared spectrum were unsuccessful. The major water-soluble derivative of dimethoate on the surface 2 and 12 days after treatment was the oxy-carboxy compound (Table IV). With these three plant species only dimethyl phosphoric acid definitely increased in proportion during the 2- to 12-day period.

On the leaf surface of mature pea plants five water-soluble derivatives were found, the major one being phosphoric acid (Table IV).

Stability of Dimethoate on Glass Surfaces. The extensive hydrolysis and partial oxidation of dimethoate on the leaf surfaces might result from a general surface phenomenon or might be unique for the plant surface due to catalytic or reactive materials from the leaf or by excretion onto the surface of materials which had been produced by metabolism inside the leaf. To differentiate these mechanisms, dimethoate was added to glass surfaces, and to glass surfaces treated with a surface wash from potato leaves (see Methods). Some degradation occurred in both cases in 7 days. The plain surfaces had 11% of the total as hydrolysis products and 1.5% of the chloroform-solubles as oxygen analog. The corresponding figures for the treated surface were 19 and 1.1%. The reactions on the plant leaf are therefore largely attributable to nonspecific effects, although the possibility remains that hydrolysis is catalyzed by a component of the leaf surface. The variety of dimethoate metabolites which appear on the leaf surface probably results, at least in part, from the excretion onto the surface of metabolites formed within the leaf.

Metabolism of Dimethoate Absorbed by Leaves. Chromatography of the chloroform-soluble compounds inside the leaf at 2 and 12 days after treatment gave two radioactive peaks which were identified as dimethoate and its oxygen analog by chromatography with known carrier compounds. Based on the per cent of oxygen analog formed inside the leaf (Table II), the potato seems to have the best ability to oxidize dimethoate.

From these findings it was possible to calculate the amount of dimethoate, oxygen analog, and water-soluble metabolites inside the potato leaf 2 and 12 days after treatment (Table III). There was a decrease with time in the total amount of oxygen analog inside the leaf, but a proportionate increase with respect to dimethoate (Table II). The decrease in total micrograms of dimethoate equivalents between 2 and 12 days presumably reflects some form of redistribution, volatilization and/or excretion. The data on the chloroform-soluble materials show that it is essential for a residue analysis method to determine both dimethoate and the oxygen analog, although it may not be necessary to distinguish between them.

Analysis of the water-soluble metabolites in the leaves of corn, cotton, and potato showed the same four materials which were found for the surface wash but in different proportions (Table V). Whereas on the leaf surface the oxy-carboxy compound predominated, inside the leaf the des-methyl metabolite was found in the greatest amount. In potato there was a larger percentage of dimethyl phosphoric acid and a lesser percentage of des-methyl derivative than in cotton and corn. The major water-soluble metabolite found in mature pea leaves was phosphoric acid and the other derivatives were present in about the same proportions on the surface as within the leaf. The contribution of *O*-methyl *O* - hydrogen *S* - (*N* - methylcarbamoylmethyl) phosphorothiolate, the des-methyl derivative of the dimethoate oxygen analog, to these results is not known, as the pure compound was not available to determine its position of elution.

Dimethoate Residues in Peas. The chloroform-soluble dimethoate equivalents from pea plants treated in the flowering stage dropped from 25 p.p.m. initial over-all residue to 0.3 p.p.m. for the pea pods and 0.5 p.p.m. for the remaining plant tissue by harvest at 17 days. Water-soluble dimethoate equivalents comprised 1.8 p.p.m. of the pods and 6.7 p.p.m. of the remaining plant tissue, while the unextractable residue fraction contained 1.1 p.p.m. dimethoate equivalents for the pods and 1.4 p.p.m. for the other tissues.

Conclusions

The formation of the oxygen analog of dimethoate on the surface of leaves is probably a nonenzymatic oxidation as it can be duplicated when dimethoate is exposed to the atmosphere in a thin layer on nonbiological surfaces. However, the high levels of oxygen analog internally suggest that enzymatic oxidation also occurs. This is also suggested by the observation that oxygen analog is formed within the plant following systemic treatment via the roots (*12, 13*).

The marked quantitative difference found in the levels of the different water-soluble derivatives on the surface and in the leaves of young corn, cotton, and potato plants suggests quite different mechanisms of degradation. As no *O,O*-dimethyl phosphorodithioic acid was found in any case, both enzymatic and nonenzymatic attack on the P—S—C bond probably occurs at the P—S rather than at the S—C linkage. Alternate explanations for the absence of this dithioic acid would be that it was rapidly

oxidized after formation or that the hydrolysis of the P—S—C bond in dimethoate always follows a preliminary phosphorothionate oxidation.

Hydrolysis at the alkoxy group occurred mainly inside leaves of corn, cotton, and potato, indicating an enzymatic action at this site. Degradation at the alkoxy group has been reported for mammals (*5, 7, 9, 10*), but this is the first similar observation in plants.

Hydrolysis in and on the surface of mature pea plants was greatly different from the other plants in that phosphoric acid was the major derivative formed in peas but was absent in corn, cotton, and potatoes. The explanation of this striking difference with peas is not known.

When dimethoate was administered to mammals, the major metabolite excreted was the thio-carboxy derivative (*5*). As none of this derivative was found in or on plants, the formation of the oxy-carboxy derivative probably proceeded by oxidation of dimethoate to its oxygen analog first and then by hydrolysis of the carbamoyl group. As the oxy-carboxy derivative was found in largest amount on the surface, the carbamoyl bond cleavage was probably nonenzymatic and it is unnecessary to postulate an enzyme hydrolyzing the C—N bond of this compound in plants.

In a previous study on dimethoate metabolism in mammals (*5*), no oxygen analog was isolated although there was indirect evidence for its formation. The plant studies reported here were made with a phosphorus-32 dimethoate sample of five times greater specific activity, which may partially account for the ability to detect the oxygen analog in plants but not mammals. An unidentified dimethoate metabolite reported in the mammalian study and designated as "unknown A" (*5*) has the same properties as the oxy-carboxy derivative found in plants.

The pathway of dimethoate degradation in plants is summarized in Figure 3.

Acknowledgment

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PESTICIDE RESIDUES

Rapid Combustion and Determination of Residues of Chlorinated Pesticides Using a Modified Schöniger Method

DONALD J. LISK

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CHLORINE has been removed from chlorinated pesticides prior to their determination as residues, by combustion and sodium reduction. Schöniger (*2, 3*) used an oxygen-filled flask to burn samples in a rapid combustion method for the microdetermination of halogens and sulfur in organic compounds. In the work reported, an oxygen-filled flask with a balloon attached for pressure control is used for combustion of residues of chlorinated pesticides.

A benzene extract of alfalfa containing the pesticide is evaporated in a cone of cellulose acetate. The cone and contents are burned in the flask and the hydrogen chloride gas is absorbed in dilute sodium hydroxide. A specially designed platinum holder permits complete combustion of the residue with no carbon formation. Chloride is determined spectrophotometrically by an adaptation of the method of Bergmann and Sanik (*1*) involving displacement of thiocyanate by chloride in the presence of ferric ion.

Description of Combustion Flask

The combustion flask and platinum holder are illustrated in Figure 1 (*A* and *B*).

A 1-liter borosilicate glass Erlenmeyer flask with a 34/28 standard-taper, female ground joint sealed on the neck is used. A side arm (1.5 cm in outside diameter, 7 cm. long) is sealed to the flask at the base of the shoulder as shown. A rubber balloon about 7 cm. long is secured to the side arm with string. A small rubber band is placed on the arm as shown.

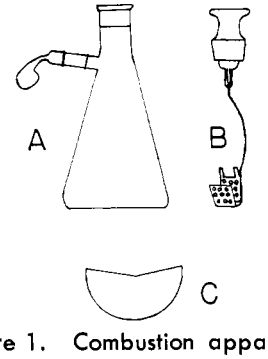

Figure 1. Combustion apparatus
A. Flask
B. Platinum holder
C. Plastic cone template

The platinum holder is constructed by sealing a 13-cm. length of No. 16 B and S gage platinum wire onto a 34/28

oxidized after formation or that the hydrolysis of the P—S—C bond in dimethoate always follows a preliminary phosphorothionate oxidation.

Hydrolysis at the alkoxy group occurred mainly inside leaves of corn, cotton, and potato, indicating an enzymatic action at this site. Degradation at the alkoxy group has been reported for mammals (*5, 7, 9, 10*), but this is the first similar observation in plants.

Hydrolysis in and on the surface of mature pea plants was greatly different from the other plants in that phosphoric acid was the major derivative formed in peas but was absent in corn, cotton, and potatoes. The explanation of this striking difference with peas is not known.

When dimethoate was administered to mammals, the major metabolite excreted was the thio-carboxy derivative (*5*). As none of this derivative was found in or on plants, the formation of the oxy-carboxy derivative probably proceeded by oxidation of dimethoate to its oxygen analog first and then by hydrolysis of the carbamoyl group. As the oxy-carboxy derivative was found in largest amount on the surface, the carbamoyl bond cleavage was probably nonenzymatic and it is unnecessary to postulate an enzyme hydrolyzing the C—N bond of this compound in plants.

In a previous study on dimethoate metabolism in mammals (*5*), no oxygen analog was isolated although there was indirect evidence for its formation. The plant studies reported here were made with a phosphorus-32 dimethoate sample of five times greater specific activity, which may partially account for the ability to detect the oxygen analog in plants but not mammals. An unidentified dimethoate metabolite reported in the mammalian study and designated as "unknown A" (*5*) has the same properties as the oxy-carboxy derivative found in plants.

The pathway of dimethoate degradation in plants is summarized in Figure 3.

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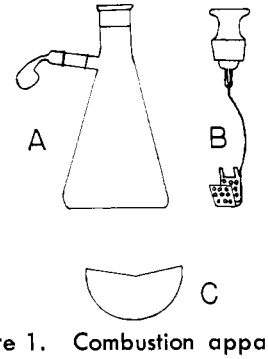

Figure 1. Combustion apparatus
A. Flask
B. Platinum holder
C. Plastic cone template

The platinum holder is constructed by sealing a 13-cm. length of No. 16 B and S gage platinum wire onto a 34/28

A method is described for the rapid combustion and determination of residues of chlorinated pesticides. Organic solvent extracts of plant samples are evaporated in a cone of cellulose acetate, which is burned in an oxygen-filled (Schöniger) flask and the hydrogen chloride gas is absorbed in dilute sodium hydroxide. The platinum holder has been specially designed and a balloon attached to the flask to permit complete combustion of the cone and its contents safely. The spectrophotometric determination of chloride is based on its displacement of thiocyanate in the presence of ferric ion. The time required for combustion and determination of chloride is about 20 minutes per sample. The recovery of DDT, Thiodan, and lindane from alfalfa by the method is described.

standard-taper, male ground joint which has been drawn out 4 cm. below the ground portion. At the lower end, 2 cm. of wire are bent to form a right angle. A piece of Style 4, perforated platinum sheet (J. Bishop and Co. Platinum Works, Malvern, Pa.), 2 cm. wide and 4 cm. long, and bent as shown is electrically welded to the wire at the lower end. The two platinum tabs (4 by 12 mm.) may be welded on or cut out as part of the sheet.

Preparation of Plastic Cones

Prepare the cones from S-600, 1-mil cellulose acetate film (Celanese Corp. of America). Wearing cloth gloves, cut the plastic using a cardboard template having the shape (4-cm. radius) shown in Figure 1, *C*. Wash each piece of plastic by dipping for 10 seconds (use forceps) in 6*N* sodium hydroxide warmed to about 60° C. in a 1-liter beaker. Rinse thoroughly in running distilled water and hang in a spring clamp, allowing excess water to drip off. When most of the water has drained, remove the plastic from the clamp using clean forceps and place on a clean sheet of aluminum foil. Remove the plastic from the foil and manually join the two edges to form a cone. Wear rubber or polyethylene gloves which have been cleaned by immersing in warm 6*N* sodium hydroxide, rinsed, and hung to dry. Heat-seal the joined edges to form a seam about 5 mm. wide. Immerse the seam for 10 seconds to about one half its width in acetone contained in a watch glass. Remove and immediately dry in the warm air stream of a hair dryer. Place each cone in a clean 65-mm., 60° funnel.

Table I. Recovery of DDT, Thiodan, and Lindane from Alfalfa

Added, P.P.M.	DDT Found, P.P.M.	Recovery, %
1.74	1.67	96.0
3.49	3.80	109.0
6.98	6.53	93.6
6.98	6.11	87.6
6.98	6.67	95.6
13.96	14.21	101.8
13.96	12.95	92.8
	Thiodan	
1.74	1.43	82.0
1.74	1.09	62.8
1.74	1.47	84.7
	Lindane	
1.74	1.47	84.2
1.74	1.66	95.5
1.74	1.66	95.5

Combustion and Determination of Residues

Procedure for Combustion and Determination of Chloride. Pipet 5 ml. of the solvent extract of the sample into the cone. Evaporate the solvent using a warm air stream. Wearing rubber gloves, remove the cone and its dry contents from the funnel. Fold the cone over and up to form a small packet about 1 sq. cm. in area. Compress and place in the center of the platinum holder, which is supported from a ring stand. Fold the tabs over to hold the packet tightly between the metal surfaces. Insert a fuse (3 mm. wide and 5 cm. long, cut from Whatman No. 42 filter paper) into the top of the holder between the folds of the packet. Pipet 30 ml. of 0.08*N* sodium hydroxide into the combustion flask. Thoroughly purge the flask with oxygen. Light the fuse and place the holder in the flask immediately after about one half of the fuse has burned away. Hold the top on until combustion is complete. Keep the flask upright throughout the combustion. As soon as the balloon deflates, fasten the end of it to the side arm with the rubber band. This prevents the balloon from being drawn into the flask by the partial vacuum created. Shake the flask vigorously for 3 minutes. Remove the holder and slide the rubber band back off the balloon. Invert the flask several times so that the balloon is rinsed through the side arm by the solution in the flask.

Pipet a 15-ml. aliquot of the solution into a 50-ml. Erlenmeyer flask. Add 5 ml. of a 0.25*M* ferric ammonium sulfate solution in 9*M* nitric acid, followed by 5 ml. of a saturated solution of mercuric thiocyanate in absolute ethyl alcohol. Mix the solution and allow it to stand for 10 minutes. Using a Beckman DU spectrophotometer, measure the absorbance of the solution at 460 mμ in a 2-cm. cell with distilled water in the reference call. To minimize chloride absorption from the air, make the measurement promptly (*1*).

The calibration curve (0 to 100 γ of chloride) is prepared as follows:

Pipet 0, 2, 4, 6, 8, and 10 ml. of a standard sodium chloride solution (10 γ of chloride per ml.) into a series of 50-ml. Erlenmeyer flasks. Make up to 15 ml. with distilled water and add 5 ml. of each of the above reagents. Determine the absorbance as described above.

Results and Discussion

The method was used to recover DDT, Thiodan, and lindane from alfalfa. Samples of alfalfa to which the insecticides were added in benzene were extracted by tumbling with benzene for 20 minutes (2 ml. of benzene per gram of crop). The benzene strip solutions were filtered (Whatman No. 1) and evaporated. A 5-ml. aliquot of each sample was placed in a plastic cone and evaporated. The recoveries obtained after combustion and evaporation of chloride are given in Table I.

In those samples fortified at 1.74 p.p.m. plant extractives representing a 28.64-gram sample were burned in each cone. In those fortified at all higher levels, extractives were burned representing 14.32 grams of alfalfa. The check value of the alfalfa was about 0.60 p.p.m. as chloride.

The cone weighed about 85 mg. and the cone plus contents of the 28.64-gram sample weighed about 150 mg. The flask and platinum holder effected complete combustion of the sample with no carbon formation. Cellulose acetate burns more vigorously than paper (*2, 3*) and the balloon was used to allow for expansion of gases. About 200 combustions have been safely conducted in the flask. Carbon forms if the flame touches glass or the platinum wire. Therefore, the platinum holder was positioned low in the flask and the wire bent so that the flame did not contact cool surfaces.

Also, with the flask remaining upright during a combustion, the platinum holder formed a bottom beneath the sample. The last fragment of the sample burned away upon it rather than falling into the solution. The perforations in the holder were adequate for admitting oxygen and still allowing for sufficient contact between the metal and sample. If the sample was not compressed in the platinum holder, a flame large enough to contact the glass or metal resulted and carbon was formed. Such samples were discarded. Normally after absorption of gases, the solution in the flask (without color reagents) showed an absorbance of 0.005 to 0.02 in a 2-cm. cell with distilled water as the reference.

The burning characteristics, solvent resistance, sealability, and chlorine content of about 20 plastic films were investigated. Cellulose acetate (S-600) was the most satisfactory. It is resistant to the solvents commonly used for extraction of residues with the exception of acetone which dissolves it. The blank value of the cone and fuse was about 11 γ as chloride. The cones did not leak when made according to the procedure. Heat-sealing was necessary to form a tight seam in order that the solvent seal would be leak-proof. The capacity of the cone is 5 ml.

The flask, platinum holder, and balloon were rinsed thoroughly with distilled water after each combustion. The balloon was replaced after about 25 combustions. The standard curve follows Beer's law as long as the mercuric thiocyanate solution remains saturated. This solution is best prepared by heating, with agitation, an excess of powdered mercuric thiocyanate in absolute ethyl alcohol and allowing it to settle for 48 hours before use. The clear solution and solid mercuric thiocyanate should be mixed, heated, and allowed to settle once every 2 weeks during use. The method should be applicable to the determination of residues of other chlorinated pesticides.

Acknowledgment

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HERBICIDE RESIDUES

Determination of Small Amounts of Arsenic in Potatoes. Extraction and Reduction of Molybdoarsenic Acid

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A method is presented for the determination of small amounts of arsenic in potatoes. Potato tissue is ashed with magnesium nitrate and the ash dissolved in acid. Acid-molybdate is added, followed by extraction with a 1-butanol–chloroform mixture to remove interfering phosphorus as molybdophosphoric acid. The aqueous solution is extracted with 1-butanol to remove arsenic as molybdoarsenic acid. A simple procedure is then used for adjusting acidity and reducing to the heteropoly blue with stannous chloride in ethyl alcohol. The effect of varying the concentration of acid and reducing agent on the blue color is shown. Silicon causes some interference. Silicon interference can be reduced by use of a 1-butanol–ethyl acetate mixture for extraction of molybdoarsenic acid. The method, used to determine possible traces of arsenic in potatoes resulting from the application of sodium arsenite to kill potato vines and weeds, yields an average recovery of 94.1%.

A MODIFICATION of the procedure of Akkseev (*1*) is described by Wadelin and Mellon (*9*) for the removal of phosphorus prior to determining arsenic. Sodium molybdate in hydrochloric acid is added to a solution containing phosphate and arsenate ions to form the respective molybdic acids. Successive extractions of the solution with a 1-butanol–chloroform mixture remove interfering phosphorus as molybdophosphoric acid. Arsenic is then removed as molybdoarsenic acid by extraction with butanol. It is determined by ultraviolet absorptiometric measurement of 12-molybdoarsenic acid. The method is not quite sensitive enough for determining traces of arsenic in biological material.

In this paper, the method of Wadelin and Mellon is used to separate phosphorus and arsenic contained in an acid solution of potato tissue–magnesium oxide ash. A sulfuric acid–ethyl alcohol solution is then added to the 1-butanol followed by a solution of stannous chloride in ethyl alcohol which reduces molybdoarsenic acid to the heteropoly blue. Spectrophotometric measurement is then made of the blue color at 740 mμ.

Method

Preparation of Standard Curve. (0 to 50 γ of arsenic.) Pipet 0-, 2-, 4-, 6-,

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Method

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Table I. Effect of Phosphorus and Silicon on Recovery of Arsenic from Synthetic Solutions

Added, γ As	P	Si	Apparent as Found
1-Butanol–Chloroform Extraction of Phosphorus. 1-Butanol Extraction of Arsenic			
0	5000		1.3
15	5000		17.1
30	5000		31.0
1-Butanol–Chloroform Extraction of Phosphorus. 1-Butanol Extraction of Arsenic			
0	1000	50	1.0
15	1000	50	17.5
30	1000	50	33.5
1-Butanol–Chloroform Extraction of Phosphorus. 1-Butanol–Ethyl Acetate Extraction of Arsenic			
0	1000	50	0.6
15	1000	50	17.7
30	1000	50	31.6
1-Butanol–Chloroform Extraction of Phosphorus. 1-Butanol Extraction of Arsenic			
0	5000	450	8.2
15	5000	450	21.0
30	5000	450	37.3
1-Butanol–Chloroform Extraction of Phosphorus. 1-Butanol–Ethyl Acetate Extraction of Arsenic			
0	5000	450	5.5
15	5000	450	18.3
30	5000	450	35.5

8-, and 10-ml. aliquots of a solution containing 5 γ of arsenic per ml. [prepared by dilution of an arsenic stock solution (*2*)] into a series of 250-ml. Squibb separatory funnels. Add water to make up the total volume to 50 ml. Treat each sample separately from this point through color development. Add 25 ml. of a 2*N* hydrochloric acid solution containing 0.03 gram of sodium molybdate per ml. Pour into the funnel 50 ml. of a 1 and 3 by volume mixture of 1-butanol–chloroform. Shake for 30 seconds and allow the layers to separate. Discard the lower layer and repeat the extraction with 1-butanol–chloroform three times more, discarding the lower layer each time. Add exactly 20 ml. of 1-butanol (117° to 118.5° C.) to the aqueous solution and shake for 30 seconds. After the layers have separated discard the lower aqueous layer and drain the 1-butanol layer into a 50-ml. Erlenmeyer flask. Add exactly 2 ml. of a 12*N* sulfuric acid solution in 95% ethyl alcohol (U. S. Industrial grade) and mix. Add 0.5 ml. of a solution containing 2 mg. of stannous chloride dihydrate per ml. of ethyl alcohol. Stopper and mix. After 15 minutes, measure the absorbance at 740 mμ using a 1-cm. cell with 1-butanol in the reference cell.

Analysis of Potatoes. The method of Evans and Bandemer (*5*) is used for the ignition of potato tissue with magnesium nitrate. Remove soil, etc., from the surface of the tubers which may contain arsenic. Thoroughly blend a representative sample of potatoes in their own liquid. Fold a disk of 12.5-cm. diameter vegetable parchment paper as for filtering. The second folding should, however, be made so that the resulting cone will just fit inside the crucible and not tip over. Fold the tip of the cone back and fit the truncated cone into a No. 4 Coors porcelain crucible. Then fold a similar disk of No. 40 Whatman filter paper and fit it inside the parchment disk. Weigh into the filter paper 15 grams of potato tissue from the blender. Blend sufficient tissue so that the sample may be taken from the top of the mix, while the blender is operating. This prevents the separation of solid and liquid in the sample. Add 6 ml. of saturated magnesium nitrate solution and mix well with the tissue using a thin stirring rod. For recovery studies, an appropriate aliquot of the arsenic standard solution is also added at this point and mixed in. Rinse any tissue remaining on the stirring rod back into the filter paper with a few drops of water. Evaporate to dryness on a steam bath. Ignite in a muffle furnace by placing in the cold furnace, slowly raising the temperature to 600° C., and heating overnight at this temperature. A light, white ash is obtained.

Wet the ash with water and add sufficient 4*N* hydrochloric acid solution to just dissolve it. By adding the acid dropwise with intermittent stirring, about 12 ml. are required. Transfer the solution quantitatively to a 50-ml. volumetric flask. Adjust the pH of the solution to between 5 and 9 with 1 *M* sodium hydroxide. Make up to volume with water and mix. Pipet a 25-ml. aliquot of this solution into a 250-ml. Squibb separatory funnel and add 25 ml. of water. Proceed as in the preparation of the standard curve.

Results and Discussion

A Model 1740 Temco muffle furnace equipped with a Wheelco Model 404 Capacitrol indicating temperature controller was used in the ignition procedure. The temperature was slowly raised to 600° C. over a 4- to 5-hour period and the crucibles were cooled down slowly after ignition.

If the procedure of Wadelin and Mellon is used, the aliquot of ash solution cannot contain more than 2 mg. of phosphorus. In the above procedure the volumes of reagents are increased by a factor of 2.5 in order that a larger portion, 25 ml., of sample solution can be taken. The upper limit for phosphorus which can be removed is, therefore, 5 mg. An absorbance of 0.123 was obtained when the extraction of 6 mg. of phosphorus was attempted in the absence of arsenic. Wooster (*10*) gives the approximate phosphorus content of raw, white potatoes as 56 mg. per 100 grams. A 25-ml. aliquot of the ash solution therefore represents 7.5 grams of potato or about 4.2 mg. of phosphorus.

Four extractions with 1-butanol–chloroform are necessary to remove virtually all of the phosphorus. An absorbance of 0.016 (Table I) was obtained when a solution containing 5 mg. of phosphorus was extracted four times with 1-butanol–chloroform. The absorbance was 0.041 when only three extractions were used. Three extractions are sufficient if larger quantities of arsenic are determined as in the Wadelin and Mellon procedure. The effect of reagent grade in place of technical chloroform is only that of reducing the amount of interfacial cuff during the extraction of phosphorus.

The sulfuric acid–ethyl alcohol mixture dissolves water droplets giving a clear blue solution. The use of a small volume of sulfuric acid–ethyl alcohol and stannous chloride dissolved in ethyl alcohol is an adaptation of the procedure of Clark (*4*) and Lindsay (*7*) for determining phosphorus in soil solutions after extraction of molybdophosphoric acid with a benzene-isobutyl alcohol solution (*8*). The stannous chloride solution in ethyl alcohol should be prepared fresh daily. The sodium molybdate–hydrochloric acid solution should be prepared fresh monthly.

A Beckman DU spectrophotometer with a slit width setting of 0.03 mm. was used to measure absorbance. Color development is not complete until 12 minutes after the addition of the stannous chloride solution. The blue color is stable for at least 24 hours. Certain lots of 95% ethyl alcohol cause color instability in the determination of phosphorus (*6*). The U. S. Industrial grade did not cause color instability in the determination of arsenic.

The relation between absorbance in the presence and absence of arsenic and

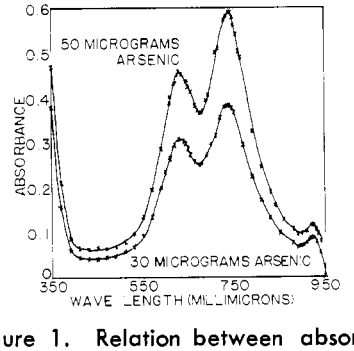

Figure 1. Relation between absorbance of heteropoly blue color and concentration of sulfuric acid in butanol

the concentration of sulfuric acid in the 1-butanol solution is shown in Figure 1. In the presence of arsenic the absorbance is constant when the concentration of sulfuric acid is between 0.2 and 0.8*M*. Higher concentrations cause a decrease in absorbance. The results of Berenblum and Chain (*3*) with phosphorus in aqueous solution are somewhat similar. The concentration of sulfuric acid in 1-butanol in this procedure is 0.54*M*. The absorbance of the control is very small at this acidity.

The absorbance of the blue color was measured and is constant when the 1-butanol solution is between 0.05 and 2.0 m*M* with respect to stannous ion. Between 0.4 and 2.0m*M*, a green color first forms which soon turns blue. The concentration of stannous ion in this procedure is 0.2m*M*.

The absorption spectrum of the heteropoly blue color at two concentrations of arsenic is shown in Figure 2. Maximum occurs at 740 mμ. The standard curve is very reproducible. The method will detect about 1 γ of arsenic using a 1-cm. cell.

Lampitt and Goldenberg (*6*) found the silicon content of potatoes to range between 7.7 and 58.7 p.p.m. Therefore, it is possible to have from about 60 to 440 γ of silicon present in 7.5 grams of potato as represented in this procedure. The method was used to recover arsenic in the presence of phosphorus and silicon in synthetic solutions. The elements were contained in 50 ml. of solution. Phosphorus was present as potassium dihydrogen phosphate and silicon as sodium metasilicate. The amount of silicon added approximately represents the limits found by Lampitt and Goldenberg (*6*). Table I shows the amount of arsenic not corrected for the blank that was recovered in each study.

The presence of 5 mg. of phosphorus causes a small increase in absorbance. The interference by silicon is more serious especially at the 450-γ level. It may be minimized by proceeding through color development with each sample as rapidly as possible after addition of the molybdate reagent (*9*). Use of a 1 and 1 by volume mixture of 1-butanol and ethyl acetate (*7*) in place of 1-butanol for extraction of molybdoarsenic acid is more selective in the presence of silicon (Table I). The absorption spectrum and standard curve of the heteropoly blue color in 1-butanol–ethyl acetate are identical with those in 1-butanol. The same solvent mixture (1-butanol–chloroform) is always used for removal of phosphorus. The recovery of arsenic (Table II) is just as satisfactory when using 1-butanol as compared to 1-butanol–ethyl acetate. There are crops (certain grasses, for example, which contain large amounts of silica) for which the 1-butanol–ethyl acetate extractant might be much more advantageous than with potatoes. For plant materials containing greater than about 8 p.p.m. of silicon, the 1-butanol–ethyl acetate mixture should therefore be used.

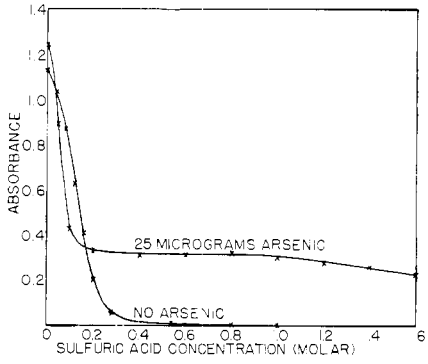

Figure 2. Absorption spectra of heteropoly blue color

The recovery of arsenic added to potato tissue is shown in Table II. Results using 1-butanol and 1-butanol–ethyl acetate are given. The average recovery using 1-butanol extraction is 94.1%. The apparent arsenic content of the potatoes averaged slightly above 1 p.p.m. This accounted, in part, for greater variability in the recovery of arsenic in quantities below 2 p.p.m.

Sodium arsenite is used to kill potato vines and weeds in potatoes. The method described has been used to determine possible traces of arsenic in potatoes resulting from treatment with sodium arsenite just before harvest. About 15 potatoes treated with sodium arsenite were blended in their own liquid. Twelve samples of the blended mixture were taken for the determination of arsenic using 1-butanol extraction. The average content of arsenic found was 1.11 p.p.m. with a range of 0.86 to 1.40. The average content of arsenic found in seven analyses of untreated potatoes was 1.24 p.p.m. with a range of 0.93 to 1.34. There was no significant difference in the arsenic found in the treated and untreated potatoes. The standard deviation of a single determination is 0.18%. The untreated potatoes may have contained arsenic, because the soil in which they were grown had received about five applications of sodium arsenite in the previous 8 years for potato vine and weed control. Also, many soils contain small amounts of arsenic. Interference by silicon contained in the potatoes probably accounted for part of the apparent arsenic found in the untreated potatoes. The method should be applicable to the determination of arsenic in other biological materials.

Table II. Recovery of Arsenic from Potatoes

Added, P.P.M.	Found, P.P.M.	Recovery, %
1-Butanol Extraction		
1.00	1.05	105.3
1.00	0.68	68.0
1.33	0.93	69.5
1.33	1.55	116.0
1.33	0.93	70.0
1.33	1.17	87.5
1.33	1.53	115.0
2.00	1.95	97.7
2.00	2.17	108.3
2.00	1.87	93.3
2.00	2.27	113.3
2.00	1.60	80.0
2.00	1.56	78.0
2.00	2.03	101.7
2.00	1.70	85.0
2.00	1.69	84.7
2.00	2.08	104.0
2.00	1.68	84.0
2.00	1.93	96.7
2.00	1.99	99.3
8.00[a]	8.59	107.3
8.00[a]	8.09	101.2
8.00[a]	7.82	97.8
1-Butanol–Ethyl Acetate Extraction		
1.00	0.76	76.0
1.33	1.41	106.0
1.33	1.31	98.0

[a] A 10-ml. aliquot of ash solution was analyzed.

Acknowledgment

This experiment was done in cooperation with Robert D. Sweet of the Department of Vegetable Crops, New York State College of Agriculture, Ithaca, N. Y. The author thanks Gregor Melnyk and V. N. Krukovsky for translating the Russian publication of Akkseev.

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Synthesis of Carbon-14–Labeled Dalapon and Trial Applications to Soybean and Corn Plants

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Sodium 2,2-dichloropropionate-2-C^{14} (sodium dalapon) at 1 mc. per mmole has been prepared by direct chlorination of propionic acid. Separation from other chlorinated acids was achieved by gradient elution from a phosphate-buffered silica gel column. A yield of 32 mole % based on the propionic acid was obtained. Radiochemical purity was 96%. This material was applied to corn and soybean plants. The distribution of radioactivity was measured. Radioactivity recovered by extractions was identified as dalapon by paper chromatography. No breakdown products were found.

DALAPON (2,2-DICHLOROPROPIONIC ACID) is a useful herbicide for the selective control of grasses. It is therefore of considerable importance to study the residues in crop plants and to learn something about the uptake, distribution, and stability of the compound in various plants. In order to provide a radiotracer for such studies sodium dalapon-2-C^{14} has been prepared. Brief tracer studies with corn and soybean plants are included in this paper.

Synthesis

The measurement of potentially small residues and even smaller amounts of possible breakdown products necessitated a specific activity of 1 mc. per mmole. This required a microscale synthesis.

Two procedures were investigated. In the first of these pyruvamide was hydrolyzed (*5*, *6*) in an acid medium and the pyruvic acid recovered by continuous ether extraction. After ether was removed, the pyruvic acid was treated with phosphorus pentachloride (*12*). Under optimum reaction conditions the maximum yield of dalapon was 20%. By-products included unreacted pyruvic acid, acetic acid, 2-chloroacrylic acid, and 2,2,3-trichloropropionic acid.

A second method involving the direct chlorination of propionic acid was investigated and was used in preparing the tracer material. The procedure is an adaptation of a microscale method described by Bass (*1*), Bass and Burlew (*2*), and Brust and Senkbeil (*3*).

Chlorination. The chlorination is carried out in two steps. One mole of chlorine reacts at 121° C. to yield principally 2-chloropropionic acid. The second mole of chlorine is introduced at 173° C. to yield predominantly 2,2-dichloropropionic acid. Phosphorus trichloride is employed as a catalyst.

Special equipment (Figure 1) had to be designed to maintain a flow of a small volume of chlorine gas through an exceedingly small quantity of liquid at controlled, elevated temperatures.

Chlorine (Dow) was forced under a head of 85% phosphoric acid from a graduated reservoir, through a drying tube containing phosphoric acid anhydride, into a reactor through a small capillary supply tube. The reactor was fabricated from glass tubing (2-mm. inside diameter), with a slight enlargement at the closed end. The chlorine supply tube was made from a 1-mm. bore capillary tube by drawing it down to a terminal cross section of about 50 microns inside diameter. Proper reaction temperatures were maintained by refluxing solvents of suitable boiling points. A dry ice trap was employed to catch volatile by-products, while a calcium chloride tube protected the system from atmospheric moisture.

The optimum conditions of chlorine supply, catalyst, time, and temperature of reaction were determined by analysis of the products obtained from 25 cold runs. The manner in which the phosphorus trichloride was supplied to the reactor was very important. When it was distilled into the reactor by vacuum transfer, frequent stoppages of the chlorine flow occurred from plugging of the capillary tube with resinous by-products. When the phosphorus trichloride was added as two or three drops from a micropipet with attendant exposure to atmospheric moisture, satisfactory flow was obtained throughout the chlorination.

The variation in composition of the crude reaction product as a function of chlorination time is shown in Figure 2. The reaction was not stepwise as at no time was there a single product found.

The composition of these crude chlorination products was determined by infrared analysis. A typical spectrum scanned on a double-beam infrared-spectrometer with sodium chloride optics is shown in Figure 3. This particular sample was prepared by dissolving 89 mg. of reaction product in 1 ml. of carbon disulfide. It was scanned in a 0.1-mm. thick cell. Absorption bands for 2,2-dichloropropionic acid (DCP), 2,2,3-trichloropropionic acid (TCP), 2-chloropropionic acid (MCP), and several unidentified materials (x) can be seen. Absorptions at 4.34 and 4.64 microns and in the 6-micron region are due to the solvent.

Purification. Isolation of 2,2-dichloropropionic acid from the mixture of acidic materials in the crude reaction product was achieved with a phosphate-buffered silica gel chromatographic column. The column was prepared as follows: To 60 grams of silica gel (Davison) were added 38 ml. of a saturated aqueous solution of sodium dihydrogen orthophosphate adjusted to a pH of 4. (A lower pH gives a faster column, but somewhat poorer resolution.) The resulting mixture was stirred well; then chloroform was added. Two-milliliter portions of this suspension were added successively to a 100-ml. buret filled with chloroform. As the silica gel settled, it was packed with a glass rod enlarged at the end to nearly the diameter of the buret. After the silica gel was added, the column was rinsed with 200 ml. of water-washed chloroform.

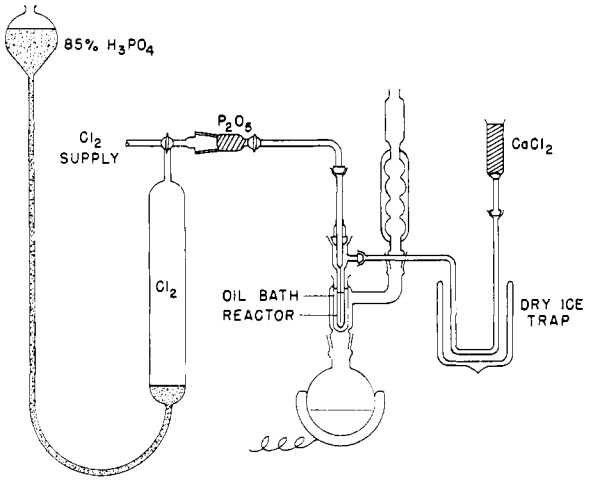

Figure 1. Propionic acid chlorination apparatus

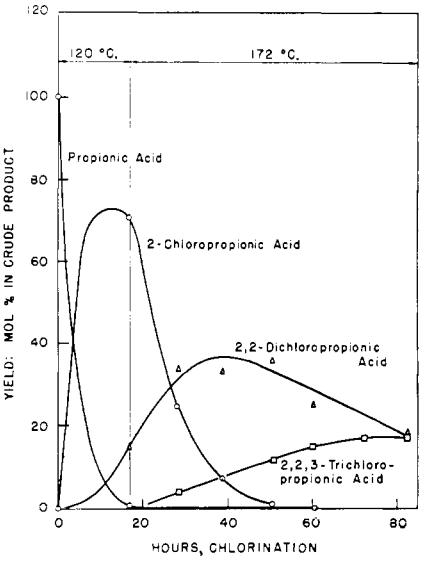

Figure 2. Composition of reaction products obtained during chlorination of propionic acid

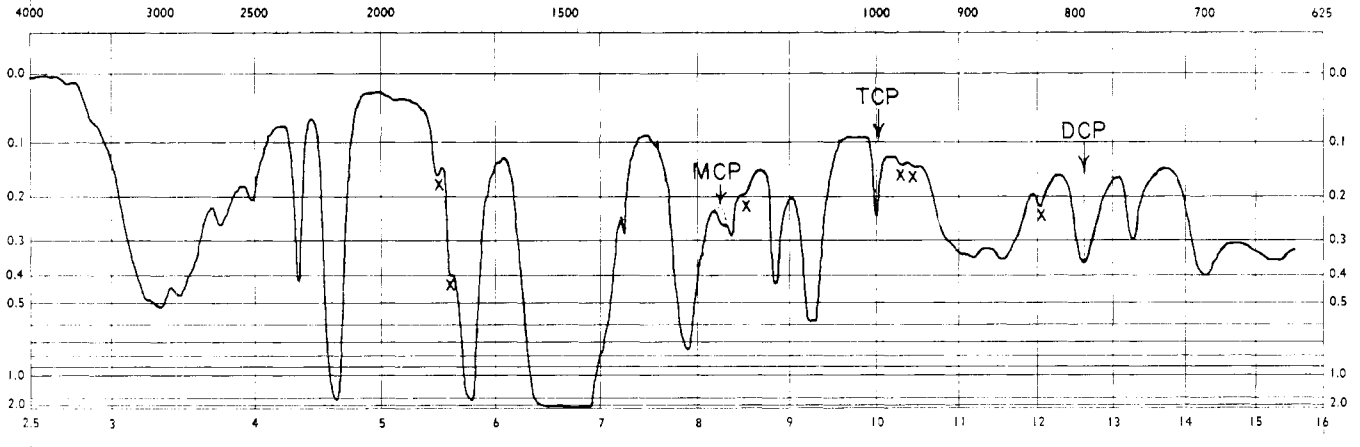

Figure 3. Infrared spectrum of propionic acid chlorination product

After the sample to be separated had been placed on the column, elution was effected with chloroform containing progressively increasing amounts of 1-pentanol. This gradient elution system was fashioned after the one described by Donaldson, Tulane, and Marshall (*9*). Chloroform, water washed, was added to the column and to a 500-ml. Erlenmeyer mixing flask which had a top outlet connected to the column. An inlet line from an elevated reservoir passed down into the mixing flask and emptied near the bottom. A magnetic stirrer was used to assure good mixing. Six hundred twenty milliliters of 2% 1-pentanol in chloroform were added to the reservoir. Fractions of 20 to 40 ml. were collected. The 2% 1-pentanol was followed by 60 ml. of 8% 1-pentanol in chloroform, 160 ml. of 16%, 260 ml. of 20%, and 300 ml of 40%. A total of 1400 ml. of solvent were used. Each fraction was titrated.

An elution curve for a mixture of 0.19 meq. of MCP, 1.84 meq. of DCP, and 0.70 meq. of TCP is given in Figure 4. The respective recoveries were 0.26, 1.67, and 0.71 meq.

Radioactive Preparation. Propionic 2-C^{14} acid (Nuclear-Chicago Corp.) (170 mg.) with a specific activity of 2.06 mc. per mmole was transferred via a vacuum manifold from the break-seal shipping vial into the reaction vessel submerged in a liquid nitrogen bath. The vessel was removed from the vacuum line and 21.5 mg. of phosphorus trichloride were added from a micropipet followed by 186 mg. of inactive propionic acid. The chlorine

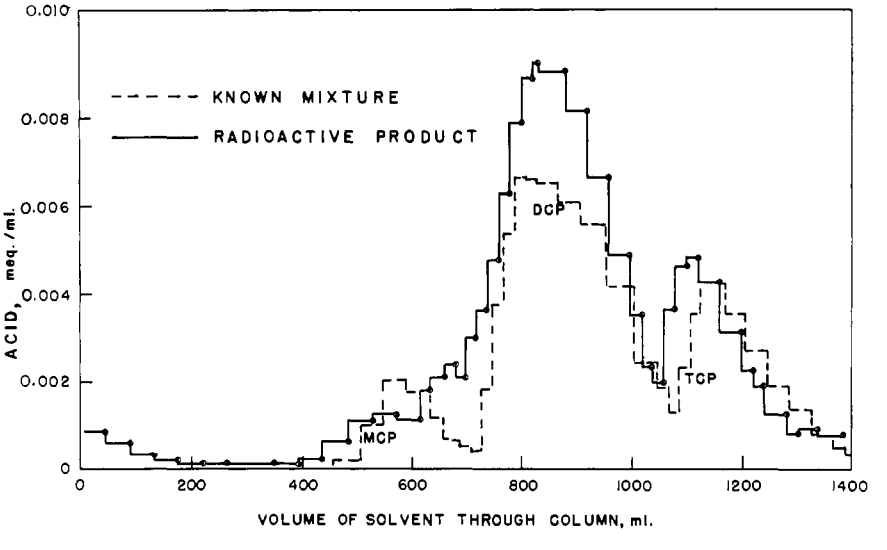

Figure 4. Chromatographic separation of chloropropionic acids

MCP 2-Chloropropionic acid. DCP 2,2-Dichloropropionic acid. TCP 2,2,3-Trichloropropionic acid

Table I. C^{14} in a Soybean Plant Treated with Dalapon-C^{14} by Root[a]

Plant Sample	Plant Tissue Wet Weight, G.	Activity in Gas Counting Aliquot, Net C.P.M.[b]	Total, D.P.M.	Dalapon, as Acid γ	Dalapon, as Acid P.p.m.
Root	1	2455 ± 9[c]	9,270	31	31
Stem, below 1st node	1.193	1865 ± 31	7,530	26	22
1st internode	0.291	1332 ± 14	3,220	11	38
2nd internode	0.236	3678 ± 61	15,000	51	216
Leaf, primary	0.913	263 ± 6	535	1.8	2.0
opposite primary	1.286	249 ± 5	592	2.0	1.6
1st trifoliolate	0.475	40 ± 2	350	1.2	2.5
2nd trifoliolate	0.285	620 ± 11	1,330	4.4	15.5
			Total recovered	128.4	

[a] Total activity applied was 1.45×10^6 d.p.m. (disintegrations per minute). Concentration of dalapon in nutrient was 32 p.p.m.
[b] Above background of 43–48 c.p.m.
[c] Standard deviation of net count rate, std. dev. $= \sqrt{\frac{\text{c.p.m.}}{\text{time counted, min.}}}$

Table II. C^{14} in Corn Plants Treated with Dalapon-C^{14} by Root[a]

Position	No. of Plants	Extract, Ml.	C.P.M./ Ml.	Total, C.P.M.	C.P.M./ Plant	D.P.M.	% of Applied
Tops	7	Water, 172	1800	310,000	44,200		
	1	Alcohol, 44	1000	44,000	44,000		
					Av. 44,100	184,000	9
Roots	7	Water, 118	170	20,100	2,870		
	1	Alcohol, 33	60	1,980	1,980		
					Av. 2,425	10,100	0.5

[a] Supplied per plant: 1.95×10^6 d.p.m.

Table III. C^{14} in Soybean Plants Treated by Spotting One Leaf with Dalapon-C^{14}

Plant Sample	Plant Tissue Wet Weight, G.	Net C.P.M. in Aliquot	Total D.P.M.	Dalapon, as Acid γ	Dalapon, as Acid % of applied	Dalapon, as Acid P.p.m.
		Treated with 6.3 γ				
Root	1	30 ± 3[a]	60	0.00390	0.06	0.004
Stem, below 1st node	1.226	102 ± 3	410	0.02670	0.42	0.022
1st internode	0.546	177 ± 4	355	0.02310	0.37	0.042
2nd internode	0.171	97 ± 3	193	0.01256	0.20	0.074
Leaf, primary, treated part	0.12	281 ± 6	91800	5.96000	94.80	50
primary, adjacent part	0.769	1001 ± 10	2300	0.14970	2.38	0.195
primary, distal part	0.864	...	...	...	...	...
primary, petiole	0.209	74 ± 4	150	0.00975	0.16	0.046
untreated primary	1.418	23 ± 5	90	0.00585	0.10	0.004
1st trifoliolate	0.564	69 ± 3	137	0.00891	0.14	0.016
2nd trifoliolate	0.279	152 ± 4	305	0.01985	0.32	0.071
3rd trifoliolate	0.079	74 ± 4	150	0.00975	0.16	0.123
			Total recovered		99.11	
		Treated with 464 γ				
Root	2.673	726 ± 6[b]	99833	6.54	1.41	2.45
Stem, below 1st node	1.367	232 ± 4	9576	0.63	0.14	0.458
1st internode	0.422	603 ± 11	20463	1.34	0.29	3.18
2nd internode	0.258	484 ± 7	15960	1.05	0.23	4.05
3rd internode	0.242	798 ± 12	26163	1.71	0.37	7.08
4th internode and growth tip	0.082	1080 ± 10	33340	2.20	0.47	26.6
Leaf, primary, treated portion	0.315	12977 ± 33	5136750	336.60	72.54	1065
primary, untreated portion	1.103	16930 ± 40	1616950	106.96	22.80	96.0
untreated primary	1.300	167 ± 3	6612	0.43	0.09	0.33
1st trifoliolate	0.987	417 ± 2	15675	1.03	0.22	1.04
2nd trifoliolate	0.399	756 ± 6	25650	1.68	0.36	4.22
3rd trifoliolate	0.242	393 ± 2	12454	0.82	0.18	3.37
			Total recovered		99.10	

[a] Above background of 46–48 c.p.m. Counted as carbon dioxide. Error given as standard deviation of net count rate.
[b] Above background of 20–21 c.p.m. Counted as barium carbonate. Error given as standard deviation of net count rate.

arm of the reactor was connected to the dry ice trap.

Monochlorination was achieved by passing 8.77 mmoles of chlorine into the reactor over a 17-hour period. The reactor temperature was controlled at 121° C. with vapors from refluxing perchloroethylene. At this temperature, 2-chloropropionic acid was the predominant reaction product.

Further substitution with chlorine was carried out at the temperature of refluxing *sec*-butylbenzene vapors (ca. 173° C.). Eleven millimoles of chlorine were passed into the reaction mixture during 31 hours.

The crude reaction product was transferred directly from the reactor to the liquid-free surface of the silica gel column. Elution and titration were carried out as described previously. The elution curve for a hot run is given in Figure 4. There is an indication of an unknown material coming through the column between the mono- and dichloropropionic acids and possibly another between the di- and trichloropropionic acids. Therefore, although the elution curves gave a good analysis of the mono-, di-, and trichloropropionic acid composition it was felt desirable to obtain further characterization of radiochemical purity. However, because the products at this point were in the form of aqueous sodium salts they were not amenable to good quantitative infrared analysis. A paper chromatographic system was therefore employed.

Ascending chromatograms were developed on Whatman No. 1 filter paper with a solvent made up of 50 parts of chloroform, 50 parts of *tert*-butyl alcohol, 5 parts of ethyl alcohol, 1 part of diethylamine, and saturated with water (somewhat less than 7 parts). An improved resolution of compounds of about the same R_f as chloropropionic and dichloropropionic acids was obtained by increasing the moisture content of the paper to about 30% of the paper's dry weight by a careful light spraying with water. After development, the residual solvent was removed by hanging the sheets over a steam bath while keeping them dry with a heat lamp (*8*). The dried chromatograms were sprayed with 0.1*N* silver nitrate and air dried. After 12 hours in the dark, the paper was exposed to room light. The chlorinated acids developed as purple spots on a tan background. Typical R_f values were: 0.19 for 2-chloro-, 0.38 for 2,2-dichloro-, and 0.51 for 2,2,3-trichloropropionic acid.

Autoradiograms were made on Kodak Type K industrial x-ray film. These confirmed the presence of additional compounds with R_f values of 0.27, 0.06, and 0.66. The first was eluted from the column between the mono- and dichloropropionic acids; the second along with the dichloropropionic acid; and the

supply tube was inserted, and the side third between the dichloro- and trichloropropionic acids. These have not been identified.

Based on the titration curves and paper chromatography, various groups of the extracts were selected, filtered, pooled, and dried. The dry sodium salts of mono-, di-, and trichloropropionic acids were isolated in this manner. The yield of 96% radiochemically pure sodium-2,2-dichloropropionate was 32 mole % based on propionic acid. The specific activity as determined by combustion to carbon dioxide and internal Geiger-Müller gas counting was 0.982 mc. per mmole.

In addition, 3.8 mole % were recovered as 95% radiochemically pure 2-chloropropionic acid and 13.7 mole % as 97% radiochemically pure 2,2,3-trichloropropionic acid.

Trial Applications of Dalapon-2-C^{14} to Plants

Corn and soybean plants were treated with the sodium dalapon-2-C^{14}; allowed to grow for a period of time estimated to be sufficient for the herbicide to enter the plant and be distributed throughout; and then they were harvested. Radioautographs were prepared to obtain a general indication of the distribution of radioactivity. Other plants were sectioned and analyzed for radioactivity. Similar studies have been reported (*7, 11*). In order to identify radioactive compounds in the plant tissues, extracts were examined by paper chromatography.

The plants were grown in the laboratory under artificial light. Four fluorescent tubes (Sylvania 40-watt cool white) and two flood bulbs (General Electric 150-watt) were operated 14 to 16 hours per day. Plants for foliar application experiments were grown in pots. Others were grown in a mineral culture solution (*13*).

Soybean plants were treated by root with the dalapon-C^{14} by growing them in a nutrient with a dalapon concentration of 38 p.p.m. Specific activity used was 0.02 mc. per mmole. The plants were harvested after 4 days.

Foliar application was made to other soybean plants. An aqueous solution of the dalapon-C^{14} at 1 mc. per mmole was spotted by micropipet to one of the primary leaves of the plants. One dose was 6.3 γ in 10 μl. There was no noticeable surface burn at this low dosage rate. The applied spot had dried on the leaf in about 10 minutes after application. The plant was harvested after 4 days. Another was treated with three 10-μl. spots containing a total of 464 γ. The spots remained wet for 1 hour. After 3 days at 85° C. and 55% relative humidity, this plant was harvested.

Corn plants in the four- to six-leaf stage were treated similarly by root, but at a concentration of 2.5 p.p.m., and a specific activity of 1 mc. per mmole. They were harvested at 11 days.

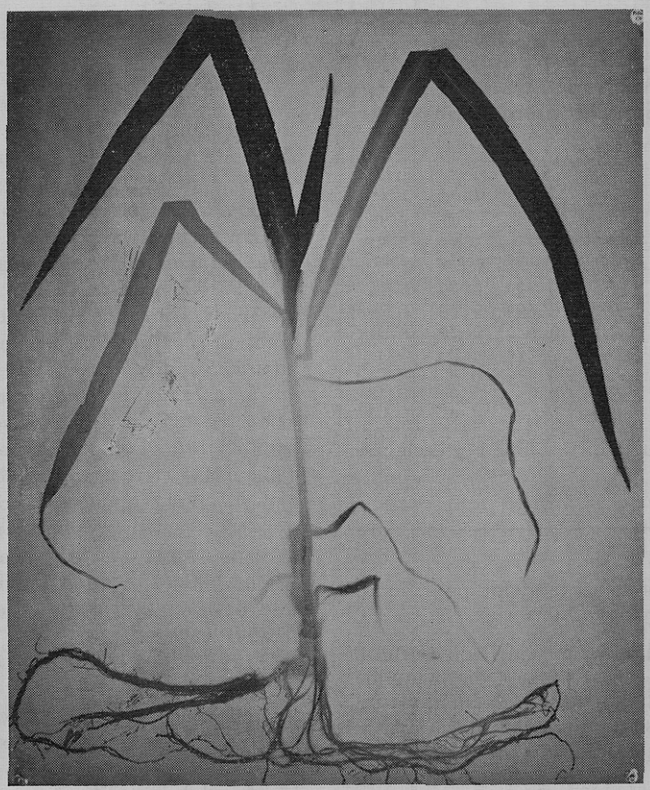

Figure 5. Radioautogram of a corn plant grown in a culture solution containing dalapon-C^{14}

Analyses

Radioautograms of whole plants were made by placing them between sheets of 0.5-mil saran film and pressing them against Eastman Type K industrial x-ray film in a cardboard film holder with weights. The films were developed after about 1 month of exposure.

Radioactivity analyses of extracts were made by drying aliquots on planchets and counting these under an end window Geiger-Müller tube.

For quantitative determination of the distribution of radioactivity, the plants were sectioned for analysis. The wet weight of each section was determined before the sample was combusted using a modification of the Van Slyke-Folch procedure (*14, 15*). The resulting carbon dioxide was collected and counted in a Geiger-Müller tube using the method described by Eidinoff (*10*) or was plated as barium carbonate and counted with an end window Geiger-Müller tube.

Water extracts of the plants were made with a Waring Blendor. The activity was extracted from the acidified water extracts with diethyl ether. Less than 2% remained in the water. The ether extracts were concentrated and chromatogrammed.

Alcohol extracts of a corn plant were obtained by a 24-hour Soxhlet extraction of the tops and roots with 80% aqueous ethyl alcohol. One-milliliter aliquots of each were dried on planchets and counted. The leaf extract was filtered, made slightly alkaline, and washed with diethyl ether. The original ethyl alcohol extract and the final water solution were chromatogrammed.

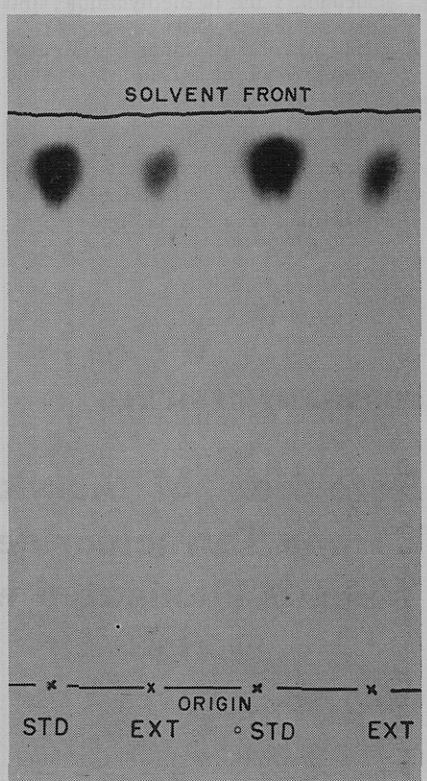

Figure 6. Radioautogram of a chromatogram of an extract from a corn plant grown in a culture solution containing dalapon-C^{14}

Table IV. Identification of C^{14} in Plant Extracts as Dalapon-C^{14} by Paper Chromatography

Plant	Solvent System	R_f Extract[a]	R_f Dalapon reference	R_f Pyruvic acid reference
Soybean	*A*	0.72[b]	0.73	0.26
	B	0.69[b]	0.70	0.56
	C	0.64[b]	0.72	0.18
Corn	*D*	0.27[b]	0.25	
	E	0.9[b]	0.9	0.6
	E	0.9[c]	0.9	0.6
	E	0.9[c]	0.9	0.6

[a] Only one spot was found in each case.
[b] Water.
[c] Alcohol.

Solvent systems used for paper chromatography of extracts were:

A. 100 ml. of *n*-butyl alcohol, 15 ml. of water, and 10 ml. of diethylamine.
B. 250 ml. of methyl alcohol, and 1 ml. of 1*N* sodium ethylate.
C. 100 ml. of *n*-butyl alcohol equilibrated with 100 ml. of 1.5*N* ammonium hydroxide.
D. 50 ml. of chloroform, 50 ml. of *tert*-butyl alcohol, 5 ml. of ethyl alcohol, 1 ml. of diethylamine, and saturated with water.
E. 100 ml. of 1-pentanol equilibrated with 100 ml. of 5*M* aqueous formic acid (*4*).

R_f values of radioactive components on the chromatograms were computed from loci determined by radioautography or Geiger-Müller tube scanning.

Results of Plant Experiments

Radioactivity entered soybean and corn plants whose roots were exposed to dalapon-C^{14}. It was distributed throughout the plants as indicated in Tables I and II and Figure 5. There were higher concentrations in young tissues. The percentages of applied activity taken into the roots and into the tops for soybean were 0.6 and 2, respectively. For corn, under slightly different conditions, percentages were about 0.5 and 9.

Radioactivity entered soybean plants, when spotted on a leaf, and was distributed throughout the plants as shown in Table III. Here again the young tissues had the highest concentrations of activity. The leaf opposite the treated leaf and the roots had the least activity. About 2% of the applied activity moved into parts of the plants other than the treated leaves.

Examination by paper chromatography of extracts prepared from root-treated soybean plants showed the presence of radioactivity which moved with R_f values corresponding to the R_f of known dalapon-C^{14}. No other radioactive spots were observed (Table IV).

From water and alcohol extracts of corn plants, radioactivity was recovered. This activity moved on chromatograms with R_f values corresponding to that of dalapon (Table IV, Figure 6). No other compounds were found. Two plausible breakdown products, lactic and pyruvic acids, move in system *E* with R_f values (0.62 and 0.65, respectively) well below the R_f of dalapon (0.9).

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FUMIGANT RESIDUES

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IN EXPLORATORY TESTS with four fumigants to control infestations of the Indian-meal moth, *Plodia interpunctella* (Hbn.), in imported shelled walnuts, Acrylon provided 100% control with no undesirable effects on flavor, odor, or appearance (*1*). A feature of the experiment was the successful use of polyethylene bags as fumigation chambers as described below. Acrylon is also known as Acritet (Stauffer Chemical Co., New York) and consists of acrylonitrile and carbon tetrachloride, 34 to 66% by volume, or 20.4 to 79.6% by weight.

Acrylon applied at the rate of 3 ml. per 55 pounds (4.1 grams per 25 kg.) of nut meats for an exposure period of 16 hours, was consistently successful in controlling infestations during a 3-year period in which some 500,000 pounds of shelled walnuts were treated (*13*). However, information was needed on the residues of acrylonitrile and of carbon tetrachloride that remained after aeration and packaging of the treated nuts. This report deals with concentration-time relationships for the Acrylon components over a period of 38 days.

Methods

Fumigation under Atmospheric Pressure. Double-walled polyethylene bags, large enough to accommodate the contents of a 55-pound box of shelled walnuts, served as small and inexpensive

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Methods

Fumigation under Atmospheric Pressure. Double-walled polyethylene bags, large enough to accommodate the contents of a 55-pound box of shelled walnuts, served as small and inexpensive

Imported shelled walnuts, in 55-pound batches, were successfully fumigated in polyethylene bags to control insect infestation by injecting each bag with 3 ml. of Acrylon. Desorption rates of acrylonitrile and carbon tetrachloride during a 38-day storage period showed that their residual amounts were affected by fumigant dosage, duration of exposure, and aeration by fan. Fumigation under reduced pressure, followed by storage for 30 days, resulted in acrylonitrile and carbon tetrachloride residues which were lower than those obtained by fumigation at atmospheric pressure. Acrylonitrile was determined polarographically and carbon tetrachloride spectrophotometrically with satisfactory specificity and sensitivity.

fumigation chambers. A wad of cotton batting backed by a double layer of aluminum foil was placed with cotton side out in the center of one of the sides of the bag. The bag opening was folded and tied tightly with flexible metal ties, and each bag was placed horizontally on a platform in the vicinity of an exhaust fan.

Acrylon was dispensed with a 5-ml. glass syringe fitted with a mechanical stop at a precalibrated 3-ml. mark, after Chaney (*3*), and with a No. 18 stainless steel hypodermic needle to pierce the bag at the target center. After the liquid was discharged, the needle was withdrawn, the hole sealed with Scotch cellulose tape, and the process repeated for each bag.

After exposure to the fumigant for 16 hours at 70° to 75° F., the walnuts were spread out on a slat platform covered with cheesecloth and aerated for 4 hours in front of a floor-type fan with 24-inch diameter blades and adjustable oscillation within a maximum arc of 90°. The aeration was followed by air-cleaning with a fanning mill to remove loose nut tissues, insect fragments, etc. Finally, the walnuts were mechanically packed in cellophane bags in half-pound lots and stored on a laboratory shelf at room temperature. Samples from different bags were analyzed at predetermined intervals. The standard or normal conditions that were tentatively adopted were 3 ml. of Acrylon per 55 pounds of shelled walnuts, 16 hours' exposure in the sealed polyethylene fumigation chambers, and 4 hours' aeration by fan. Variations of these conditions were studied to find the effect of doubling the normal dosage rate, tripling the normal exposure period, and eliminating the aeration by fan.

Vacuum Fumigation. In vacuum fumigation, wherein the air pressure is reduced before the fumigant is released, diffusion and penetration proceed rapidly; therefore, the period of exposure may be shortened considerably. Although methyl bromide appears to be the most widely used fumigant for vacuum fumigation, Monro suggested (*11*) determining acrylonitrile and carbon tetrachloride residues of walnuts fumigated with Acrylon under reduced pressure.

A 25-quart cast aluminum pressure canner, *A*, was converted for vacuum fumigation, following suggestions by Lynn (*9*) and Monro (*11*). The pressure fittings were removed from the cover and were replaced with cemented glass and metal fittings as shown in Figure 1. A cheesecloth bag, *C*, containing 10 pounds of shelled walnuts and six cages, each of which contained 50 adults of confused flour beetles, *Tribolium confusum* Duv., 2 weeks old, was placed on the wire rack, *B*. To promote uniformity of the gas-air mixture an improvised fan made of a thin brass sheet, *D*, soldered to a flexible metal strip was attached to a swivel that was suspended on the underside of the cover, *E*. The fan was operated by an Alnico magnet moved manually on the dome of the chamber.

With the cover in position a weighed amount of Acrylon liquid corresponding to 78 mg. per liter of space in the fumigation chamber was contained in the borosilicate glass vessel, *F*. After the vessel was evacuated, *J*, to 75 mm. of mercury, Acrylon was introduced as a gas through butt joint *H*, and the copper gas-inlet tube, *I*. The evaporation of Acrylon liquid was accelerated by immersing the glass vessel in lukewarm water. When evaporation was visibly complete, the air was admitted, *G*, until a vacuum of 100 mm. of mercury was reached (*K*, vacuum gage). The Alnico magnet was moved to and fro for 5 minutes to cause movement of the improvised fan to assist admixture of gas and air. After 3 hours of fumigation at 70° F., air was admitted, the cover was removed, and the nut meats were sampled for analysis and the effect of aeration.

Method for Carbon Tetrachloride. The residual amounts of carbon tetrachloride retained by the fumigated walnuts were determined spectrophotometrically by the Ramsey method (*12*), employing the Fujiwara reaction.

Exploratory tests with carbon tetrachloride and acrylonitrile standards added to untreated walnuts showed that the efficiency of recovery of carbon tetrachloride was 85 to 90%, and that acrylonitrile did not interfere, even if present at 50 times the carbon tetrachloride levels. Amounts of carbon tetrachloride as low as 0.1 mg. were readily measured by the Fujiwara reaction, with a variation between triplicates not exceeding 0.7% transmittance.

A 100-gram sample of shelled walnuts was lightly rolled with a glass bottle to reduce the particle size and was placed in a two-necked 1-liter round-bottomed flask to which 265 ml. of distilled water, 30 ml. of 1*N* sulfuric acid, and 5 ml. of 20% phosphotungstic acid were added. From this point Ramsey's procedure (*12*)

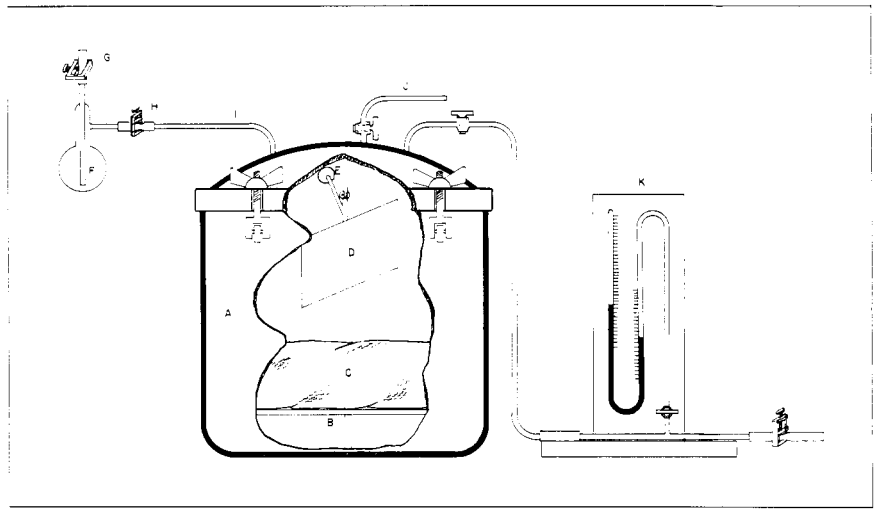

Figure 1. Vacuum fumigation assembly

was followed, except that a more rapid rate of air flow of approximately 100 ml. per minute was preferred. Also, a constant air suction source regulated with a needle valve and rotameter provided steadier air flow than was attained with a Mariotte bottle. A Beckman B spectrophotometer with 10-mm. matched cells was used to measure the color at a wave length of 530 mμ. As the slope of the standard curve varied somewhat on different days, for each group of determinations a fresh standard curve was used, along with carbon tetrachloride and carbon tetrachloride–acrylonitrile check tests, to cancel out small differences between days. Slight turbidity of the pyridine-alkali reagent sometimes occurred, and this was corrected by filtering through a dry, folded filter paper. The analytical results were corrected for the value of blank (untreated) samples and for recovery of carbon tetrachloride from added standards.

Acrylonitrile. The polarographic method of Fuller and Norris (*5*) was adapted for the determination of acrylonitrile retained by the Acrylon-fumigated walnuts. The method depends on the fact that acrylonitrile forms an azeotropic mixture with water and can thus be concentrated and codistilled out of aqueous suspensions of foodstuffs, etc. Acrylonitrile reduces at about −1.90 volts in a polarographic cell employing a dropping mercury electrode and a mercury pool anode, with 0.1*M* tetramethylammonium hydroxide as the supporting electrolyte.

The Gawron polarographic cell (*6*) used was simple and convenient for routine work and gave reproducible results. Although acrylonitrile may be measured in the presence of dissolved oxygen, the recorded waves were more reproducible when the cell solution was deaerated with nitrogen (it was not necessary to deaerate the anode solution). All measurements were made with a Radiometer Model PO_3 recording polarograph (Radiometer Co., Copenhagen, Denmark).

Standard solutions of acrylonitrile in water were prepared from a purified and freshly distilled sample of acrylonitrile provided by the Research Division, American Cyanamid Co., Stamford, Conn. Amounts of acrylonitrile as low as 0.025 mg. were readily measured. The efficiency of recovery of acrylonitrile standards added to aqueous suspensions of walnuts was 95 to 100%, with no interference from carbon tetrachloride. Calibration data showed a linear relationship between concentrations of acrylonitrile in the original solution and the diffusion current of a given fraction or aliquot.

For separating acrylonitrile from the treated walnuts, distillation from an acidified water suspension was employed. Thus, a 100-gram sample was placed in a 24/40 1-liter round-bottomed flask, to be heated with an electrical heating mantle. A solution of 280 ml. of distilled water, 15 ml. of 1*N* sulfuric acid, and 5 ml. of 20% phosphotungstic acid was added. A 250-mm. Vigreux fractionating column, a 75°-angle condenser head, a 300-mm. Liebig condenser with adapter, and a graduated 25-ml. receiver immersed in an ice bath were attached. The distillation was regulated to about 1 ml. per minute with a voltage regulator, until about 23 ml. of distillate were collected. This was diluted to 25 ml. in a volumetric flask, from which a suitable aliquot (between 1 and 5 ml. generally provided an adequate sample) was transferred to a 10-ml. volumetric flask. One milliliter of 1*M* tetramethylammonium hydroxide, polarographic grade (Southwestern Analytical Chemicals, Austin, Tex.) was added to the aliquot, and then diluted to the mark with water, and mixed and transferred to the Gawron cell (*9*) containing 0.1*M* tetramethylammonium hydroxide in the anode compartment. The cell solution was deaerated with nitrogen presaturated by passing through a separate portion of a blank solution. Current-voltage curves from −1.4 to −2.2 volts were recorded in the usual manner, measuring the diffusion current as the difference between the base line (residual current) and the plateau of the acrylonitrile wave (at about −2.0 volts).

Results

The concentration-time relationships of carbon tetrachloride and acrylonitrile that resulted from the various treatments applied to shelled walnuts are shown in Tables I and II.

The mortality of test insects in all cases was complete. The mortality resulting from vacuum fumigation with the relatively short exposure period of 3 hours was interesting in that examination of the test cages 1/2 hour after opening the chamber showed that the cages located at the perimeter of the load had a larger percentage of affected insects. However, all the test insects were dead 7 hours after removal from the toxic atmosphere.

Discussion

Table I, dealing with carbon tetrachloride and acrylonitrile levels of walnuts fumigated in polyethylene bags under atmospheric pressure, shows that dosage, period of exposure, and aeration influence the amounts retained by the packaged walnuts. Thus, when the dosage was doubled and the exposure period was increased 2.7 times without supplementary aeration by fan, the carbon tetrachloride residue levels over the 38-day period were appreciably higher, although not in direct proportion to the increase in dosage and exposure period. Aeration by fan, however, lowered the carbon tetrachloride residues in every instance. Although for this experiment both the fan and the aeration period were arbitrarily chosen, nevertheless it is suggested that lower residues might be obtained in commercial practice by adapting "fluidized solid bed" columns (*10*) for forced aeration. In any event, the normal or standard conditions that were chosen for commercial application (*1*)

Table I. Amount of Acrylon Components Found on Atmospheric-Fumigated Walnuts

(Interaction of 2 dosages × 2 exposure periods × 2 aeration periods)

Nature of Treatment			Days after Packaging of Walnuts											
			Carbon Tetrachloride, P.P.M.						Acrylonitrile, P.P.M.					
Acrylon added,[a] ml.	Exposure, hr.	Fan, hr.	2	9	18	24	30	38	2	9	18	24	30	38
3	48	4	28.0	25.5	16.0	13.0	9.6	4.8	7.5	5.0	0.0	0.0	1.0	2.5
3	48	0	40.5	32.2	24.5	20.9	23.1	24.0	10.0	5.0	0.8	1.0	1.0	2.0
6	48	4	33.0	30.0	10.0	17.5	9.6	14.4	10.0	6.0	2.5	2.5	3.5	3.7
6	48	0	50.4	33.0	35.2	...	33.7	34.5	17.5	11.0	6.8	10.0	10.5	8.5
3	18	4	15.7	6.7	6.0	4.7	2.9	5.7	5.0	1.0	0.0	1.0	0.0	0.0
3	18	0	18.0	18.5	17.2	15.1	13.5	13.0	6.0	1.0	0.0	2.5	1.0	0.5
6	18	4	18.0	24.0	18.0	13.0	11.5	13.3	11.0	12.5	2.4	4.0	3.5	1.0
6	18	0	28.2	39.5	35.0	31.8	24.0	21.6	13.5	12.5	4.1	5.0	7.5	5.0

[a] Normal dosage = 3 ml. Acrylon per 55 pounds of walnuts, or 164 mg. per kg. of walnuts.

Table II. Amount of Acrylon[a] Components Found on Vacuum-Fumigated Walnuts[b]

Nature of Treatment	Days after Packaging of Walnuts													
	3/4	1	4	8	14	21	30	3/4	1	4	8	14	21	30
	Carbon Tetrachloride, P.P.M.							Acrylonitrile, P.P.M.						
Treatment A[c]	17.5	16.0	13.5	9.3	10.5	7.3	5.3	12.5	16.6	16.6	14.9	6.8	4.1	1.3
Treatment B[d]	16.5	12.0	5.0	1.9	2.4	0.5	0.5	16.0	12.5	10.0	6.0	2.5	0.8	0.0

[a] Total weight of Acrylon applied = 1.882 grams; applied dosage = 1882/4.55 = 413 mg./kg.
[b] Total amount of nuts in 24.1-liter vacuum-fumigation chamber = 10 lb. or 4.55 kg.
[c] Vacuum-fumigated for 3 hours at 110 mm. of mercury at a relative concentration per liter of empty space = 1882/24.1 = 78 mg./liter.
[d] Same as Treatment A, but aerated in laboratory fume hood for 16 hours.

(3 ml. of Acrylon per 55 pounds of walnuts, 16 hours' exposure, and 4 hours' aeration by fan) gave the lowest carbon tetrachloride residues.

Table I also indicates that the residue levels of acrylonitrile are appreciably lower than those of carbon tetrachloride, and that aeration assists the lowering. With due regard to the greater relative toxicity of acrylonitrile, and also to the 1 to 4 ratio of acrylonitrile to carbon tetrachloride as initially applied, the comparative rate data shown herein confirm the author's findings with wheat (*2*) that the ratio of the components of a fumigant mixture may change considerably after application because of differential sorption.

Table II, pertaining to vacuum fumigation, shows generally lower carbon tetrachloride residues than those of atmospheric-fumigated walnuts, despite the greater amount of fumigant that was applied. Evidently the shortened exposure period was a major factor herein. Supplementary aeration, as in treatment B, aided the lowering of residues of both carbon tetrachloride and acrylonitrile, and confirms the trend shown in Table I. The vacuum of 110 mm. of mercury that was used in this experiment approximates the 25-inch vacuum used commercially.

As indicated previously, methyl bromide is widely used for fumigation of nut meats and other foodstuffs. Because of differences in conditions and methods of fumigation, it is not possible to make valid comparisons of the concentration-time relationships between the bromide residues of walnuts reported by others (*4*, *7*, *8*) and those of acrylonitrile and carbon tetrachloride reported herein. It would appear, nevertheless, that the desorption patterns for methyl bromide residues of vacuum-fumigated walnuts show a sharp drop within 24 hours from relatively high values and become almost constant thereafter to levels of 30 to 40 p.p.m. (*7*).

Polyethylene bags proved to be simple, inexpensive, and convenient as fumigation chambers for fumigation of 25-kg. batches of walnuts that had been stored in their original boxes in commercial cold storage at 34° F., and which were treated as required at the packaging warehouse (*13*). The method of application by syringe is considered to be simple, inexpensive, and safe, if adequate precautions are taken in the application. This method would not be suitable for extremely volatile fumigants such as methyl bromide.

Forced aeration applied within several hours after the exposure period should be investigated in greater detail as a means of lowering volatile fumigant residues.

Acknowledgment

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FISH STORAGE EFFECTS

Sodium Ion, Potassium Ion, and Weight Changes in Fish Held in Refrigerated Sea Water and Other Solutions

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When sockeye salmon, halibut, herring, lemon sole, and brill were stored in refrigerated sea water for a week, analyses of the flesh revealed a rise in sodium ion and a drop in potassium ion over the natural levels. Weight increases in the fish ranging from 2.4% for salmon to 17.5% for a group of lemon sole were recorded after storage. The outer layer of the flesh of sockeye salmon showed a slow rise in sodium ion and a drop in potassium ion content over the first 48 hours of immersion in chilled sea water, after which time much more rapid changes in the flesh levels of these ions occurred. When solutions were prepared containing sodium and potassium ions at concentrations approximating those calculated for the total tissue water of the fish, the sodium and potassium ions level of the flesh of fish immersed in them remained close to their natural values. It was found that the weight changes of the fish could be prevented by adding 2% of polyvinylpyrrolidone to the immersing solutions.

HOLDING SALMON AND HALIBUT in chilled sea water prior to processing is a procedure which is rapidly gaining acceptance in the fishing industry of the northwest Pacific coast. Chilling units and reefer tanks have been installed on trollers, seiners, tenders, and packers, and in canneries in Canada and the United States, including Alaska (*3*, *14*).

A French patent assigned to LeDanois in 1920 (*9*) covered the holding of fish on boats in sea water or brine, but at −4° C. (24.8° F.) a temperature which would freeze the fish. Hess reported in 1933 that fish kept twice as well at 30° F., the freezing point of fish muscle, as at 36° to 37° F. and that a temperature of 30° F. could be attained using circulating sea water containing ice (*7*). The engineering, bacteriological, chemical, and biochemical aspects of holding fish in refrigerated sea water have been the subject of considerable study at the Technological Station over the past several years.

The concentrations of sodium and potassium ions in the flesh of living fish differ little from those prevailing in animal tissues (*11*). Sodium and potassium ions in sea water are present at approximately 20 and 0.07 times, respectively, the concentration of these ions in fish flesh. Living fish maintain these concentration gradients by expending energy. Dead fish, on the other hand, if maintained in sea water, tend to equilibrate with their surroundings. The sodium ion concentration of the flesh rises, the potassium ion drops (*13*), and the fish increase in weight (*2*).

In this paper the sodium and potassium ions and the weight changes in fish stored in refrigerated sea water are recorded for various species. The effect of varying the salt concentrations and osmotic pressure of the immersing solutions on the extent of the changes observed is also reported.

Methods

Unless otherwise indicated, fish were cleaned and then sampled according to a slight modification of the official method (*1*). Samples of homogenized fish were dissolved in nitric acid and analyzed for sodium and potassium ions using a flame photometer attachment for a Beckman DU spectrophotometer. Details of the sampling and analytical procedures used have been described (*11*). Two methods were employed to maintain fish in refrigerated sea water or other salt solutions for the desired length of time. In one case a laboratory scale model of equipment designed for use on a fishing boat was employed. Fish were held in the immersing solution in a plywood box painted on the inside with Terecote, a Thiokol-type water-resistant coating (Technical Research Co., Seattle, Wash.). The immersing solution was continuously circulated at a rate of 4.5 gallons (U. S.) per hour through the box and over pipes containing refrigerant in an outside chilling unit. The temperature of the immersing solution was maintained at 30° ± 0.5° F. When the effect of a number of different immersing solutions on fish was being compared, 5-gallon plastic pails equipped with stirrers were used and the experiments were conducted in a refrigerated room maintained at approximately 32° F.

The ratio of fish to immersing solution was maintained as closely as possible to one part by weight of fish to four of solution. Chlortetracycline was added as an antibacterial agent to all immersing solutions at a level of 10 p.p.m. Artificial rather than natural sea water was used throughout this study because the salt concentrations were more readily reproducible than with the natural sea water available to this laboratory. The artificial sea water had the following composition (*10*) in grams per kg.: NaCl, 23.476; $NaSO_4$, 3.917; $NaHCO_3$, 0.192; KCl, 0.664; KBr, 0.096; $MgCl_2$, 4.981; $CaCl_2$, 1.102; $SrCl_2$, 0.024; H_3BO_3, 0.026. Half-strength sea water was prepared by diluting the artificial

sea water with an equal volume of fresh water.

Results

The effect on the sodium and potassium ion content of the flesh of storing eviscerated coho salmon (*Oncorhynchus kisutch*) in refrigerated sea water for periods up to 4 weeks has been reported (*13*).

In the present study, the earlier observations were extended in the following manner. Sockeye salmon (*Oncorhynchus nerka*) were used in place of coho. Artificial rather than natural sea water was employed as the immersing solution to provide more readily reproducible salt concentrations. As sea water along the coastline is frequently diluted with fresh water, particularly near points where rivers discharge into the sea, the artificial sea water was tested at both full and half strength. As fish are frequently eviscerated immediately after capture, a comparison was made of the extent of the changes occurring in whole and eviscerated fish. Weight changes in the fish were also determined. The results are recorded in Table I. For this experiment, sockeye salmon freshly caught in a gill net at the mouth of the Fraser River were quickly transported to the laboratory and immediately immersed in previously prepared solutions. Less than 4 to 6 hours elapsed between the time of capture of the fish and the beginning of the experiment.

The results show (Table I) that half-strength sea water raised the sodium ion level of the flesh of the fish approximately half as high as full-strength sea water, but produced little difference in the potassium ion concentration. Although the eviscerated fish presented a greater surface exposed to the salt solution than did the whole fish, surprisingly little difference was observed in the sodium and potassium ion concentrations of the flesh from the two groups. Fish held in full-strength sea water tended to gain more weight than those in the half-strength solution, while the increase observed in eviscerated fish exceeded that in whole ones.

In the experiment just described, the first analyses were made after immersion of the fish for 1 week in the sea-water solutions. In previous experiments with coho salmon in which fish were immersed for short intervals in sea water, the natural levels of sodium and potassium ions were maintained in the fish for a period of approximately 24 hours, after which time a rather abrupt rise in sodium ion and fall in potassium ion occurred (*13*).

To determine whether a similar phenomenon could be observed in another species, an experiment was conducted using sockeye salmon. In an effort to cast further light on the nature of the phenomenon, the flesh in a 1-inch slice taken through the center section of the fish was divided into inner and outer layers. The inner layer represented the flesh from the backbone to a point midway between the backbone and the outer surface of the fish, while the outer layer was made up of the flesh from the mid-point to the outer surface. The layers were rendered skin- and bone-free and then homogenized. The flesh from a second 1-inch slice through the fish taken at a point adjacent to the first was pooled in the usual manner for analysis. For this experiment sockeye salmon were caught in gill nets at the mouth of the Fraser River and transported immediately to the laboratory. Approximately 4 hours elapsed from the time of capture of the fish until the whole fish were immersed in the experimental sea water tank. At each 6-hour interval three fish were removed from the tank for sampling and analysis. The results obtained are recorded in Figure 1. The outer layer of flesh showed a slow rise in sodium ion concentration and fall in potassium ion which was evident from the earliest analyses. After approximately 72 hours these changes began to take place much more rapidly. No change occurred in the ion levels of the inner layer of flesh during the first 48-hour period. As can be seen, the increase in the rate of change of the ion concentrations in the outer layer occurred after changes in sodium and potassium ion levels had begun to take place in the inner layer of flesh. As might be expected, values obtained from

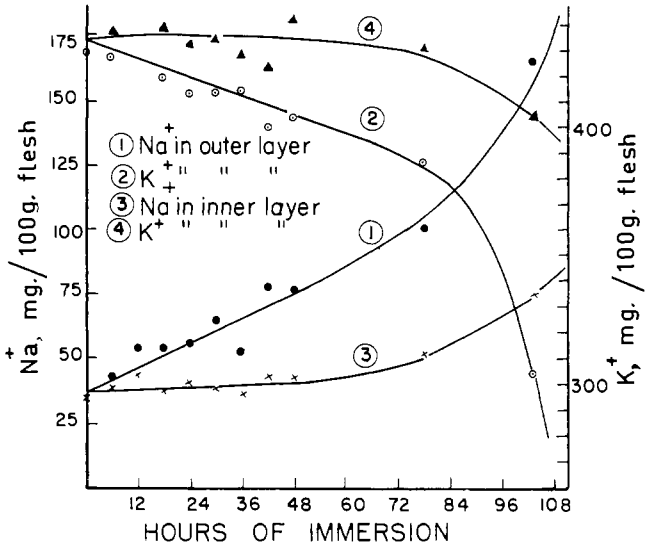

Figure 1. Na^+ and K^+ changes in inner and outer layers of muscle tissue of sockeye salmon at various intervals after immersion of the fish in full strength refrigerated sea water

Each recorded point represents average value for three fish

Table I. Sodium and Potassium Ions and Weight Changes in Sockeye Salmon Held Whole and Eviscerated in Refrigerated Sea Water

Sea Water Concentration	Immersion Time, Weeks	Mg./100 G.[b] Na^+	Mg./100 G.[b] K^+	Wt. Increase,[a] %
Control (not immersed)	...	33.5 ± 1.1	411 ± 15	...
		Whole fish		
Full strength	1	277 ± 13	276 ± 5	2.98 ± 0.79
	2	422 ± 18	198 ± 6	4.33 ± 1.25
Half strength	1	149 ± 12	265 ± 9	2.38 ± 0.58
	2	221 ± 20	201 ± 17	3.45 ± 0.65
		Eviscerated Fish		
Full strength	1	297 ± 38	272 ± 19	4.63 ± 1.39
	2	487 ± 26	193 ± 19	8.16 ± 1.69
Half strength	1	178 ± 29	257 ± 34	3.89 ± 0.99
	2	250 ± 21	178 ± 25	5.60 ± 1.00

[a] Of entire fish.
[b] Based on wet weight of muscle tissue. Each value represents average and average deviation of four fish.

a flesh sample representing a composite of the inner and outer layers, were approximately the average of the two sets of values shown.

The initial slow rise in sodium ion and concomitant fall in potassium ion in the outer layer of the flesh (Figure 1) may well have been due to equilibration of the immersing solution with the extracellular fluid of the tissue, while the more rapid changes occurring subsequently could represent equilibration with intracellular fluid. Studies with much smaller sections of tissue would be required to test this hypothesis further.

Information on ion and weight changes in fish immersed in refrigerated sea water has so far been restricted to studies on various species of salmon. As a step toward extending these observations to include other commercially important species of fish, an experiment was conducted with halibut (*Hippoglossus stenolepsis*). For this purpose, halibut ranging in weight from 10 to 20 pounds were caught at sea and packed in ice for transport to the laboratory. The experiment was begun within 24 hours of the time of capture of the fish. In this study a slice 1 inch wide was taken from the center section of the fish and divided into inner and outer layers. As in the previous salmon experiment, the inner layer represented the flesh from the backbone to a point midway between the backbone and the outer surface, while the outer layer was composed of flesh from this mid-point to the outer surface. The layers were freed of skin and bone and then homogenized. The results (Table II) show that after 1 week in half-strength sea water, the inner layer of flesh of the halibut was almost unchanged with respect to sodium and potassium ion concentration, while the values for the outer layer had changed very appreciably. After 2 weeks the two layers had still not equilibrated. The weight increases recorded after 1 and 2 weeks are slightly higher than for sockeye salmon held under comparable conditions.

It was of interest to know whether sea water could be modified or solutions devised which would minimize or eliminate the changes observed in the preceding experiments. To investigate this possibility, smaller species of fish were selected to enable a reasonable number of different solutions to be tested at the same time with the facilities available.

In Table III are recorded the results obtained when herring were immersed for 1 week in various solutions. For experiments with herring the fish were obtained from seine boats as soon as possible after capture, which was usually within 24 to 36 hours. January-caught herring were used. The whole herring were weighed individually before being immersed in the various solutions. In the case of immersion in full-strength sea water the results show (Table III) that the uptake of sodium ion was somewhat higher for herring than had been observed for sockeye salmon under comparable conditions (Table I). The potassium ion loss for herring was a little less and the weight changes were slightly higher.

As the sodium ion uptake by and the potassium ion loss from the cells could possibly be due to exchange (*6*), it seemed not unlikely that if potassium ion loss could be prevented, sodium ion uptake might not occur. Accordingly potassium ion was added to the sea water until the concentration equaled that calculated for the potassium ion concentration of the total tissue water in the flesh of the fish. Although this concentration of potassium ion in the immersing fluid not only maintained but somewhat increased the potassium ion concentration in the flesh, it failed to prevent the uptake of sodium ion (Table III). Similar results were obtained when potassium ion was added to half-strength sea water.

From a knowledge of the moisture content of the flesh and its content of sodium and potassium ions, one can calculate the concentration of sodium and potassium ions in the total tissue water. If the immersing fluid contained sodium and potassium ions in this same concentration, it should be possible to maintain the flesh levels of these ions approximately constant. Solution 5 in Table III was prepared to test this possibility. The total tissue water of an average herring was calculated to contain 1.09 grams of sodium ion and 6.44 grams of potassium ion per liter. These levels were provided in the immersing solution using appropriate amounts of sodium chloride and potassium chloride. Although some changes did occur as a result of immersion of the fish in this solution, a reasonable approximation of the starting values was maintained (Table III).

A series of solutions was tested using lemon sole (*Parophrys vetulus*) as the experimental fish. This species was chosen because it is a small flat fish with a high ratio of surface area to body weight. In addition, as it never accumulates any

Table II. Sodium and Potassium Ion Changes in Inner and Outer Layers of Muscle Tissue of Halibut and Weight Increases in Fish Preserved in Half-Strength Refrigerated Sea Water

Time, Weeks	Portion of Tissue Analyzed	Mg./100 G. Na^+	Mg./100 G. K^+	Wt. Increase,[a] %
0	Inner layer	45.6 ± 6.1	510 ± 9	
	Outer layer	47.7 ± 5.1	493 ± 15	
	Average	46.7 ± 5.6	501 + 12	
1	Inner layer	67.4 ± 5.1	501 ± 15	3.7 ± 0.7
	Outer layer	149.3 ± 22.9	389 ± 17	
	Average	108.4 ± 14	445 ± 16	
2	Inner layer	137.4 ± 19.1	413 ± 22	5.1 ± 0.9
	Outer layer	210.8 ± 19.1	302 ± 23	
	Average	174.1 ± 19.1	358 ± 23	

[a] Of entire fish.
[b] Based on wet weight of muscle tissue. Each value represents average and average deviation of four fish.

Table III. Changes in the Sodium and Potassium Ion Content of the Muscle Tissue and in the Weight of Herring Held for 1 Week in Various Immersing Solutions

Solution No.	Composition of Solution[a]	Mg./100 G.[b] Na^+	Mg./100 G.[b] K^+	Wt. Increase, %
...	Control, not immersed	68.4 ± 1.9	446 ± 8	
1	Sea water (full strength)	365 ± 20	305 ± 23	3.4 ± 0.6
2	Solution 1 + K^+	412 ± 21	522 ± 3	3.8 ± 0.6
3	Sea water (half strength)	227 ± 30	267 ± 10	4.3 ± 1.0
4	Solution 3 + K^+	229 ± 9	554 ± 7	5.3 ± 0.6
5	Na^+ and K^- to maintain flesh level constant	79 ± 5	542 ± 19	7.7 ± 0.8

[a] Solution 2. Sea water + 11.61 grams/l. of KCl. Solution 4. Half-strength sea water + 11.97 grams/l. KCl. Solution 5. 2.76 grams/l. of NaCl + 12.33 grams/l. of KCl.
[b] Based on wet weight of muscle tissue. Each value represents average and average deviation of six fish.

appreciable levels of fat as compared to herring or salmon, it can be considered also as representative of the nonfatty fish. When lemon sole were immersed in half-strength sea water for 1 week (Table IV), the sodium ion uptake and potassium ion loss greatly exceeded those for sockeye salmon and herring held under comparable conditions (Tables III and VI) and the weight increase, 17.5%, far exceeded that for any of the other species of fish examined. A solution of sodium and potassium chlorides designed to maintain the flesh levels constant again came close to achieving the desired effect (solution 2). Such a solution can be readily prepared from sea water by mixing 1 part of sea water with 9 parts of fresh water and adding potassium chloride. When tested (solution 3) it caused some rise in sodium ion, but maintained the potassium ion level in the flesh of the lemon sole close to the value at the start of the experiment.

Little difficulty is experienced in preparing solutions which will maintain the level of sodium and potassium ions in the flesh at or near their natural values. Fish immersed in such solutions, however, usually gained more weight than when stored in sea water. As no significant net uptake of salt was involved when these solutions were used, the weight increase observed could only be due to uptake of water by the flesh. Water uptake would be expected to occur if the osmotic pressure of the immersing solution was lower than that of the tissue fluids of the fish. It was therefore desirable to find an agent which would be sufficiently soluble to provide the required osmotic pressure in the solution but be impermeable to the tissue membranes of the fish. Such an agent would be expected to have properties similar to those of the compounds employed to maintain osmotic pressure in plasma substitutes (*4*). A compound used for the latter purpose, polyvinylpyrrolidone (PVP) was tested to determine its capacity to prevent weight increases in lemon sole (Table V). Only 2% of this compound prevented water uptake from a solution of sodium and potassium chlorides and from full and half-strength sea water. Separate experiments showed that higher concentrations of polyvinylpyrrolidone actually caused the fish to lose weight.

Discussion

To compare the extent of the changes occurring in various species of fish immersed in refrigerated sea water, the results for fish held under comparable conditions for 1 week are summarized in Table VI. The smallest changes in sodium and potassium ions occurred in halibut. As the changes recorded represent the average for the flesh of the fish taken in cross section, the larger the fish the less the change that would be expected in such values, if insufficient time had elapsed for complete equilibration of the flesh with the immersing solution. As Table II showed, the inner layer of the flesh of halibut had not reached the sodium ion content of the outer layer at the end even of the second week of immersion.

For fish of the same weight one might expect the greatest changes to occur with fish having the highest ratio of surface area to body weight. Although this would appear to be borne out by a comparison of the changes in herring and the first group of lemon sole (Table VI), a second group of lemon sole showed a weight increase similar to that in the herring and much less than in the first group of fish. The first group of lemon sole were caught in March and contained no visible milt or roe, while the second group were captured in November and contained well developed gonads. Such marked differences in the physiological state of the living fish could well have an influence on the extent of changes occurring in storage. It may be of significance that the skin of herring (*12*) and salmon (*15*, *17*) has been observed to

Table IV. Variations in Sodium and Potassium Ion Content of Flesh and Weight Changes in Lemon Sole Held for 1 Week in Solutions of Various Compositions

Solution, No.	Composition of Solution[a]	Mg./100 G.[b] Na^+	Mg./100 G.[b] K^+	Wt. Increase, %
...	Control (not immersed)	89 ± 1.0	387 ± 2	...
1	Sea water (half strength)	311 ± 9	138 ± 4	17.5 ± 1.6
2	Na^+ and K^+ to maintain flesh level constant	113 ± 1.4	405 ± 3	18.2 ± 1.4
3	1 part sea water + 9 parts fresh water + K^+ to maintain flesh level constant	115 ± 2	378 ± 18	22.9 ± 4.3

[a] Solution 2. 2.35 grams of NaCl and 10.24 grams of KCl per l. Solution 3. Indicated amounts of sea water + 10.3 grams/l. of KCl.
[b] See Table III.

Table V. Capacity of Polyvinylpyrrolidone to Prevent Weight Increases in Lemon Sole Immersed in Salt Solutions

Solution, No.	Salt Composition of Immersing Solution[a]	Polyvinylpyrrolidone Addition, %	Wt. Change[b]
1	Na^+ and K^+ to maintain flesh level constant	0	+ 8.5 ± 1.3
2	Same as solution 1	2	+ 0.7 ± 0.9
3	Sea water (half strength)	0	+ 7.8 ± 1.5
4	Sea water (half strength)	2	+ 0.1 ± 1.4
5	Sea water (full strength)	0	+11.5 + 2.4
6	Sea water (full strength)	2	+ 0.8 ± 0.6

[a] Solution. 1. 2.37 grams of NaCl and 7.9 grams of KCl per l.
[b] Each value represents average and average deviation of 10 fish.

Table VI. Sodium and Potassium Ions and Weight Changes in Various Species of Fish Held 1 Week in Half-Strength Refrigerated Sea Water

Species	Weight, Lb.	Change Recorded,[a] Mg./100 G. Na^+	Change Recorded,[a] Mg./100 G. K^+	Wt. Increase, %
Sockeye salmon	5.29 ± 0.23	+115	−146	2.4 ± 0.6
Halibut	15.2 ± 1.7	+ 61	− 56	3.7 ± 0.7
Herring	0.33 ± 0.04	+159	−179	4.3 ± 1.0
Lemon sole				
1	0.65 ± 0.08	+222	−249	17.5 ± 1.6
2	1.0 ± 0.05	+273	−266	5.5 ± 1.9
Brill (*Eopsetta jordani*)	1.7 ± 0.27	+223	−213	7.8 ± 1.4

[a] Change recorded in Na^+ and K^+ levels represents difference between amounts present before and after immersion for 1 week in half-strength sea water.

constitute a greater proportion of the body weight of the fish just prior to spawning than at other stages in their cycle of maturation.

In relation to the other fish (Table VI) both the herring and salmon appear to have increased in weight less than might have been expected from the extent of the changes in their sodium and potassium levels. Whether this means that the osmotic pressure of the intracellular fluids is lower in these fish than in the others or that the higher fat content of the two species specifically impeded the uptake of water cannot be determined from the data.

The fact that storage of fish in refrigerated sea water produces an uneven distribution of sodium ion in the flesh indicates that analyses based on the official method of sampling (*1*) in this case provide insufficient information for marketing purposes. A situation could conceivably arise wherein the outer layer of flesh of a fish contained an unpalatable level of sodium ion, the inner layer remained almost unchanged, while a value based on the cross-sectional sample specified in the official method indicated a level of sodium ion within the limits of palatability.

There have been many studies in which hydration of mammalian tissues has been observed in salt solutions isoosmotic with blood and tissue fluids, and there are conflicting explanations to account for the phenomenon (*8, 16, 18*). In this study, where the ion concentrations of the immersing solution were adjusted to maintain the flesh levels constant, water uptake must have been due primarily to the hypertonicity of the intracellular fluids of the flesh. This was further substantiated by the observation that a nondiffusible substance capable of increasing the osmotic pressure of the immersing solution prevented water uptake by the fish.

Polyvinylpyrrolidone is a chemically inert, hydrophilic, water-soluble macromolecule essentially nontoxic for humans by all routes of administration. The compound is widely used in the formulation of pharmaceuticals and has been recommended for use as a beverage clarifier (*5*).

In the practical application of the results reported here it would be feasible to premix and package sodium chloride, potassium chloride, and polyvinylpyrrolidone for convenient addition to tanks of fresh water on fishing vessels or in shore plants.

Acknowledgment

The authors are indebted to R. J. Bose and Bir Mullick, who assisted in carrying out certain phases of this investigation.

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COFFEE VOLATILES

Analysis of the Volatile Constituents of Coffee

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A METHOD OF SAMPLING roast and ground coffee for analysis of coffee volatiles by gas chromatography has previously been reported (*3*). A few modifications in procedure and equipment have made it possible to obtain quantitative data on 19 volatile constituents of coffee.

Data on the green beans and beans roasted at various temperatures have been obtained for seven trade varieties of coffee. Analysis of 13 retail brands of coffee has been performed and the data presented for comparative purposes.

Brewed coffee has been analyzed using the same sampling technique. By use of comparative analysis of brewed coffee and coffee suspended in water, it has been possible to calculate the percentage of each constituent which is extracted and retained in freshly brewed coffee. Concentrations of volatiles in brewed coffee are shown to be in the range of a few parts per billion to 1 to 2 p.p.m.

Eighteen of the chromatogram peaks have been assigned tentative identifications.

Evaluation of the final roast data for the seven trade varieties indicates a possible relationship between the degree of roasting and the ratio of diacetyl to acetyl propionyl. The green bean analysis shows differences which may be helpful in typing or grading green coffee.

Experimental

Apparatus and Procedure. The apparatus used in this work is essentially that previously described (*3*). The major modifications are: Original column replaced by a 4-meter column of 15% Carbowax 1500 on 30- to 60-mesh Chromosorb. Recorder sensitivity increased from 10 mv. full scale to 2.5 mv. full scale.

The sampling procedure was changed only to the extent of a decreased carbon

constitute a greater proportion of the body weight of the fish just prior to spawning than at other stages in their cycle of maturation.

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COFFEE VOLATILES

Analysis of the Volatile Constituents of Coffee

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A METHOD OF SAMPLING roast and ground coffee for analysis of coffee volatiles by gas chromatography has previously been reported (*3*). A few modifications in procedure and equipment have made it possible to obtain quantitative data on 19 volatile constituents of coffee.

Data on the green beans and beans roasted at various temperatures have been obtained for seven trade varieties of coffee. Analysis of 13 retail brands of coffee has been performed and the data presented for comparative purposes.

Brewed coffee has been analyzed using the same sampling technique. By use of comparative analysis of brewed coffee and coffee suspended in water, it has been possible to calculate the percentage of each constituent which is extracted and retained in freshly brewed coffee. Concentrations of volatiles in brewed coffee are shown to be in the range of a few parts per billion to 1 to 2 p.p.m.

Eighteen of the chromatogram peaks have been assigned tentative identifications.

Evaluation of the final roast data for the seven trade varieties indicates a possible relationship between the degree of roasting and the ratio of diacetyl to acetyl propionyl. The green bean analysis shows differences which may be helpful in typing or grading green coffee.

Experimental

Apparatus and Procedure. The apparatus used in this work is essentially that previously described (*3*). The major modifications are: Original column replaced by a 4-meter column of 15% Carbowax 1500 on 30- to 60-mesh Chromosorb. Recorder sensitivity increased from 10 mv. full scale to 2.5 mv. full scale.

The sampling procedure was changed only to the extent of a decreased carbon

Studies on the roasting of seven varieties of coffee show that generally the concentration of volatiles in the bean increases with increased roasting. Hydrogen sulfide, methyl formate, and acetyl propionyl reach a maximum concentration within the normal roasting temperatures. The concentration of dimethyl sulfide remains essentially constant during the roasting process. Analysis of green beans shows differences which may be useful in typing or grading green coffee. In roasted coffee, the ratio of diacetyl to acetyl propionyl may be indicative of the degree of roast. Analysis of brewed coffee shows the concentration of volatiles to be in the range of 1 to 2000 p.p.b.

dioxide venting time, which then allowed a recollection of hydrogen sulfide without excessive recollection of carbon dioxide. The over-all sensitivity of the instrument was checked by use of a standard injection of acetone prior to the analysis of a coffee sample instead of using toluene as an internal standard.

The trade variety coffee beans were prepared by use of the following equipment. Gas-fired Thermalo roaster (Burns) with Foxboro controllers of 500-pound green bean capacity. Stir-flex coolers (Jabez Burns and Sons, Inc.), vacuum coffee can closing machine (Canco).

The beans were extracted from the roaster at predetermined temperatures, cooled, and the whole beans were vacuum packed under approximately 29 inches of vacuum.

The cans were opened and the beans were ground, using a coffee grinding attachment on a Waring Blendor, just prior to analysis. The green beans, being too tough for the coffee grinding attachment, were reduced to 20- to 40-mesh with the conventional Waring Blendor blades.

A 10-gram sample was used for the analysis of the green bean, the 300°, and the 325° F. roast. All other analyses were performed using a 5-gram sample.

Chromatograms and Calculations. The method of determining peak areas by multiplying the peak height by the peak width at half peak height requires prior knowledge of the sample so that each peak may be properly attenuated for best chart presentation. Many of the coffee samples would therefore have required a separate determination merely to establish the attenuator settings to be used in the actual analysis. Attenuation of peaks, when the instrument is not at recorder zero, results in base line shifts and makes peak height determinations difficult, especially when separations are not complete.

In order to eliminate these problems and to avoid time-consuming reruns, the chromatograms were obtained with peak bases always recorded at the most sensitive setting, and attenuating as necessary to obtain the peak maximums. With the peak bases all recorded at the same sensitivity, it is then relatively simple to establish the base line for the entire chromatogram. By knowing the recorder zero position, it is a simple matter to establish what the base line would be at the attenuation setting used to record a peak maximum. If a peak maximum was obtained at an attenuation factor of four, then the base line to use for measurement of peak height would be one-quarter of the distance up from the recorder zero to the base line established at the most sensitive setting. The true peak height is then determined by multiplying the measured peak height by the attenuation factor. The relative peak areas are calculated from the product of peak height and retention time.

Results obtained by this method were in good agreement with the results of a rerun of the same coffee in which the areas were determined by peak height times peak width at half peak height.

Materials. The trade varieties of coffee were roasted and vacuum packed (Jos. Martinson and Co., Inc.) under the direction of personnel of the Coffee Brewing Institute. The 13 retail brands of coffee were obtained from the Coffee Brewing Institute and coffee for brewed analysis was purchased locally.

Analysis was performed on the green bean and on samples roasted to 300°, 325°, 350°, 375°, and 400° F. and at varying final temperatures for each trade variety. The varieties and final roasting temperature are as follow:

Variety	Final Roasting Temperature, ° F.
Santos	402
Maracaibo Cuban	419
Medellin Armenia	421
Past crop Colombia	430
Old crop Bucaramanga	431
Peruvian	413
Central America	407

Results and Discussion

Acetaldehyde, acetone, and methanol have been definitely identified previously by infrared spectra of the collected component (*3*). Methyl formate, furan, propionaldehyde, isobutyraldehyde, methylfuran, butyraldehyde, methyl ethyl ketone, isovaleraldehyde, and ethyl alcohol were tentatively identified on the basis of retention times. Hydrogen sulfide, isoprene, methyl mercaptan, dimethyl sulfide, diacetyl, and acetyl propionyl were tentatively identified on the basis of their distinctive odors and retention times.

A study of the complete data indicates that the concentration increases with increasing roasting temperature for a majority of the compounds analyzed. The exceptions to this steady increase are: Hydrogen sulfide reaches a maximum concentration at about 350° F. and then rapidly decreases. Methyl mercaptan shows a slight decrease be-

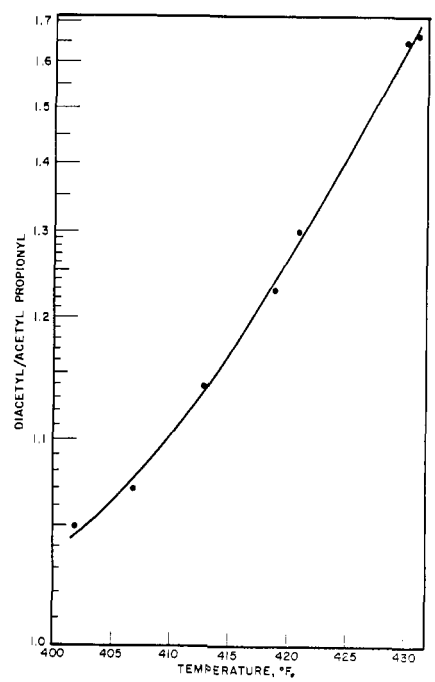

Figure 1. Relationship between degree of roast and the ratio of diacetyl to acetyl propionyl

tween 350° and 375° F. and then continues to increase. This decrease is attributed to the onset of physical changes in the bean which permits a greater rate of loss. Dimethyl sulfide shows a constant concentration during roasting. Methyl formate reaches a maximum at about 400° F. in four of the seven varieties. Acetyl propionyl appears to reach a maximum concentration at about 425° F.

Prior to the assignment of tentative identifications, the peaks were assigned numbers according to their relative retention times, on the basis of toluene as 1000. Table I, consisting of only a portion of the data, shows the results obtained for the green bean, the 350° F. roast, and the final roast, for four of the varieties analyzed.

Although there are insufficient data on the green beans to form any definite conclusions, a few interesting results are evident. Of the 19 compounds found in roasted coffee, 16 were detected in the green bean of one or more of the seven varieties analyzed. Dimethyl sulfide, although occurring at a constant concentration during the roasting process for a given variety (exception is old crop Bucaramanga), differs considerably in amount from one variety to another. Medellin, noted for its aromatic nature, has seven times as much as Santos and would indicate that a measure of the dimethyl sulfide content of green beans may be indicative of the aromatic nature of the beans in question. The green bean data for Maracaibo, Medellin, Peruvian, and Central America is sufficiently different from the other varieties for a number of compounds as to indicate a type or class difference. Only a statistical analysis of green bean data and organoleptic tests of the roasted coffee could satisfactorily determine if a relationship exists between green bean data and flavor or aroma.

A study of the final roast data indicates that the degree of roasting cannot be determined by the absolute amount of any particular volatile constituent. For the 400° F. roasts of six of the varieties studied, the ratio of diacetyl to acetyl propionyl is constant at 0.84 ± 0.04. Santos was not sampled at 400° F. The ratio of diacetyl to acetyl propionyl constantly increases with roasting for temperatures over 400° F. as shown below:

Variety	Final Roast, ° F.	Ratio Diacetyl/ Acetyl Propianyl
Santos	402	1.05
Central America	407	1.07
Peruvian	413	1.14
Maricaibo, Cuban	419	1.23
Medellin, Armenia	421	1.30
Past crop Colombia	430	1.65
Old crop Bucaramanga	431	1.67

This relationship is graphically presented in Figure 1 and indicates a possible method for determining the degree of roast. For a double-roasted demitasse coffee (data not included) the ratio was 2.77. An approximate assignment of these ratio values might be made as follows:

Ratio	Degree of Roast
<1.1	Light
1.1 to 1.3	Medium
>1.3	Dark

Table I. Results of Analyses for Green Beans, 350° F. Roast, and Final Roast for Four Varieties

Source[a]	° F.	Peaks and Compounds: 44 Hydrogen Sulfide	69 Isoprene	86 Methyl Mercaptan	105 Acet-aldehyde	130 Dimethyl Sulfide	150 Methyl Formate	170 Furan	190 Propion-aldehyde	220 Isobutyr-aldehyde	250 Acetone
		Toluene Equivalents, γ/G.									
Col.	430	1.64	1.01	3.82	48.8	1.21	10.9	4.40	8.58	19.9	80.0
Med.	421	1.98	1.21	2.43	55.1	3.44	13.2	4.95	10.1	16.3	79.1
Cen.	407	1.53	1.06	1.56	56.9	0.71	12.5	3.42	8.16	12.8	53.7
San.	402	4.26	0.69	2.30	50.3	0.48	9.70	2.20	6.20	15.0	42.2
Col.	350	12.2	0.04	3.02	12.6	1.13	0.71	0.37	0.73	19.0	8.17
Med.	350	6.46	0.10	1.75	12.6	3.74	1.50	0.56	0.91	14.2	11.2
Cen.	350	4.88	0.13	1.35	13.3	0.72	2.09	0.57	0.93	10.8	11.5
San.	350	13.2	0.08	2.30	15.0	0.46	1.30	0.35	0.73	13.0	9.10
Col.	Green	0.50	...	0.26	3.97	1.05	...	0.03	0.08	1.10	4.02
Med.	Green	0.56	...	0.10	1.60	3.54	...	...	0.05	0.18	0.90
Cen.	Green	0.93	...	0.12	1.86	0.55	...	0.02	0.08	0.20	0.90
San.	Green	0.28	...	0.19	3.70	0.50	...	0.02	0.14	0.66	4.90

		Peaks and Compounds: 290 Methyl-furan	350 Butyr-aldehyde	450 Methyl Ethyl Ketone	470 Isovaler-aldehyde	540 Unknown	630 Methanol	780 Diacetyl	850 Ethanol	1420 Acetyl Propionyl
	° F.	Toluene Equivalents, γ/G.								
Col.[a]	430	13.8	0.68	22.5	2.82	0.87	100	16.5	1.95	10.0
Med.	421	14.7	0.73	19.9	3.60	1.00	91.0	18.8	4.00	14.5
Cen.	407	8.79	0.51	14.9	2.47	0.53	87.9	19.7	1.93	18.4
San.	402	4.90	0.34	15.1	2.00	0.32	119	16.0	0.60	15.2
Col.	350	0.39	0.06	15.3	1.79	...	77.5	1.24	1.06	1.59
Med.	350	0.67	0.07	12.2	1.97	0.07	101	1.73	4.08	2.53
Cen.	350	0.83	0.10	9.87	1.18	...	107	1.77	2.20	3.24
San.	350	0.42	0.07	10.2	1.20	0.07	96.1	1.60	0.26	2.10
Col.	Green	...	0.03	1.10	1.57	...	7.41	0.27	0.65	...
Med.	Green	...	0.04	0.19	0.28	...	12.4	0.08	3.51	...
Cen.	Green	0.06	0.06	0.21	0.25	...	13.4	0.09	1.72	...
San.	Green	0.16	0.07	0.62	0.80	0.17	14.0	0.27	0.46	...

[a] Col., past crop Colombia; Med., Medellin Armenia; Cen., Central America; San., Santos.

The complete data on the 13 retail brands of coffee are presented in Table II and include the diacetyl-acetyl propionyl ratio for each coffee. No attempt has been made to evaluate these data as no information on the roasting of these coffees was available.

Table III summarizes the data on green beans and several roasted coffees giving the normal concentration ranges of the various volatile compounds in the dry coffee.

Brewed Coffee. Experimental Results

The ability of helium sweep gas to strip coffee volatiles from an aqueous solution was demonstrated as follows: A 5-gram sample of dry coffee was first analyzed to determine the total amount of each volatile constituent present. The dry coffee sampler was then replaced with a 100-ml. flask containing 5 grams of the coffee in 80 ml. of distilled water. The flask and contents were heated to 100° C. and the helium sweep gas was bubbled through the suspension of coffee in water at the same rate as with the dry coffee sample. The stripped volatiles were collected and analyzed in the usual manner. The data from these two analyses are presented in Table IV (A-1, A-2). Neglecting alcohols, the recovery of volatiles is comparable with the exception of an increase in hydrogen sulfide from the aqueous sample, and a slight decrease in diacetyl and acetyl propionyl from the aqueous sample. The general agreement in recovery of volatiles from these two analyses indicates the sampling method is applicable to aqueous coffee solutions within the afore-mentioned limitations.

By increasing the sampling time of the aqueous sample to one and one half hours (50% increase), the diacetyl and acetyl propionyl values approach the values obtained by analysis of the dry coffee. The hydrogen sulfide value, however, increased by 50%, or in direct proportion to the increased sampling time. It would therefore appear that hydrogen sulfide may well be a product of hydrolysis in the brewing of coffee.

Analysis of 5 grams of coffee in 80 ml. of distilled water and 80 ml. of tap water was performed to determine the effect of different water on coffee volatiles. The data on this test are shown in Table IV (B-1, B-2). The results of the two analyses are very similar with the single exception of hydrogen sulfide, which is markedly reduced in the tap water sample. This reduction in hydrogen sulfide is attributed to the type and/or amount of dissolved solids in the tap water.

Brewed coffee was prepared in a clean household drip coffee pot using 50 grams of coffee and 800 ml. of boiling distilled water. Two types of brewed coffee were prepared, one from a freshly opened can of coffee, and the other from a can which had been open for 6 days. The recovery of brewed coffee in each case was 665 ± 5 ml. An 80-ml. aliquot of each brewed coffee was then analyzed to determine the volatiles retained in the brew. A 5-gram sample of each coffee in 80 ml. of distilled water was also analyzed to determine the volatile content of the dry coffee equivalent to that required for the 80-ml. aliquot of the brewed coffee. The total amount of each volatile constituent in the 80-ml. aliquot expressed as a percentage of the total amount found in the corresponding 5 grams of dry coffee can then be calculated, and has been designated "per cent retained."

The results of the above analyses are reported in Table IV. Although the per cent retained in the brewed coffee

Table II. Data on Thirteen Retail Brands of Coffee

Brand	Peaks and Compounds									
	44 Hydrogen Sulfide	68 Isoprene	86 Methyl Mercaptan	105 Acet-aldehyde	130 Dimethyl Sulfide	150 Methyl Formate	170 Furan	190 Propion-aldehyde	220 Isobutyr-aldehyde	250 Acetone
	Toluene Equivalents, γ/G.									
A	0.46	0.48	1.33	45.0	0.98	6.92	1.85	5.38	11.5	38.0
B	1.64	0.50	1.61	44.1	0.98	8.42	1.98	6.20	10.6	38.8
C	1.30	0.39	1.40	56.1	0.15	7.20	2.07	6.30	13.0	44.1
D	1.95	0.58	2.75	60.0	0.54	8.95	2.58	8.37	11.8	53.8
E	2.65	0.11	2.10	43.6	0.49	8.64	3.10	6.61	13.3	51.2
F	2.06	0.46	1.76	46.6	1.58	8.28	1.94	6.78	11.4	42 0
G	2.28	0.64	1.61	45.0	0.82	9.15	2.22	6.93	12.5	46.3
H	3.31	0.43	2.09	49.1	0.24	9.25	1.86	6.26	12.3	42.2
I	2.18	0.41	1.26	59.5	0.58	8.36	2.08	6.46	9.86	44.0
J	2.50	0.53	1.85	45.0	0.88	9.15	2.15	6.41	12.6	44.4
K	1.46	0.68	2.32	49.2	0.81	10.9	3.29	8.10	13.9	65.0
L	2.61	0.42	1.48	44.8	1.73	5.54	1.79	5.22	13.5	35.8
M	3.28	0.59	2.36	63.0	1.17	5.32	2.49	7.20	15.0	50.4

Brand	Peaks and Compounds									
	290 Methylfuran	350 Butyr-aldehyde	450 Methyl Ethyl Ketone	470 Isovaler-aldehyde	540 Unknown	630 Methanol	780 Diacetyl	850 Ethanol	1430 Acetyl Propionyl	Ratio 780/1420
	Toluene Equivalents, γ/G.									
A	4.65	0.32	12.9	1.86	0.30	87.4	12.6	4.55	11.9	1.06
B	4.96	0.34	13.1	1.84	0.33	80.6	12.6	3.60	14.0	0.90
C	4.50	0.34	14.4	2.25	0.30	98.0	15.8	2.30	13.1	1.20
D	7.30	0.51	14.4	2.72	0.56	80.1	16.6	2.56	13.1	1.27
E	7.35	0.41	15.4	2.02	0.41	75.0	11.0	2.85	8.11	1.36
F	5.54	0.37	14.1	2.88	0.41	82.8	13.6	6.00	14.7	0.93
G	6.56	0.39	15.3	1.99	0.48	80.6	14.1	2.42	15.7	0.90
H	4.87	0.36	14.5	1.72	0.37	76.5	14.2	1.72	12.9	1.10
I	4.96	0.41	13.0	2.15	0.33	81.6	17.5	3.15	14.4	1.21
J	5.85	0.39	15.2	2.00	0.41	72.0	14.1	3.12	14.4	0.98
K	9.97	0.65	16.9	2.46	0.75	69.3	15.3	2.48	13.3	1.15
L	4.16	0.30	14.2	2.25	0.30	66.2	13.1	3.23	13.3	0.99
M	5.85	0.44	16.7	2.70	0.41	84.4	18.9	3.82	15.8	1.20

is variable for the different compounds, for the same compound the per cent retained in the two samples is generally in agreement. From these per cent-retained figures, it should therefore be possible to calculate, with reasonable accuracy, the amount of each constituent which would occur in a freshly prepared cup of coffee from the dry coffee data of Tables I and II.

Table V presents the data for brewed coffee, both fresh and 6-day-old, and the data for instant coffee at the concentrations found in normal preparation. Patton and Josephson (*2*) have determined the following taste threshold concentrations: methyl mercaptan, 0.002 p.p.m.; acetaldehyde, 1.3 p.p.m.; and dimethyl sulfide, 0.012 p.p.m.

Wales and Harmon (*5*) have determined diacetyl in cottage cheese in the range of 0.7 to 10.0 p.p.m. and Campbell *et al.* (*1*) give the taste threshold concentration of hydrogen sulfide as 0.05 p.p.m. in water and 0.12 p.p.m. in brewed coffee. These materials apparently occur in brewed coffee in the concentration ranges which could affect flavor or aroma, especially if one considers that the instrument was not calibrated for specific compounds.

Since completion of the work reported here, papers on the analysis of coffee volatiles have been presented by Sullivan, Robertson, and Merritt (*4*) and Zlatkis and Sivetz (*6*). Although Sullivan and Zlatkis each report the identity of about 30 compounds, only 14 compounds were identified by both. Twelve of these 14 compounds were also identified by this investigator. There are many quantitative differences which are probably due to the different methods of sample preparation and collection, as

Table III. Data on Concentrations of Volatile Compounds of Green Beans, Several Roasted Coffees, and Dry Coffee

Peak	Compound	Trade Varieties: Green bean	Trade Varieties: Final roast	Retail Bronds
		Concentration, γ/G.		
44	Hydrogen sulfide	0.1 - 1.0	0.7- 4.3	0.4- 3.3
69	Isoprene	0	0.6- 1.4	0.1- 0.7
86	Methyl mercaptan	0.1 - 0.3	1.5- 4.3	1.2- 2.8
105	Acetaldehyde	1.6 - 5.0	45.0- 70.0	44.0- 65.0
130	Dimethyl sulfide	0.1 - 3.5	0.5- 3.5	0.5- 1.8
150	Methyl formate	0	9.0- 13.5	5.0- 11.0
170	Furan	0 - 0.03	2.0- 6.0	1.8- 3.3
190	Propionaldehyde	0.05- 0.4	6.0- 11.0	5.0- 8.5
220	Isobutyraldehyde	0.2 - 1.2	15.0- 20.0	10.0- 14.0
250	Acetone	0.6 - 5.0	40.0-100.0	35.0- 55.0
290	Methylfuran	0 - 0.2	5.0- 20.0	4.5- 10.0
350	Butyraldehyde	0.02- 0.07	0.3- 1.0	0.3- 0.5
450	Methyl ethyl ketone	0.2 - 1.3	15.0- 25.0	13.0- 17.0
470	Isovaleraldehyde	0.2 - 2.0	2.0- 4.0	1.7- 2.8
540	Unknown	0 - 0.2	0.3- 1.0	0.3- 0.8
630	Methanol[a]	5.0 -15.0	60.0-100.0	65.0-100.0
780	Diacetyl	0.07- 0.3	16.0- 20.0	11.0- 19.0
850	Ethyl alcohol[a]	0.2 - 4.0	0.6- 4.0	1.7- 6.0
1420	Acetyl propionyl	0	10.0- 19.0	8.0- 16.0

[a] Data unreliable.

Table IV. Analysis of Stripped Coffee Volatiles

	Peaks and Compounds: 44 Hydrogen Sulfide	69 Isoprene	86 Methyl Mercaptan	105 Acetaldehyde	130 Dimethyl Sulfide	150 Methyl Formate	170 Furan	190 Propionaldehyde	220 Isobutyraldehyde	250 Acetone
	Total Toluene Equivalents, γ									
A-1	8.46	1.51	8.05	199	6.40	22.6	6.71	24.6	54.7	162
A-2	13.7	1.65	9.16	185	6.59	20.8	6.71	22.7	54.2	154
B-1	13.9	1.51	9.08	187	6.40	20.2	6.40	22.0	51.6	162
B-2	2.15	1.43	9.50	188	6.04	20.7	6.40	22.0	51.6	154
C-1	11.4	2.51	9.66	190	10.7	26.0	9.00	23.2	57.8	152
C-2	4.82	0.21	4.46	139	5.00	17.5	3.58	16.1	37.6	156
Retained, %	42	8	46	73	47	68	40	69	65	103
D-1	9.90	0.96	8.75	159	2.76	14.5	5.10	18.6	41.3	108
D-2	4.30	0.12	4.24	111	1.59	10.3	2.11	11.9	33.4	100
Retained, %	44	12	49	70	58	70	41	64	81	93

	Peaks and Compounds: 290 Methylfuran	350 Butyraldehyde	450 Methyl Ethyl Ketone	470 Isovaleraldehyde	540 Unknown	630 Methanol	780 Diacetyl	850 Ethanol	1420 Acetyl Propionyl
	Total Toluene Equivalents, γ								
A-1	17.7	1.35	62.0	10.9	1.12	292	69.1	10.2	64.2
A-2	17.2	1.11	60.0	10.5	1.05	50	49.3	2.36	54.7
B-1	16.0	1.10	58.3	10.3	0.92	47.0	50.8	1.75	57.8
B-2	15.7	0.98	55.3	9.95	0.76	51.0	49.8	2.06	51.1
C-1	21.9	1.22	60.4	10.5	0.94	57.5	46.0	2.34	49.3
C-2	6.20	0.49	39.0	5.80	0.00	66.0	50.8	2.66	53.6
Retained, %	28	40	65	55	0	115	110	114	109
D-1	11.7	0.73	42.0	7.22	0.0	38.2	32.5	1.75	32.8
D-2	3.10	0.0	25.2	3.65	0.0	36.8	32.8	1.78	32.0
Retained, %	26	0	60	50	0	97	101	102	98

Table V. Data for Brewed and Instant Coffee

Preparation	Peaks and Compounds: 44 Hydrogen Sulfide	69 Isoprene	86 Methyl Mercaptan	105 Acetaldehyde	130 Dimethyl Sulfide	150 Methyl Formate	170 Furan	190 Propionaldehyde	220 Isobutyraldehyde	250 Acetone
	Concentration, P.P.M.									
1[a]	0.060	0.0026	0.056	1.74	0.063	0.22	0.045	0.20	0.47	1.95
2[b]	0.054	0.0015	0.053	1.39	0.020	0.13	0.026	0.15	0.42	1.25
3[c]	0.010	0.0000	0.022	0.71	0.000	0.02	0.004	0.038	0.14	0.55

	Peaks and Compounds: 290 Methylfuran	350 Butyraldehyde	450 Methyl Ethyl Ketone	470 Isovaleraldehyde	540 Unknown	630 Methanol	780 Diacetyl	850 Ethanol	1420 Acetyl Propionyl
	Concentration, P.P.M.								
1[a]	0.078	0.006	0.49	0.073	0	0.83	0.64	0.032	0.67
2[b]	0.039	0.000	0.33	0.047	0	0.46	0.41	0.022	0.40
3[c]	0.008	0.000	0.11	0.019	0	0.03	0.24	0.000	0.11

[a] Prepared from fresh coffee using 10.6 grams of coffee per 170 ml. of boiling, distilled water.
[b] Preparred from 6-day-old coffee using same proportion as 1.
[c] Prepared from an instant coffee using 1.91 grams per 170 ml. of distilled water.

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VEGETABLE FLAVORS

Flash Exchange Gas Chromatography for the Analysis of Potential Flavor Components of Peas

JACK W. RALLS

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agriculture Research Service, U. S. Department of Agriculture, Albany 10, Calif.

The technique of flash exchange gas chromatography was applied to the determination of aldehydes, ketones, and acids in the steam distillate of peas. The compounds identified were acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, biacetyl, formic acid, acetic acid, and isovaleric acid. All these components occurred at a concentration of less than 3 p.p.m. on a fresh-weight basis. A mixture of these compounds at the concentration levels found did not reproduce the characteristic odor of pea steam distillate.

THE LONG-RANGE GOAL of flavor research is to improve flavor so that abundant, nutritious foods will be consumed at an increased rate. It is most desirable that rapid techniques be developed which could be applied to small, experimental quantities of material. Accuracy need not be greater than that required for a starting point in taste panel evaluation. With this type of research tool, studies of plant breeding, crop selection, harvesting practice, processing, and storage could be made with improved flavor as an objective.

A useful technique for the determination of carbonyl compounds and acids was developed which required only a few milligrams of compounds (*3*). Aldehydes, ketones, and acids are isolated as solid derivatives, regenerated in rapid exchange reactions, and volatilized

Table V. Data for Brewed and Instant Coffee

Preparation	Peaks and Compounds: 44 Hydrogen Sulfide	69 Isoprene	86 Methyl Mercaptan	105 Acetaldehyde	130 Dimethyl Sulfide	150 Methyl Formate	170 Furan	190 Propionaldehyde	220 Isobutyraldehyde	250 Acetone
	Concentration, P.P.M.									
1[a]	0.060	0.0026	0.056	1.74	0.063	0.22	0.045	0.20	0.47	1.95
2[b]	0.054	0.0015	0.053	1.39	0.020	0.13	0.026	0.15	0.42	1.25
3[c]	0.010	0.0000	0.022	0.71	0.000	0.02	0.004	0.038	0.14	0.55

	Peaks and Compounds: 290 Methylfuran	350 Butyraldehyde	450 Methyl Ethyl Ketone	470 Isovaleraldehyde	540 Unknown	630 Methanol	780 Diacetyl	850 Ethanol	1420 Acetyl Propionyl
	Concentration, P.P.M.								
1[a]	0.078	0.006	0.49	0.073	0	0.83	0.64	0.032	0.67
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A useful technique for the determination of carbonyl compounds and acids was developed which required only a few milligrams of compounds (*3*). Aldehydes, ketones, and acids are isolated as solid derivatives, regenerated in rapid exchange reactions, and volatilized

Table I. 2,4-DNPH Prepared from Steam Distillate of Peas

Run No.	2,4-DNPH, Mg. 1958[a]	1959[b]
1	25.4	11.7
2	21.2	11.5
3	23.6	8.9
4	15.1	11.3

[a] Insoluble 2,4-DNPH collected by filtration.
[b] Extracted. Filtration gave 0.7 to 2.8 mg. of 2,4-DNPH.

Table II. Concentration of Volatile Carbonyl Compounds in Initial Steam Distillate of Peas

Compound	Av. Concn., Mg./Kg. Fresh Wt. 1958	1959
Acetaldehyde	2.4	0.56
Acetone	0.35	0.12
Propionaldehyde	0.17	0.00
n-Butyraldehyde	0.13	0.06
n-Valeraldehyde	0.00	0.44

Table III. Change in Volatile Carbonyl Composition of Steam Distillate of Peas (1958) with Time

Time, Min.	Volume Distillate, Ml.	Weight 2,4-DNPH, Mg.	Weight Due to Biacetyl	Carbonyl Content, Mg./Kg., Fresh Wt.	
				Biacetyl	0.61
				Acetaldehyde	0.62 (0.70)
				Acetone	0.14 (0.52)
				Propionaldehyde	0.046 (0.10)
				n-Butyraldehyde	0.055 (0.11)
33	275	15.1	6.27		
57	263	5.05	4.04	Biacetyl	0.39
81	310	3.59	3.58	Biacetyl	0.34
99	258	1.96	3.02	Biacetyl	0.29

directly into a gas chromatography unit for separation and identification. Aldehydes and ketones are determined by regeneration from 2,4-dinitrophenylhydrazones (2,4-DNPH) with α-ketoglutaric acid. Monobasic, carboxylic acids are transformed from their potassium salts into ethyl esters by heating with potassium ethylsulfate.

Experimental

The peas used were portions of large lots of blanched, frozen, Perfection (canners) peas prepared on June 25, 1958, and June 19, 1959, at Milton, Ore. The only difference in the two lots was the earlier maturity and longer fluming time for the 1959 lot. The peas (2-kg. samples) were thawed for 4 hours, ground with 1000 ml. of distilled water in a food blender, and steam distilled at atmospheric pressure with a boiling water bath as a heat source. A heating period of 1 hour was necessary to reach distillation temperature. A first distillate of 250 ml. was collected by passing in clean steam for about a half hour. A gas trap containing 2,4-dinitrophenylhydrazine reagent was used to ensure that all volatile carbonyl compounds were trapped. The distillate was treated with a solution of 200 mg. of 2,4-dinitrophenylhydrazine in 20 ml. of concentrated hydrochloric acid. The turbid suspension was heated to boiling for 5 minutes, cooled slowly to room temperature, and stored overnight in a refrigerator. The 2,4-DNPH was collected by filtration, washed with 2*N* hydrochloric acid, then water, and dried to constant weight at reduced pressure over phosphorus pentoxide. The volatile C_2 to C_6 aldehydes and ketones in the mixed 2,4-DNPH were determined by the flash exchange reaction with excess α-ketoglutaric acid (*3*).

After the predominant derivatives had been removed by filtration, the filtrate and washings were diluted to 450 ml. and extracted continuously with purified petroleum ether for 16 hours. The yellow-colored extract, 250 ml., was washed with three 50-ml. portions of water, dried over anhydrous sodium sulfate, and evaporated.

For the determination of acids, the steam distillate was treated with 0.1*N* potassium hydroxide to pH 8.5 and evaporated. The resulting mixture of potassium salts was dried over phosphorus pentoxide at room temperature and pressure. The volatile C_1 to C_6 monobasic, carboxylic acids were determined by flash exchange with potassium ethylsulfate.

Results and Discussion

Carbonyl compounds are present in the steam distillate of peas at a very low concentration. Indeed, if blanched, frozen, intact peas are heated with water, there is almost no detectable carbonyl compound in the distillate. It was necessary to steam-distill the pulverized peas to get sufficient carbonyl derivative for analysis. The results of several such steam distillations are shown in Table I. These results are in marked contrast to those reported by Silberstein (*5*) and Wager (*6*). Silberstein obtained 60 to 300 mg. of 2,4-DNPH from 2 kg. of peas, by steam distillation under pressure, which may account for part of the difference in results. The discrepancy is due in part to such factors as variety of pea, maturity at harvest, type of blanching used, and storage conditions. Silberstein demonstrated that blanched peas had a much lower volatile carbonyl content than fresh peas.

The results of the analyses of the mixed 2,4-DNPH from pea distillates are shown in Table II. The predominant carbonyl compounds are acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, and biacetyl. The latter compound was determined separately by colorimetric methods and the results are shown in Table III. The difference in amount and distribution of the carbonyl compounds in the 1958 and 1959 pea lots is interesting. The 1959 material had a much lower volatile carbonyl content and was different from the 1958 material by the absence of propionaldehyde and the presence of *n*-valeraldehyde.

A brief study of the variation of carbonyl content with time of distillation was made and the data are shown in Table III. It was found that after the first distillation fraction, the major part of the weight of the precipitated 2,4-DNPH was due to biacetyl bis-(2,4-dinitrophenylhydrazine). The concentration of the biacetyl appears to decrease to a limiting value with time. A separate study (*4*) of acetoin and biacetyl in peas showed that acetoin is the predominant material with a concentration of 260 mg. per kg. of fresh weight. The acetoin is not volatile with steam under the conditions of the distillation.

Extraction of the filtrate after collection of the precipitated 2,4-DNPH produced considerable material (11.7 mg. from the first fraction of 1958 run No. 4). Analysis of the extracted material is shown within parentheses in Table III. It was expected that some trace constituents would appear in the extracted material. This did not occur, because only the four primary carbonyl compounds were found at both the 90° and the 150° C. column temperatures.

Volatile acids in the steam distillate

Table IV. Volatile Acids in Steam Distillate of Peas (1959)

Acid	Concn., Mg./Kg. Fresh Wt. Run No. 1	2	Av.
Formic	0.33	0.46	0.39
Acetic	0.024	0.029	0.027
Isovaleric	0.067	0.060	0.064

of 1959 peas are shown in Table IV. The acid composition is relatively simple. Formic acid is the primary constituent with small amounts of acetic and isovaleric acids also present. Formic acid has been detected previously in canned peas (*1*).

The steam distillate of peas has the pleasant, characteristic odor of cooked peas. If the compounds responsible for the odor can be identified, a substantial part of pea flavor would be understood. Earlier work in this field has indicated that carbonyl compounds are an important part of pea odor (*2*). Mixtures of the carbonyl compounds identified in this study do not approach the cooked pea odor. Work on the isolation and identification of the odorous components of pea steam distillate is being continued.

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LACTOSE BINDING IN MILK

Free and Bound Lactose in Milk

R. C. DUTRA, W. G. JENNINGS, and N. P. TARASSUK

Department of Food Science and Technology, University of California, Davis, Calif.

By using both isotope dilution methods and a conventional protein precipitation technique, it was demonstrated that in unheated milk 0.54% lactose is associated with milk protein(s). The association is apparently weak, as all lactose is removed from unheated milk by dialysis for 72 hours. The results suggest an equilibrium, free lactose ⇄ bound lactose, favoring a free-bound ratio of about 8 to 1. Binding of lactose by heated proteins, which is apparently a carbonyl-amino reaction, was investigated at 80° and 100° C. The activation energy of the reaction, probably the first step in the browning reaction, is of the order of 11 × 10^3 calories per mole.

WHILE lactose binding by the proteins of heated milk (browning reaction) has been the object of much research and the subject of a comprehensive review (*10*), little attention has been directed to bound lactose in unheated milk.

Various carbohydrates (glucose and galactose among others) have been reported to be associated with casein (*7, 8*), with some minor protein components (*13*), and with amino acids from tryptic hydrolyzates (*1*). Goulden, studying freeze-dried model systems, interpreted infrared spectra as indicating an association between casein and lactose (*3*). Other workers (*9*) disagreed with this interpretation, and Goulden subsequently attributed the irregularities in the spectra to the polymorphism of lactose (*4*). Schober and Christ (*12*) studied the binding of glucose by casein as influenced by heat, and concluded that two different types of association occurred—a reversible binding in protein that was native or subjected to low heat treatments, and at higher temperatures an irreversible binding that was a part of the browning reaction. The existence of bound lactose in unheated milk has not been definitely established nor has the extent of binding been measured.

The objects of this investigation were to determine the amount of bound lactose in unheated milk, the nature of the binding, and the energy requirements of the process in the temperature range 80° to 100° C.

Materials and Methods

Heating Trials. Aliquots (150 ml.) of fresh, standardized (3.5% fat) mixed-herd raw milk were placed in 14½-ounce evaporated milk cans. The cans were soldered closed, placed in a constant-temperature water bath for the indicated periods, and cooled in running tap water for 15 minutes. They were stored in an ice-water bath overnight and the milk was analyzed the next day.

Dialysis Trials. Separate aliquots of the milk were placed in dialysis bags and dialyzed against a pH 6.60 buffer (3.75 grams of anhydrous monohydrogen sodium phosphate and 5.41 grams of anhydrous dihydrogen potassium phosphate per liter of distilled water). The volume ratio of milk to buffer during these trials was about 1 to 10.

Lactose Determinations. Total lactose was determined by the zinc acetate–phosphotungstic acid reagent method of Grimbleby (*5*). Free lactose was determined by two methods, with excellent agreement in results.

Isotope Dilution Methods. To reduce coincidence losses, a 6-mg. portion

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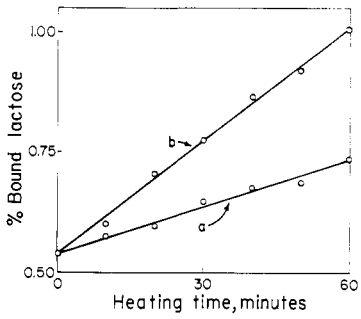

Figure 1. Influence of heating time on amount of bound lactose

a. 80°C.
b. 100°C.

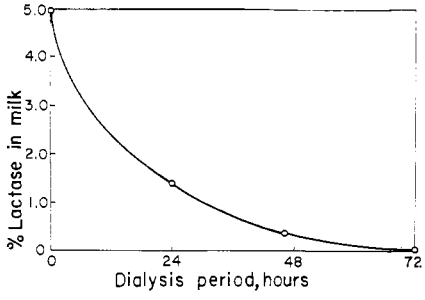

Figure 2. Amount of lactose in unheated milk as a function of dialysis time

of high - activity uniformly labeled carbon-14 lactose was added to 55 mg. of recrystallized carbon-12 lactose, and the two were completely mixed by dissolving in water and allowing slow evaporation at room temperature. When the original weight was realized, the specific activity of this "attenuated" tracer was determined. A 61-mg. portion was dissolved in 20.511 grams of fresh, standardized, raw milk, and the mixture placed in a dialysis bag for isolation of the centrifugal diffusate (*6*). The bag, suspended in a large centrifuge tube, was centrifuged 3 hours at 1500 r.p.m. in a centrifuge with a peripheral diameter of 38 cm. The centrifugal diffusate was evaporated on a steam bath until lactose crystallization occurred. The lactose was recovered by filtration and purified by paper chromatography; a mixture of methyl ethyl ketone, propionic acid, and water (75 to 25 to 30) was used as the developing solvent. After the specific activity of the recovered lactose was measured, isotope dilution was calculated by Paneth's equation as described by Willard, Merritt, and Dean (*14*).

Dialyzed Iron Method. In this simple procedure for free lactose, 15 ml. of dialyzed iron was added to 20 ml. of milk previously diluted with 20 ml. of distilled water. After standing for a few minutes the mixture was stirred well with a glass rod, made up to 100 ml., filtered, and polarized in a 2-dm. tube.

Results and Discussion

The binding of lactose at 80° and 100° C. is shown in Figure 1. From the slopes, the enthalpy of activation can be obtained with the integrated form of Arrhenius equation:

$$\log \frac{k_2}{k_1} = \frac{\Delta H^{\ddagger} (T_2 - T_1)}{2.303\, R T_2 T_1}$$

As the concentration of the reactants was the same at both temperatures, the ratio of the rate constants is equal to that of the slopes, as shown by the differential equations:

$$\left(\frac{dl}{dt}\right) 80° = k_{80°} (\text{lactose})^a (X)^b$$

$$\left(\frac{dl}{dt}\right) 100° = k_{100°} (\text{lactose})^a (X)^b$$

Therefore, as the ratio of the slopes is 2.235,

$$\log 2.235 = \frac{\Delta H^{\ddagger}}{(2.30)(1.99)(373)(353)}$$

Hence

$$\Delta H^{\ddagger} = 11 \times 10^3 \text{ calories per mole}$$

This value probably represents the energy of the first step of carbonyl-amino interaction, as there were no significant changes in color as measured by reflectance (*2*). This conclusion is supported by the fact that this value is in good agreement with the same thermodynamic quantity as determined from Patton and Flipse's data (*11*) for the binding of radioactive lactose by milk protein, and confirms Patton's results, indicating that the binding of lactose is initiated prior to color development.

In Figure 1, the intercept indicates that about 0.54% of the lactose of milk is bound before any heat treatment is applied. Grimbleby (*5*) predicted that the binding of lactose by protein could be a significant source of variation in lactose determination, as different methods of protein precipitation would liberate varying amounts of bound lactose. These data indicate how large such variations could be. It is conceivable that the glucose and galactose reported in casein hydrolyzates (*7, 8*) are actually hydrolytic products from bound lactose, particularly as the casein in question had been resuspended, reprecipitated, and washed to remove "free" impurities.

Figure 2 shows that dialysis of unheated milk for 72 hours removes essentially all lactose—both free and bound. It would seem that bound lactose in unheated milk is held through a weak linkage, probably not of carbonyl-amino nature as in heated milk. Prolonged dialysis disrupts this weak association, which may be due to hydrogen bonding, so that all lactose is dialyzable. This suggests that lactose in unheated milk occurs in a state of dynamic equilibrium:

$$\text{Bound lactose} \leftrightarrows \text{free lactose}$$

and the data indicate that the equilibrium favors a free-bound ratio of about 8 to 1.

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Colorimetric Determination of Phosphate in Sugar Products

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A fairly rapid colorimetric method for determining both inorganic orthophosphate and total phosphate in sugar products has been developed. The test is sufficiently accurate for quality control work and suited to series determinations in both colored and colorless products. This method is based on the observation that a yellow color proportional to the amount of orthophosphate present forms when an excess of ammonium molybdate is added to an acidified solution of ammonium vanadate and orthophosphate. The color is measured at a wave length of 420 mμ.

No adequate rapid method for the quantitative determination of the amount of inorganic and total phosphate present in sugar products is available in the literature. In this study, a photometric ammonium molybdivanadophosphate test was used to develop a method suitable for this purpose. Such a method has been used for phosphorus determinations in steel (*10*), iron ore (*3*), limestone (*2, 11*), phosphate rock (*1*), plants (*8*), and fertilizers (*4*). Kitson and Mellon (*7*) have made an extensive investigation of this method.

Apparatus

Photovolt Lumetron Photoelectric Colorimeter, Model 402-E. A 420-mμ Corning Glass filter was used. Standard colorimetric test tubes, 18 mm. in outside diameter, were employed as sample holders.

Palo multipurpose shaking machine.

Reagents

Standard Phosphate Solution. Dissolve an appropriate amount of pure potassium dihydrogen phosphate in distilled water so that 1 ml. of the solution contains 0.10 mg. of phosphorus pentoxide.

Ammonium Vanadate Solution Containing 0.20% of Vanadate. Cautiously add 2.35 grams of ammonium metavanadate, NH_4VO_3, reagent grade, to 500 ml. of boiling distilled water. Next add 100 ml. of dilute sulfuric acid (1 to 12). After cooling the solution, transfer it to a 1-liter volumetric flask and dilute to 1 liter with distilled water.

Ammonium Molybdate Solution Containing 5% of Molybdenum Trioxide (MoO_3). Dissolve an appropriate amount of pure ammonium molybdate, $(NH_4)_6MoO_{24}.6H_2O$, reagent grade in distilled water.

Concentrated nitric acid, concentrated sulfuric acid, and dilute sulfuric acid (1 to 1).

Procedure

Preparation of Calibration Curve. Add 0 to 15 ml. of standard phosphate solution with a buret, covering the range from 0 to 1.50 mg. of phosphorus pentoxide, into sixteen 100-ml. volumetric flasks. Dilute each flask to about 50 ml. with distilled water. Pipet 7 ml. of dilute sulfuric acid (1 to 1) into each flask and shake. Pipet 10 ml. of vanadate solution and then 20 ml. of molybdate solution with shaking into each flask and finally dilute to 100 ml. with water. After allowing the standard solutions to stand for 15 minutes, determine the absorbance at 420 mμ for each solution after setting the instrument to read zero absorbance using the reagent blank. Finally, plot the absorbance readings *vs.* milligrams of phosphorus pentoxide on rectangular graph paper and draw the best straight line through the points.

Determination of Total Phosphate (Both Inorganic and Organic). Pipet 10 ml. of distilled water containing 1 gram of sugar into a 100-ml. borosilicate glass volumetric flask. Digest the sample with 30 ml. of concentrated nitric acid and shake continuously over a low flame until the volume of solution is reduced to about 5 ml. (about 20 minutes). Cool, add 7 ml. of concentrated sulfuric acid, and heat over a low flame with continuous shaking until the solution turns pale yellow to colorless and sulfur trioxide fumes are evolved (about 10 minutes). If charring occurs at this point (very rare), it is the result of incomplete destruction of the organic matter present. The best procedure in this case is to repeat the digestion being careful to heat the nitric acid off more slowly, to use more nitric acid, or to use a smaller amount of sample initially. When sulfur trioxide fumes are evolved, cool the flask, cautiously add 25 ml. of water, and boil the solution for about 10 minutes with continuous shaking. Cool and dilute the solution to mark with water. Pipet 50 ml. of this solution and no additional acid into another 100-ml. volumetric flask and then prepare a reagent blank solution by adding 50 ml. of water and 7 ml. of dilute sulfuric acid (1 to 1) to a third 100-ml. volumetric flask. If the sample to be tested is very low in phosphate content, add a known amount of phosphate at this point with a standardized buret in order to bring the absorbance of the test solution into the more sensitive range of the calibration curve. Next, add the vanadate and molybdate reagents and obtain the absorbance of the test solution as described under "Preparation of Calibration Curve."

Determination of Inorganic Orthophosphate. Follow the same procedure as for total phosphate, but omit the acid digestion steps and add 7 ml. of dilute sulfuric acid (1 to 1) to the test solution before adding the vanadate and molybdate reagents to the flask.

Discussion and Results

Absorption Behavior of Colored Ammonium Molybdivanadophosphate Complex. The colored complex was found to follow the Beer-Lambert law for the range 0 to 1.50 mg. of phosphorus pentoxide at a wave length of 420 mμ. This wave length was used because it is close to the wave length of maximum spread between a solution containing 0.5 mg. of phosphorus pentoxide

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per 100 ml. of solution and a blank solution. The full color intensity is developed in 15 minutes and then remains stable for at least 1 hour. Because this method was designed as a rapid test, this point was not studied further. The color is not very sensitive to temperature variations, as the absorbances of test solutions were measured at room temperature which varied to the extent of 4° with negligible error.

Stability of Reagents. All the reagents used in this method remain stable for at least 3 months.

Diverse Ions. Silicate, pyrophosphate, sulfate, chloride, nitrate, calcium, ferrous, ferric, magnesium, potassium, and sodium ions do not interfere with the method. To test for diverse ions, 1 mg. of each of the above ions was added to a solution containing 0.5 mg. of phosphorus pentoxide. It was observed that 1 mg. of each of these ions was equivalent to not more than 0.01 mg. of phosphorus pentoxide. These ions were added in the sodium or chloride form, except for ferrous which was added in the sulfate form.

However, silicate ion interferes seriously with the method if an acidity of 0.5*N*, which Kitson and Mellon found to be the optimum acidity, is employed. At an acidity of 0.5*N* 1 mg. of silicon dioxide added as sodium metasilicate is equivalent to about 0.7 mg. of phosphorus pentoxide. In the method described an acidity of 1.2*N* was used, thereby eliminating silicate interference. The presence of up to 20 grams of sucrose per 100 ml. of solution does not cause any interference with color development.

Acid Concentration. An acidity of 1.2*N* was employed. Sulfuric, hydrochloric, nitric, or perchloric acids may be used to obtain the proper acidity. In view of these properties, it was decided to use sulfuric and nitric acids as a suitable digestion medium rather than the more dangerous perchloric and nitric acids. Ma and McKinley (*9*) have found that digestion with a mixture of sulfuric and nitric acid is sufficient for the decomposition of various types of organic phosphate compounds. Hardin (*6*) has reported that the sulfuric-nitric acid digestion adequately effects complete solution and ionization of the phosphorus content in fertilizer.

Acid digestion rather than ashing is preferred as a method for destroying all organic matter in test samples because the former procedure converts all phosphates to orthophosphate at the same time as it removes all organic matter. Acid digestion is also more suited to series determinations, in that as many as five digestions may be carried out simultaneously, with very little attention on the part of the analyst, if a Palo multipurpose shaking machine is employed. The ashing method, on the other hand, converts some of the phosphates present to meta- or pyrophosphate, rather than orthophosphate. This leads to low total phosphate results, since only ortho-

Table I. Extent of Hydrolysis of Some Sugar Phosphates to Inorganic Orthophosphate after 15 Minutes' Standing at Room Temperature and on Acidity of 1.20*N*

Compound	% Hydrolyzed Range	% Hydrolyzed Average
Glucose-1-phosphate	5.5–6.2	5.8
Fructose-6-phosphate	6.0–6.5	6.4
Fructose 1,6-phosphate	9.0–9.8	9.5

Table II. Precision of Method for Total Phosphate and Inorganic Orthophosphate

Product[a]	Total Phosphate: Av. P_2O_5 content, mg.[b]	Total Phosphate: Av. dev., mg.	Inorganic Orthophosphate: Av. P_2O_5 content, mg.[b]	Inorganic Orthophosphate: Av. dev., mg.
Philippine raw sugar				
A	0.063	0.011	0.083[c]	0.015
B	0.107	0.008	0.190[c]	0.012
C	0.159	0.007	0.300[c]	0.010
D	0.210	0.004	0.295[c]	0.012
E	0.110	0.009	0.120[c]	0.015
F	0.104	0.010	0.132[c]	0.014
Cuban raw sugar				
A	0.026	0.012	...	...
B	0.038	0.014	0.061[c]	0.019
C	0.024	0.012	0.016[c]	0.016
D	0.052	0.011	0.080[c]	0.018
E	0.071	0.009	0.083[c]	0.023
Puerto Rican raw sugar				
A	0.044	0.013	0.068[c]	0.019
B	0.054	0.010	0.010[c]	0.008
Peruvian raw sugar	0.050	0.012	0.012[c]	0.008
Affination magma				
A	0.087	0.013	0.010	0.007
B	0.221	0.007	0.021	0.008
Affination sirup				
A	0.172	0.010	0.105	0.015
B	0.257	0.006	0.172	0.013
Phosphoric acid treated molasses, A	1.108	0.006	0.926	0.012
Phosphoric acid treated molasses, B	1.004	0.007	0.819	0.014
High remelt sirup				
A	0.496	0.013	0.298	0.017
B	0.434	0.010	0.276	0.019
Low remelt sirup				
A	0.778	0.012	0.479	0.013
B	0.852	0.010	0.506	0.012
Final molasses				
A	1.025	0.006	0.674	0.012
B	1.184	0.007	0.703	0.015
Brown sugar				
A	0.364	0.010	0.288	0.014
B	0.316	0.011	0.262	0.016
Granulated sugar				
A	...	...	0.058[d]	0.006
B	...	...	0.071[d]	0.005
C	...	...	0.120[e]	0.003
Partial invert sirup				
A	...	...	0.046[d]	0.004
B	...	...	0.051[d]	0.005
C	...	...	0.102[e]	0.003

[a] 0.500-gram samples used for all analyses unless otherwise indicated.
[b] Average of three analyses.
[c] 1.000-gram samples used.
[d] 10.000-gram samples used because sample colorless initially.
[e] 20.000-gram samples used because sample colorless initially.

phosphates are determined by the photometric method.

In testing for total phosphate, 7 ml. of concentrated sulfuric acid were used because it provides a suitable volume of digestion medium for a 1-gram sample of sugar and results in an acidity (1.2*N*) which eliminates silicate interference.

When testing for inorganic phosphate in the presence of organic phosphates as in the case for sugar products, the acid concentration of the test solution, along with its temperature and standing time, is of the utmost importance. Gee, Domingues, and Deitz (*5*) used an acidity of about 0.25*N* for their inorganic phosphate test. Spencer and Meade's method for inorganic phosphate (*12*) employs solutions of about 0.38*N*. The molybdivanadophosphate inorganic test can also be carried out at an acidity of 0.25*N* with good accuracy, provided silicates are absent. The calibration curves obtained using 0.25*N* and 1.2*N* acidity almost coincide.

To obtain an estimate of the extent of hydrolysis of sugar phosphates to inorganic phosphate using 1.2*N* acidity, the author selected the three compounds listed in Table I as representative sugar phosphates. Employing the currently described method, he then determined the inorganic phosphate content of each of these compounds four times using acidity of 0.25*N* and of 1.20*N*. From the literature it appears that negligible hydrolysis of sugar phosphates occurs at 0.25*N*. Therefore, the difference between the inorganic phosphate content found at these two acidities was taken as a measure of the extent of hydrolysis of these compounds due to the higher acidity in Table I.

Correction for Initial Yellow Color of Some Sugar Solutions. If a colored solution such as a raw sugar is to be tested for inorganic phosphate, consideration must be given to the fact that raw sugar solutions themselves are initially yellow in color. This can be accomplished by measuring the absorbance of 100 ml. of the raw sugar solution containing 7 ml. of dilute (1 to 1) sulfuric acid after setting the colorimeter at zero absorbance using distilled water at the same acidity. If this absorbance value is subtracted from the absorbance value obtained by adding vanadate and molybdate to a similar raw sugar solution, the corrected absorbance value is obtained.

Precision of Total Phosphate and Inorganic Orthophosphate Methods. To evaluate the precision of the total phosphate method 28 samples of various sugar products were analyzed in triplicate. As can be readily calculated from Table II, the standard deviation of total phosphate method is ± 0.010 mg. The precision of the inorganic orthophosphate method was evaluated by analyzing 27 samples of various colored sugar products and six colorless products. The standard deviation for colored products was found to be ± 0.015 mg., while that for colorless samples was calculated to be ± 0.005 mg.

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SEVERAL PROCESSES ARE BEING investigated in the Cottonseed Products Research Laboratory for removing gossypol from cottonseed meats with the aid of aliphatic amines during the ordinary extraction of oil from meats. Primary *n*-octylamine is the preferred amine so far. In all of the processes varying amounts of residue amine remain in the meal. The amounts are estimated to range from less than 0.1% up to about 0.4% of the weight of the meal.

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This is a report on a preliminary investigation into the toxicity of a commercial primary *n*-octylamine, Armeen 8D (Armour & Co.) (*3, 4*). This amine has been used in much of the process development so far.

A considerable amount of toxicological data on aliphatic amines appears in the literature. With one exception (*6*), however, none of it refers specifically to octylamine. Most acute toxicological data appear to have been summarized in the "Handbook of Toxicology" (*7*). Some data from this handbook which are useful to the present investigation are reproduced in Table I.

Acute oral toxicity tests have been carried out on several acetate salts of aliphatic amines (*2*). The compounds tested were Armour's Armac C, Armac T, Armac TD, and Armac HT. The descriptions of these compounds have been given (*2*). They are manufactured from amines containing from 8 to 18 carbon atoms.

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A considerable amount of toxicological data on aliphatic amines appears in the literature. With one exception (*6*), however, none of it refers specifically to octylamine. Most acute toxicological data appear to have been summarized in the "Handbook of Toxicology" (*7*). Some data from this handbook which are useful to the present investigation are reproduced in Table I.

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Two hundred broiler-type chickens, including both sexes, were for 9 weeks fed rations containing different levels of commercial primary *n*-octylamine. Levels of amine were 0.036, 0.072, 0.144, and 0.36% of the rations. A control ration containing no amine, but otherwise similar to the other four, was also fed. No definitely toxic effects were observed. Consistent lesions were produced only by force feeding of amine at levels above those which the chickens would voluntarily accept in the ration. Many of these birds died. Prominent changes included hardening and peeling of the crop lining, inflammation of the intestine, edema and congestion of the lungs, and swelling of the anterior lobes of the kidneys.

Table I. Acute Oral Toxicities of Primary Aliphatic Amines[a]

No.	Compound	Animal	Dosage,[b] LD_{50}, Mg./Kg.
851	Ethylamine	Rat	400
1141	Isopropylamine	Rat	820
320	*n*-Butylamine	Rat	500
1027	Hexylamine	Rat	670
153	Aniline[c]	GP[d]	2500
	Aniline	Cat	100–200
	Aniline	Dog	500

[a] Data from "Handbook of Toxicology" (*7*).
[b] Milligrams of chemical administered per kilogram of body weight.
[c] The dosage values given for aniline are lethal doses—i.e., *LD* rather than LD_{50}. These data are included for comparative purposes.
[d] Guinea pig.

Table II. Composition of Rations Fed to Birds in Feeding Test

Ingredient		Per Cent
Ground yellow corn		30.0
Ground milo		19.0
Solvent process soybean meal, 45% protein		36.0
Dehydrated alfalfa meal, 17% protein		2.5
Condensed fish solubles		2.5
Dried whey		2.5
Soybean oil		2.5
Defluorinated rock phosphate		2.0
Oyster shell flour		1.5
Salt		0.5
Manganese sulfate, 70% (11.4 grams)		
	Grams[a]	
Vitamin mix		1.0
Riboflavin	0.2	
Niacin	1.3	
Calcium pantothenate	0.5	
Choline chloride	160.0	
Vitamin B_{12}	1.2	
Vitamin A	40.0	
Vitamin D_3	3.0	
Vitamin K	0.2	
Methionine	22.5	
Aureomycin	2.5	
BHT[b]	11.3	
Ground milo	210.9	
Total	453.6	100.0
Protein, %	22.5	
Calories/lb.	930	
Calories/% protein	41.3	

[a] Grams/lb.
[b] Butylated hydroxytoluene.

LD_{50} toxicity for Armac C was 600 to 800 mg. per kg. in rabbits. LD_{50} toxicities for the other three compounds were greater than 2000 mg. per kg. in rats. The compounds were administered in water solution by stomach tube. Surviving animals were observed for 5 days, by which time maximum mortality had been reached.

A modified subacute toxicity test was carried out on rabbits with Armac C and with Armac HT. One dose per day, 5 days per week for 37 days, was fed by stomach tube. The size of the dose was one third the LD_{50} as determined in the acute toxicity test. For both materials, the only observed effects of this treatment were slight weight losses.

Subacute and chronic toxicity tests have been carried out with octadecylamine (Armour's Armeen 18D) (*3*). No toxic effects of any kind were observed when octadecylamine was fed to rats for 2 years at levels of up to 0.05%. However rats fed this amine at a level of 0.3% of their diet for a shorter time developed an accumulation of histiocytes with pale or foamy cytoplasm in the mucosa of the small intestine and mesenteric lymph nodes. Focal granulomas were seen in the livers of some of these animals. Anorexia, weight loss, and increased mortality also occurred.

Dogs were fed octadecylamine at levels of 0.6 and 3.0 mg. per kg. per day for 1 year without toxic effects. One of three additional dogs receiving 15.0 mg. per kg. per day died after 22 weeks. The other two dogs gained less weight than did the controls. They also showed slight changes in the mucosa of the small intestine. Although the death of one dog may not have been caused by the amine, octadecylamine fed to dogs at a level of 15 mg. per kg. per day did not appear to be completely innocuous.

Experimental

Poultry feeds are the principal market toward which the low gossypol meal being developed is pointed. A complete evaluation of the toxicity of octylamine was not considered to be appropriate until the technical and economic successes of the processes under development were closer to realization. For these reasons, a practical feeding test on chickens could be expected to yield the most useful information. This test could be considered to be in the nature of a subacute toxicity test.

Rations to be fed were to be alike except for the quantities of amine they contained. The composition of the diet used is known to affect the results of toxicity studies (*8*). Therefore the basal ration was to be as complete as possible in all known nutritional requirements in order for symptoms of amine toxicity not to be confused with symptoms of nutritional deficiency.

To incorporate amine into a ration, a solution of amine in *n*-hexane was added to soybean meal before the meal was mixed into the ration. Sufficient commercial (Phillips Petroleum Co.) *n*-hexane was used to wet the meal thoroughly. After being mixed, the mixture was spread on wrapping paper on the floor and left until the *n*-hexane had evaporated. Using *n*-hexane as carrier associated the amine intimately with the soybean meal in a manner similar to the intimate association of amine and meal in amine-treated cottonseed meal.

The quantities of amine added to soybean meal by this procedure were 0.1, 0.2, 0.4, and 1.0% of the weight of the meal. The Armeen 8D used was a distilled grade of a commercial primary amine having the following analysis (*2, 4, 5*).

Primary amine content	89.2%
Hexylamine (approximate % of primary amine by weight)	3
Octylamine (approximate % of primary amine by weight)	90
Decylamine (approximate % of primary amine by weight)	7
Secondary amine	3.9
Moisture	2.0
Iodine value	1 5

Nitriles were other impurities.

Principal impurities in the amine beside water were secondary amines and nitriles.

Most of the process development work has been carried out with the technical, undistilled grade of amine designated Armeen 8. Because toxic effects of amine might be confused with toxic effects of impurities, the purer grade was used for the toxicity studies. This of course did not allow the potential toxicity of the impurities in the technical grade to be evaluated.

Eighty-pound batches of each ration were mixed at one time. This size of batch required 28.8 pounds of soybean meal. About 27 pounds of meal at a time were treated with amine for each batch. Feeding of each newly mixed batch of feed to the chickens was usually started within 2 days after mixing. Four batches of each ration were consumed during the test.

The composition of the basal ration is shown in Table II. The ration numbers and corresponding amine contents in the soybean meal and in the complete ration are as follows: No. 1: 0, 0; No. 2: 0.1, 0.036; No. 3: 0.2, 0.072; No. 4: 0.4, 0.144; and No. 5: 1.0, 0.36.

Sexed, 1-day-old chicks were procured from a commercial hatchery. The chicks were of a commercial broiler strain designated Peterson cross. They were vaccinated for Newcastle disease. All were then fed for 7 days on No. 1 ration.

At 8 days of age, 100 birds were selected at random from each sex, were wing banded, weighed, and distributed to 10 cages, 10 of each sex in each cage. Cages were Oakes No. 8 wire brooder cages, five per battery. Batteries were arranged side by side in the same position relative to walls, windows, radiator, etc.

One of the two cages for testing each ration was selected from each battery. Relative positions of the two cages were selected either at extremes of top and bottom of the batteries, or in the middle. This was done as an attempt to minimize the possible effects of cage position on growth.

Feeding of test rations was started as soon as the birds were placed in the test cages on the 8th day. The birds usually were weighed once each week thereafter, throughout the test. During much of the test careful feed consumption data were kept. Paper envelopes were installed under feed troughs on brooder cages to decrease the feed loss and thus to improve the reliability of calculated feed utilization.

At 30 days of age, the birds were transferred to larger wire growing cages which contained eight cages in two stacks per battery. Two batteries, side by side, were used. The birds in the two brooder cages were divided into three groups of 13 or 14 birds each and each group was placed in a separate growing cage. Thus the birds in each ration were redistributed among three cages instead of two. The birds were maintained in these growing cages until the end of the test when the birds were 9 weeks old.

Several birds were sacrified for post-mortem examination at the end of the feeding test. Eleven birds on ration 1, five on ration 4, and 10 on ration 5 were sacrified.

Because no signs of any toxic effects had been observed during the feeding test, some forced feedings were made at higher levels of amine to see whether any symptoms of toxicity could be induced. Birds from ration 1 were used.

By the same technique used to add amine to soybean meal, enough amine was added to some mixed ration to raise the amine content to 4.89% of the weight of the ration. This ration mixture was ground so that about 95% would pass a 60-mesh screen. When this feed was mixed with two and a half times as much water the resulting slurry could be easily poured down a 1/8-inch inside diameter rubber tube placed down a chicken's throat with the lower end of the tube about 3/4 inch above the opening into its crop. A funnel was attached to the upper end of the tube. A satisfactory dose size was found to be 20 grams of meal mixed with 50 ml. of water, followed by another 10 to 25 ml. of water, as required, to rinse out the beaker and funnel. This intubation was necessary, because the birds would not voluntarily eat the feed containing 4.89% of amine and having a strong amine odor.

Seven birds were fed this mixture twice daily until death or until they were sacrified for post-mortem examination.

Some of the above feed was mixed with additional *n*-hexane and amine to give six different amine contents ranging from 7.5 to 20%. One dose of each feed was given to a different bird, and the birds were observed for 5 days before sacrifice. These birds were fasted overnight without feed, but with water before they were dosed.

Because none of the birds in the above group died with single doses of amine mixed in feed, four birds were fed with amine emulsified in water and without feed. The emulsifier was Hercules cellulose gum equivalent to 0.5% of the weight of the water.

Post-mortem examination for gross lesions were made on all force-fed birds (those in Table VI), with the exception of birds 3C and 4P.

Results and Discussion

Evaluation of the effects of amine in the rations were made by observation of

Table III. Mean Weights of Birds on Different Rations during Feeding Test

Age of Birds, Weeks	Ration No. 1	2	3	4	5	Rank[a]
	Mean Weights of Pullets, G.					
1	74	74	76	75	74	
2	153	156	153	157	148	4, 2, 3, 1, 5
3	259	264	259	266	240	4, 2, 3, 1, 5
4	413	419	414	414	383	2, 3, 4, 1, 5
6	766	768	768	762	718	2, 3, 1, 4, 5
7	965	969	975	956	908	3, 2, 1, 4, 5
8	1153	1169	1183	1151	1106	3, 2, 1, 4, 5
9	1316	1343	1363	1323	1280	3, 2, 4, 1, 5
	Mean Weights of Cockerels, G.					
1	75	77	75	76	74	
2	157	163	163	162	151	2, 3, 4, 1, 5
3	268	284	283	278	256	2, 3, 4, 1, 5
4	439	460	461	447	420	3, 2, 4, 1, 5
6	855	886	891	869	833	3, 2, 4, 1, 5
7	1101	1139	1136	1105	1069	2, 3, 4, 1, 5
8	1344	1390	1384	1370	1306	2, 3, 4, 1, 5
9	1564	1615	1597	1582	1519	2, 3, 4, 1, 5

[a] Ranked in order of decreasing mean weight.

Table IV. Analysis of Variance of Feeding Test Data by Sex for 8 Weeks of Age

Source of Variation	Degrees of Freedom	Mean Square	F[a]
	Pullets		
Between rations	4	18,000	1.5
Between cages within rations	10	8,044	
Between birds within cages within rations (error)	87	12,037	
	Cockerels		
Between rations	4	22,395	1.39
Between cages within rations	10	4,938	
Between birds within cages within rations (error)	83	16,183	

[a] To be significant at the 5% or 1% probability levels, *F* should be as great as 2.48 or 3.56, respectively.

the external appearance of the birds, plotting of growth curves and statistical analysis of the weight data, and by post-mortem examination of randomly selected birds.

Birds on different rations did not show any differences in external appearance such as degree of feathering, crippled feet, etc.

Data on total weights of birds at the end of 8 weeks of age were chosen for statistical analysis. Because of errors in sexing the chicks and in selecting the birds from two cages to be put into three cages at 30 days of age, different numbers of each sex in each cage resulted. For this reason statistical analysis of the data was carried out on each sex separately. This method of analysis did not allow possible interactions between sex and ration to be evaluated. However, on plotted growth curves, the response by both sexes to the different rations was much the same, indicating that probably no sex-ration interaction existed.

Mean weight data are presented in Table III. The statistical analysis is summarized in Table IV.

The statistical analysis showed that the mean weights by ration were not significantly different from each other within sexes. In other words, differences in mean weight as great as those shown would be expected to occur by chance alone about 15 to 25% of the time. Therefore, while the amine content of the rations may have affected growth, the data did not prove that it did.

In spite of the lack of significance between mean weights, the consistency of the relative rank of the weights from week to week, as shown in Table III, would seem to have some meaning. In particular, ration 5 was lowest in weight for both sexes beginning with 2 weeks of age and running until the end of the test.

Feed consumption data showed no evidence that birds on any of the rations consumed more feed or utilized it more efficiently in making gains than birds on any other ration.

Table V contains data on amine intake by birds during the feeding test, while Table VI contains amine intake data on birds fed by intubation. Comparing these tables with Table I, the highest level of daily intake with ration 5 in Table V is seen to be of about the same order of magnitude as the toxic levels in Table I, while all doses in Table VI are as high or higher than toxic levels in Table I.

Although no real attempt was made to determine lethal dosages for chickens, the results with birds 14C and 15C in Table VI suggest that the lethal dose may be of the same order of magnitude as the values given in Table I for other amines.

Apparently when amine is mixed with feed, toxicity is greatly reduced. This may be the reason no deaths occurred among birds during the feeding test. Also in the latter test, daily amine intake was spread out rather than being in one dose.

In all of the tests involving forced feeding, changes in the behavior of the birds were noted. The first change was a lethargy and drowsiness which appeared within 20 minutes or less, depending upon the amount of amine administered and whether it was mixed with feed. A bird so affected would sit with its eyes closed until it was handled or otherwise disturbed. Then it would open its eyes, but would make no efforts to escape as normal birds always did. The depth of lethargy was also roughly dependent upon the amount of amine in the dose. Birds in this state had to be pushed to make them walk and then they walked uncertainly and slowly. Slavering always occurred among birds which were force-fed amine. These were the principal outward changes both in birds which did and did not die.

Before a bird died it might regurgitate and have muscular spasms. Some of the deaths appeared to have been caused by obstruction of the trachea

Table V. Daily Intake of Amine by Birds for Two 1-Week Periods during Feeding Test

Ration No.	Average Bird Weight, G.[a]	Amine Consumption, Mg./Kg./Day[b]
2-Week-Old Birds at Beginning of Period		
2	216	47
3	198	103
4	216	200
5	198	452
7-Week-Old Birds at Beginning of Period		
2	1158	25
3	1156	52
4	1153	93
5	1088	280

[a] The average weight during the feeding period is given. These weights are not comparable among rations, because of different numbers of cockerels and pullets per ration.

[b] Consumption is given in terms of milligrams per kilogram of body weight per day.

Table VI. Forced Feeding of High Levels of Amine

Bird No.[a]	Bird Weights, G. Initial	Bird Weights, G. Final	Amine in Feed, %	No. of Doses Recd.[b]	Rate of Intake of Amine, Mg./Kg./Day[c]	Total Intake of Amine G./kg.	Total Intake of Amine G.	Days Observed
1C	1891	1720[d]	4.89	3	1100	1.6	2.9	1[e]
2C	1837	1704[d]	4.89	4	1100	2.2	3.9	1 1/2[e]
3C	1968	1900[d]	4.89	3[f]	1500	2.3	4.4	1 1/2[e]
4P	1728	1672[d]	4.89	3[f]	1700	2.5	4.3	15
5C	1888	1618	4.89	9	1100	5.1	8.9	4[e]
6C	2006	1870	4.89	16	1100	8.1	15.7	10
7P	1776	1954	4.89	16	1100	8.4	15.7	10
8P	1494	1547	7.5	1[g]	1000	1.0	1.5	5
9P	1683	1603	10.0	1[g]	1200	1.2	2.0	5
10P	1744	1762	12.5	1[g]	1400	1.4	2.5	5
11C	1648	1468	15.0	1[g]	1800	1.8	3.0	5
12C	1721	1461	17.5	1[g]	2000	2.0	3.5	5
13C	1899	1542	20.0	1[g]	2100	2.1	4.0	5
14C	1804	1576	[h]	1	600	0.6	1.0	5
15C	1878	...	[h]	1	1100	1.1	2.0	5 hr.[e]
16C	1930	...	[h]	1	2100	2.1	4.0	30 min.[e]
17C	2051	...	[h]	1	9800	9.8	20.0	10 min.[e]

[a] C or P indicates bird was cockerel or pullet.
[b] When more than one dose was administered, doses were given two per day, except on weekends when none were given.
[c] Expressed in milligrams intake per day per kilogram of body weight, based on average body weight during period of administration.
[d] Weight after 1 1/2 days, at time when last dose of amine was administered.
[e] This bird died.
[f] This bird received doses containing 30 grams instead of 20 grams of amine-feed mixture per dose.
[g] Birds fasted 16 hours with access to water before dose was administered.
[h] Amine was emulsified with water, but was not mixed with feed for dosage.

with regurgitated feed in birds which were too stupefied by the amine to fight against strangulation. All of the birds fed amine without feed shook violently for a time whether or not they died later.

Post-mortem examination of 11 birds on ration 1 revealed no significant gross lesions. In one bird, 15 to 20 red plaques were found in the small intestine. The plaques varied from 3 to 8 mm. in their longest dimension, had discrete borders, and were rectangular to ovoid in shape. From a close visual examination it was judged that the reddening resulted from capillary dilatation in the mucosa. The plaques were not associated with Peyer's patches (lymphatic tissue).

Three out of five birds examined from the ration 4 group had intestinal plaques similar to those seen in one control bird. Two of these plus one without plaques showed diffuse hyperemia (inflammation) of the upper one third to two thirds of the intestinal tract.

No lesions were found in three birds out of the 10 examined from the group fed ration 5. Reddened plaques, ranging from questionable to distinct, were found in the upper one to two thirds of the intestinal tracts of five birds. One with plaques and two of those without showed a marked diffuse hyperemia of the upper half of the intestinal tract.

The presence of reddish plaques in chickens receiving various amounts of *n*-octylamine cannot be ascribed to that compound, because such plaques were also found in the intestine of one bird which was fed only on the control ration. Hyperemia of the intestine may have been caused by amine; however, the effect was not observed in all birds from the same ration.

Hardening of the crop mucosa and enteritis (inflammation of the intestine) were consistent lesions in birds force-fed meal containing 4.89% of octylamine. In three crops out of the five examined, some areas of hardened mucosa were desquamating. Enteritis ranged from moderate catarrhal to a marked diffuse hyperemic inflammation of the upper half of the intestinal tract. The birds which died, Nos. 1C, 2C, and 5C (Table VI), had edema of the lungs. No. 5C also had hemorrhagic plaques in the lower trachea, congestion of the bronchi, and cloudiness of the pericardial sac.

Birds Nos. 8P through 13C (Table VI) all had hardening or desquamation or both of the crop mucosa. Upper intestinal changes in birds 9P through 13C included slight catarrhal enteritis, scattered petechiation, reddened plaques, and diffuse hyperemia. Healing was evident. If this group had been examined earlier than 5 days after the single dose, the lesions probably would have been more prominent.

The principal lesions found in birds force-fed an emulsion of *n*-octylamine involved the crop, upper intestines, and the respiratory system. Hardening of the crop mucosa and traces of a fibrinous pseudomembrane in the small intestine were found in No. 14C, killed 5 days after a single dose. The birds which died after a single dose, Nos. 15C, 16C, and 17C, all had swelling of the anterior lobes of the kidney and hardening and desquamation of the crop mucosa. No. 15C had a severe catarrhal enteritis with desquamation of villi. The lower trachea, bronchi, and lungs were hyperemic. The coronary vessels were congested. No. 16C had a severe catarrhal enteritis and moderate diffuse hyperemia of the upper intestine. Scattered red plaques were present in the duodenum. The lungs were congested and edematous. No. 17C showed swelling of the villi in the upper intestine. The surface appeared chalky. The pharynx and esophagus were necrotic. A marked diffuse hyperemia was present in the proventriculus and anterior portion of the gizzard.

The results of this investigation indicate that *n*-octylamine is not definitely toxic to poultry when it is fed in a practical ration unless the concentration of amine is greater than 0.36% of the ration. Concentrations of 5% of amine are toxic.

The subacute toxicity of *n*-octylamine for poultry appears to be considerably less than the toxicity of octadecylamine for dogs. The lowest level fed to poultry in this test was about twice the highest level fed to dogs in the Armour investigation. Some toxic effects were noted in the latter.

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Water-Soluble Hemicelluloses of Grass Holocellulose

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Hemicelluloses were prepared from five species of forage grasses by extracting them from the holocelluloses by hot water. They were constituted in descending order of abundance by xylose, glucose, arabinose, galactose, and rhamnose, and by some uronic acids. Slight quantitative differences existed among species and stages of growth, particularly with respect to glucose, the most variable constituent.

FORAGES DIFFER from one another in nutritive value, and species and stage of maturity are important factors in this respect. An objective in the study of hemicelluloses is the uncovering of differences which might be associated with these variables. Binger, Sullivan, and Jensen (*1*) found that almost three fourths of the total hemicelluloses of orchard grass could be extracted from chlorite holocellulose by hot water. It seemed probable that this fraction of the hemicelluloses might show differences associated with species and stage of growth.

Materials and Methods

Water-soluble hemicelluloses were prepared from five grasses by the method described by Routley and Sullivan (*6*). It consists of preparing chlorite holocellulose, removing the hemicelluloses by boiling water, and recovering the hemicelluloses from the water by concentration, dialysis, and precipitation by alcohol.

The hemicelluloses were analyzed for their component sugars as follows: A 0.1-gram sample was heated in a vial with 5 ml. of 1*N* sulfuric acid in a boiling water bath under an air condenser for 6 hours, cooled, transferred to a 50-ml. centrifuge tube, and neutralized to the mixed methyl red–bromocresol green indicator with calcium carbonate. After centrifuging and washing the solid matter the solution was made slightly acid, concentrated on the steam bath, diluted to 5 ml., again centrifuged to clarify, and preserved with thymol. The method of hydrolysis described gave the maximum yield of reducing sugars, with little destruction of xylose. Biuronides, but not oligosaccharides, were found in the hydrolyzate.

The component sugars were separated by paper chromatography, using sheets of Whatman No. 1 filter paper, 7.5 inches wide. A 1.5-inch strip was marked off on each side for staining, and an additional 1.25-inch strip for a paper blank. Spottings of the hemicellulose hydrolyzate were made on the side strips and on the remaining 3.25 inches. Xylose, arabinose, glucose, and galactose were separated by three 16-hour descending runs, with drying between, with a slight modification of the solvent proposed by Masamune and Maki (*4*), *n*-amyl alcohol, pyridine, and water, 135 : 145 : 120. Rhamnose, which moved more rapidly with this solvent, was separated by a single run of 16 hours. The 1.5-inch side strips were removed with shears and dipped in a solution composed of 100 ml. of isopropyl alcohol (85%), 1.66 grams of phthalic acid, and 0.75 ml. of aniline. The strips were blotted and heated at 100° to 102° C. for 15 minutes. The sheets were reassembled. With the stained outer strips as guides the center strips containing the intact sugars and a paper blank 3.25 inches long were cut out and eluted by the Dent method, essentially as modified by Dimler *et al.* (*2*). Between 0.5 and 1.0 ml. of eluate was collected, diluted if necessary to 1.0 ml., directly into observation test tubes of the Klett-Summerson photoelectric colorimeter marked at 1 and 8 ml. Determination of reducing power was made in the same tubes by the Nelson colorimetric modification (*5*) of the Somogyi method, with dilution to 8 ml. Readings were made with the No. 50 filter. Equations for each sugar were developed, 5 to 50 γ in 1-ml. volume. The accuracy of the method was checked with mixtures of 40 γ each of the sugars which were separated on papers, eluted, and determined as above. Recoveries were 94 to 104%.

Separation of uronic acids was attempted with the solvent system proposed by Fischer and Dörfel (*3*).

Table I. Sugars in Hemicelluloses of Orchard Grass at Three Stages of Maturity in Percentage of Total Sugars

Stage of Maturity	No. of Samples in Average	Rhamnose	Galactose	Glucose	Arabinose	Xylose
Pre-emergence	6	2.2	7.5	23.7	12.9	53.7
Half-emergence	5	1.6	6.7	25.4	12.5	53.8
Flowering	3	1.3	7.3	14.2	13.0	64.2

Table II. Sugars in Hemicelluloses of Different Species of Forage Grasses in Percentage of Total Sugars

Species	No. of Samples in Average	Rhamnose	Galactose	Glucose	Arabinose	Xylose
Orchard grass	14	1.8	7.2	22.4	12.8	55.7
Reed canary, 1st cut	3	0.8	6.5	10.1	11.1	71.5
Reed canary, fall cut	1	1.3	6.2	19.8	11.2	61.5
Tall fescue	2	1.6	4.7	11.1	9.6	72.9
Timothy	2	0.9	4.8	16.3	9.1	68 8
Kentucky blue	2	1.5	6.8	12.2	12.2	67.3

Results

Five sugars were found in all hemicellulose preparations. Xylose was always the most abundant, and in decreasing order were glucose, arabinose, galactose, and rhamnose, the last always being present in minor amount. The results with uronic acids were inconclusive. Spots corresponding in location

to galacturonic acid appeared by visual comparison to amount to 12 to 14% of the different hemicelluloses. This corresponds closely to the amounts found by Binger, Sullivan, and Jensen (*1*) in similar samples of orchard grass by densitometer methods. However, the reducing power of these spots after elution corresponded to only 1 to 3% as galacturonic acid. It is probable that these spots were of biuronides. Two or three other similar spots appeared on some chromatograms.

Fourteen samples of orchard grass represented three stages of maturity: pre-emergence, half emergence, and blooming. The weights of crude hemicellulose obtained from the three stages averaged 7.5, 10.7, and 11.2% of the grass, respectively, and of these approximately 70, 80, and 63%, respectively, were recovered as sugars. Weighted averages of the amounts of the sugars, in per cent of total sugars recovered, are given in Table I. Rhamnose declined slightly with maturity of the grass. A marked decrease in glucose also occurred between the second and third stage of maturity, while xylose was correspondingly higher. A similar decrease in the glucose content of the hemicelluloses of the stems and leaves of brome grass during the course of maturity was noted by Routley and Sullivan (*6*).

The weighted average composition by species appears in Table II. In reed canary grass one sample represented mixed late summer and early fall cuttings and it contained a higher proportion of glucose than the first cuttings, all of which were advanced in maturity and were very similar to one another in composition. In other grasses no great differences were found between samples of the same species, and the results reported for them are averages. In comparisons among species of grasses differences were noted only in the glucose and in the corresponding xylose contents. Orchard grass was distinctly higher than all the other grasses in the glucose content of the water-soluble hemicelluloses. A glucosan may be a constituent of the hemicellulose preparations, especially of those from orchard grass.

It is recognized that losses occurred both in the preparation of the holocelluloses and in the extraction and precipitation of the hemicelluloses and that these losses may have had some influence on the composition of the final hemicellulose preparations. The place of occurrence and the extent of these losses will be an object of future study.

Summary

Hemicelluloses were prepared from five species of forage grasses by extracting them from the holocelluloses by hot water. They were constituted in descending order of abundance by xylose, glucose, arabinose, galactose, and rhamnose, and by some uronic acids. Slight quantitative differences existed among species and stages of growth, particularly in respect to glucose, the most variable constituent.

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GRASS HOLOCELLULOSE

Stepwise Hydrolysis of Grass Holocellulose

T. G. PHILLIPS, D. G. ROUTLEY, and J. T. SULLIVAN

U. S. Regional Pasture Research Laboratory, University Park, Pa., and the New Hampshire Agricultural Experiment Station, Durham, N. H.

Grass holocellulose was subjected to successive heatings with 0.01*N* sulfuric acid in a boiling water bath for 1, 2, 4, and 16 hours. Simple sugars, uronides, oligosaccharides, and hemicelluloses were extracted by each of the successive treatments. All the arabinose, galactose, and rhamnose, practically all the uronides, and 75% of the xylose were removed from the holocellulose. Glucans accompanied the other hemicelluloses, but glucose does not appear to be a constituent of the polyuronide hemicelluloses. The oligosaccharides found in the hydrolyzates indicate a considerably branched structure for the polyuronide hemicelluloses. The compounds recovered from the extracts are similar to those obtained by various workers from the hemicelluloses of other Gramineae.

MUCH OF THE PRESENT knowledge of the structure of the hemicelluloses of nonwoody plants has been obtained from the more or less highly purified xylan portion. Recently, however, partial or stepwise hydrolysis of the entire hemicellulose has produced compounds of low molecular weight which have provided some evidence as to the structure of the more highly branched and more readily soluble portion of the hemicelluloses (*6, 10*). As no forage grass hemicelluloses were included in these studies, it appeared desirable to compare the products of their stepwise hydrolysis with those of other plants, especially other Gramineae.

Material and Methods

Chlorite holocelluloses were prepared as described by Routley and Sullivan (*5*). They came from orchard grass, cut at the half-emerged stage, and brome grass, combined leaf and aftermath. Direct hydrolytic treatment of the holocellulose was used in order to avoid the losses entailed in extracting the hemicelluloses. To 8 grams of holocellulose in a 250-ml. Erlenmeyer flask, 130 ml. of 0.01*N* sulfuric acid were added. The mixture was heated under an air condenser in a boiling water bath for 1

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MUCH OF THE PRESENT knowledge of the structure of the hemicelluloses of nonwoody plants has been obtained from the more or less highly purified xylan portion. Recently, however, partial or stepwise hydrolysis of the entire hemicellulose has produced compounds of low molecular weight which have provided some evidence as to the structure of the more highly branched and more readily soluble portion of the hemicelluloses (*6, 10*). As no forage grass hemicelluloses were included in these studies, it appeared desirable to compare the products of their stepwise hydrolysis with those of other plants, especially other Gramineae.

Material and Methods

Chlorite holocelluloses were prepared as described by Routley and Sullivan (*5*). They came from orchard grass, cut at the half-emerged stage, and brome grass, combined leaf and aftermath. Direct hydrolytic treatment of the holocellulose was used in order to avoid the losses entailed in extracting the hemicelluloses. To 8 grams of holocellulose in a 250-ml. Erlenmeyer flask, 130 ml. of 0.01*N* sulfuric acid were added. The mixture was heated under an air condenser in a boiling water bath for 1

hour and filtered on a Büchner funnel. The filtrate and washings, of pH 3.5, were neutralized with saturated barium hydroxide, centrifuged, and washed, in the high speed centrifuge. The supernatant and washings were concentrated on the water bath. This solution contained sugars, oligosaccharides, and aldobiuronic acids, as well as hemicellulose which had been dissolved from the holocellulose and which was not hydrolyzed or only partially so. The hemicellulose was precipitated by ethyl alcohol and separated and washed at the centrifuge. The precipitation by ethyl alcohol was repeated, with intervening concentration of the supernatant and washings, until there was no further precipitation of hemicellulose. The hemicellulose precipitates were combined, washed with ethyl alcohol and ether, and dried. The last supernatant and washings were evaporated to a small measured volume. This, referred to as the extract, contained free sugars, oligosaccharides, and some aldobiuronic acids.

The holocellulose residue from the above treatment was heated in the boiling water bath with 130 ml. of 0.01*N* sulfuric acid for 2 hours. The mixture was treated as described above. The pH of the filtrate was 2.5. The residue from the 2-hour treatment was heated in the same way for 4 hours, and the 4-hour residue for 16 hours. Extracts and hemicelluloses from each treatment, and the final residue, were analyzed.

For the determination of the constituent sugars, the holocelluloses, hemicelluloses, and residues were hydrolyzed by heating with 1*N* sulfuric acid for 6 hours. Analyses of the hydrolyzates, and also of the extracts, were carried out as described (7) except that no quantitative determinations of rhamnose were made.

Rhamnose separated readily beyond glucose with one 16-hour descending run with *n*-amyl alcohol, pyridine, and water. Comparatively large portions of the sample are required to show this sugar.

Aliquots of the extracts were heated with 0.25*N* sulfuric acid to hydrolyze the oligosaccharides which interfere with the identification of uronides on chromatograms. The mixtures were neutralized, concentrated, and chromatographed with two descending 24-hour runs with ethyl acetate, acetic acid, formic acid, and water for qualitative separation. As the material available was not sufficient for elution and identification, preparations were made from another portion of the brome grass holocellulose. After separation in quantity, the uronides were subjected to hydrolysis by methanolic-hydrochloric acid, and the products were identified by comparison with authentic samples. These uronides were compared qualitatively as described above.

Some added information as to the occurrence and nature of the oligosaccharides and uronides was obtained by two supplementary procedures: Separate 2-gram portions of the holocelluloses were treated as described above, but only once, and for 1, 2, 4, and 16 hours. Hemicelluloses were separated from the extracts as above. Samples of 0.04 gram of the holocelluloses and of the final residues and of 0.02 gram of the hemicelluloses just prepared were heated in small stoppered vials in the water bath with 1 ml. of 0.1*N* sulfuric acid. At the end of 1, 2, 4, 7, and 12 hours, portions of the hydrolyzates were spotted on paper for chromatography with three ascending runs with *n*-amyl alcohol, pyridine, and water (135:145:120). The oligosaccharides of the extracts were also separated, in this case by repeated descending runs with the same solvent. The oligosaccharides located by spraying with aniline hydrogen phthalate on side strips were further separated after elution with other solvents as needed: ethyl acetate, acetic acid, formic acid, and water (18:3:1:4); 1-butanol, pyridine, and water (6:4:3); and 1-butanol, acetic acid, and water (4:1:4) (organic phase). Finally the oligosaccharides were eluted, subjected to stepwise hydrolysis with 0.1*N* sulfuric acid, and their components were determined chromatographically. If more than one sugar appeared, their ratios were determined.

Table I. Recovery of Solids from 8 Grams of Holocellulose

Compound	Grams	%
Hemicellulose 1	0.430	5.4
2	1.128	14.1
3	0.358	4.5
4	0.129	1.6
Total hemicelluloses	2.045	25.6
Sugars in extracts, as anhydrides	0.330	4.1
Residue	5.000	62.5
Total recovery	7.375	92.2

Table II. Per Cent Sugars[a] and Ash in the Solid Preparations

Compound	Galactose	Glucose	Arabinose	Xylose	Ash
Holocellulose	1.5	4.2	5.0	21.3	3.8
Hemicellulose 1	3.2	11.6	8.3	33.6	22.3
2	3.9	10.4	4.9	46.9	13.6
3	2.2	9.7	2.4	41.6	17.6
4		17.1		21.6	27.7
Residue		2.6		8.8	4.8

[a] Liberated by heating 6 hours with 1*N* sulfuric acid.

Table III. Recovery of Sugars from the Holocellulose

	Galactose		Glucose		Arabinose		Xylose	
Compound	Mg.	%	Mg.	%	Mg.	%	Mg.	%
Holocellulose, 8 grams	123	100.0	336	100.0	396	100.0	1706	100.0
Extract 1	2	1.6			65	16.4	4	0.2
2	6	4.9			142	35.9	22	1.3
3	4	3.3			19	4.8	26	1.5
4	4	3.2	6	1.8	10	2.5	64	3.8
Extracts, total	16	13.0	6	1.8	236	59.6	116	6.8
Hemicellulose 1	14	11.4	49	14.7	36	9.1	145	8.5
2	44	35.8	114	34.0	55	13.9	531	31.1
3	8	6.5	35	10.4	9	2.2	148	8.7
4			22	6.5			28	1.6
Hemicelluloses, total	66	53.7	220	65.5	100	25.2	852	49.9
Extracted, total	82	66.7	226	67.3	336	84.8	968	56.7
Residue			129	38.4			436	25.6
Recovery, total	82	66.7	355	105.7	336	84.8	1404	82.3

Results

No qualitative differences were found between orchard grass and brome grass. Quantitative differences were slight, except for a noticeably higher glucose content in the orchard grass preparations. As this does not affect the conclusions to be drawn, the two sets of figures have been averaged in the tables.

The 7.8% loss of solids (Table I) could arise from the following: solids other than simple sugars in the extracts, including oligosaccharides and uronides; losses in the recovery of the hemicelluloses as quantitative transfer of these is difficult; and other losses in handling.

All the hemicelluloses precipitated from the extracts were contaminated with extraneous material (Table II). The ash content was especially high. No arabinose or galactose was found in the hemicellulose from the fourth extraction or in the residue.

Arabinose was removed rapidly, galactose more slowly, but both completely, as none of either sugar was found in the hydrolyzate of the residue (Table III). Xylose was split off much more slowly. The failure to attain quantitative recovery of these sugars may be accounted for in part by their occurrence in oligosaccharides and uronides, in part by destruction during hydrolysis, and in part by technical difficulties.

Probably, the treatment with dilute acid gradually changed some glucans to forms more easily hydrolyzed than they were in the holocellulose. This would account for the high recovery of glucose.

Oligosaccharides. Eight of these were separated in amounts sufficient for hydrolysis. From their position on chromatograms and their behavior during stepwise hydrolysis, an estimate can be made of the number of sugar units in each. They are as follows:

An arabinose disaccharide was present in extracts 1 and 2. It was hydrolyzed to arabinose by 0.01*N* sulfuric acid in 4 hours.

An arabinose-galactose disaccharide was present in extracts 1 and 2, but only a slight trace, or none, was found in extracts 3 and 4. It was hydrolyzed completely by 0.1*N* sulfuric acid in 3 hours.

An arabinose-xylose disaccharide was like the disaccharide above in occurrence and rate of hydrolysis.

An arabinose-galactose-xylose trisaccharide occurred in relatively small amounts in extract 2, with little or none in extracts 3 and 4. It was hydrolyzed by 0.1*N* sulfuric acid in 1 hour.

An apparent tetrasaccharide containing one arabinose, two galactose, and one xylose units was like the above trisaccharide in occurrence, but its R_f was much lower.

A xylose disaccharide, a xylose trisaccharide, and a xylose tetrasaccharide appeared in all the extracts. They were hydrolyzed completely by 0.1*N* sulfuric acid in 7 hours.

Uronides. These were present in the holocelluloses and in all the hemicelluloses and extracts. They were absent from, or present in only slight traces in, the residues.

Five uronides were separated from brome grass hemicellulose. Their constituents as determined by hydrolysis were: galacturonic acid, at least two units; galacturonic acid and rhamnose; glucuronic acid and galactose; glucuronic acid and xylose; and methyl glucuronic acid and xylose.

The first two were present in very small amounts. They may have come from nonhemicellulose contaminants. Free galacturonic acid was also present.

Rhamnose. This sugar was present in the holocelluloses, in increasing amounts in extracts 1 to 4, and in decreasing amounts in hemicelluloses 1 to 4. None could be detected in the residue. In all cases the amounts of rhamnose were small.

Discussion

Treatment of holocellulose with dilute acid produced two distinct results, a dissolving action and a hydrolyzing action which may interact. By far the greatest part of the dissolved material was removed by the combined action of the 1- and 2-hour treatments. As the holocellulose neutralized some of the acid used in the first extraction, the final pH was 3.5. This treatment was far less effective than the second, or 2-hour extraction, in which the pH was maintained at 2.5.

The treatments removed from the holocellulose all the arabinose, galactose, and rhamnose, practically all the uronides, and three fourths of the xylose. The xylose remaining in the residue would appear to be essentially in the form of pure xylan.

A considerable part of the material dissolved by each of the successive treatments was hemicellulose, precipitated from the neutralized extract by ethyl alcohol. These four hemicellulose preparations showed changes in composition as to the relative amounts of the three sugars, galactose, arabinose, and xylose, in them. Whether this resulted from fractionation of the hemicelluloses or only from the gradual removal of galactose and arabinose by hydrolysis is uncertain.

Uronides were present in all four of the hemicelluloses, as well as in the extracts. Thus they were more resistant to removal by hydrolysis than were the sugars except xylose and glucose.

The action of dilute acids for a limited time would be expected to affect mainly the side chains of the hemicellulose molecule rather than its central core (*8*). Thus, the simple sugars and the uronides in the extracts probably formed parts of such side chains.

The uronides isolated from the holocellulose are similar to those prepared from some other hemicelluloses (*1–4, 9*). Small amounts of the uronides appeared in the extracts, but more remained in all four of the hemicellulose preparations. Since only a trace of uronides was found in the residue, it appears that the hemicelluloses containing them are more readily soluble than are the xylans free from uronides, which include the xylose remaining in the residue.

The most direct evidence as to the structure of the hemicelluloses lies in the oligosaccharides occurring in the extracts, especially those which appeared in the earlier treatments and which were easily hydrolyzed. These give some indication of the nature of the side chains attached to the main xylan chain of the polyuronide hemicellulose.

Finding an arabinose disaccharide in the products of mild hydrolysis of the grass holocelluloses shows that longer arabinose side chains may occur, unless a separate araban is present. The oligosaccharides containing arabinose and galactose, arabinose and xylose, and arabinose, galactose, and xylose also indicate side chains of some length. From the large amounts of free arabinose in the extracts it seems probable that more and perhaps longer side chains containing this sugar may occur.

The series of xylose oligosaccharides may have come either from side chains of branched molecules or from the main chain broken at random. The former would appear to be the more probable result of the mild treatment.

The behavior of glucose was quite different from that of the other sugars. Only a very small amount of it appeared in the extracts and that only in the final one, resulting from 16 hours of exposure to the hot, dilute acid. Each of the hemicellulose preparations, and also the residue, yielded glucose on hydrolysis with 1*N* sulfuric acid for 6 hours. No oligosaccharides containing glucose were found in the extracts. It seems probable, therefore, that glucose is not combined in the same molecule as are the other sugars and the uronic acids, but arises from some glucan resembling the other hemicelluloses in solubility.

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Complexometric Titration of Calcium and Magnesium in the Presence of Phosphate in Milk and Blood Plasma

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A quick and sensitive method for determining calcium and magnesium in biological materials, with particular reference to milk and blood plasma, is presented. The interference of phosphate ions in this complexometric titration was eliminated by adding the disodium salt of (ethylenedinitrilo)tetraacetic acid to the neutral system and back-titrating the excess with calcium and magnesium standard solutions. Milk and blood plasma samples were used directly without removing the phosphate ions or milk proteins.

The classical procedures for the determination of calcium and magnesium in biological materials are time-consuming and inefficient, especially when these elements are found in small amounts, as in blood plasma. There have been attempts to develop a rapid and accurate titration procedure based on the original work of Schwarzenbach and collaborators (*1*, *6*). The main difficulties encountered in analyzing biological materials are due to the interference of orthophosphate ions, which precipitate calcium and magnesium at the pH required for their determination.

These difficulties were overcome by removing phosphate ions by anion exchange resins and determining calcium in the effluent (*2*, *7*). Separation of calcium from phosphate ions by cation exchange and determination of calcium in the eluent were attempted in milk (*4*, *5*).

The application of ion exchange is, however, time-consuming, especially in milk samples where preremoval of proteins is necessary to avoid precipitation in the exchange column. Moreover, because of the several washings of the exchangers, the calcium and magnesium ions in the effluents or eluents become diluted and the end point of the titration becomes indistinct. This difficulty could be overcome in calcium determination by using a sensitive fluorescent indicator (*8*). The present study, however, determines the calcium and magnesium in milk and blood plasma directly by adding disodium salt of the (ethylenedinitrilo)tetraacetic acid to the neutral system and back-titrating the excess with calcium and magnesium standard solutions. Thus, it is not necessary to remove the milk proteins or use exchange resins to eliminate the interfering ions.

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Apparatus and Reagents

Magnetic stirring bars, Teflon coated.

Polyethylene-coated magnetic pickup rod.

Buffer Solution I, pH 13.0. Dissolve 560 grams of reagent grade potassium hydroxide and 66.0 grams of reagent grade potassium cyanide in 1 liter of glass-distilled water.

Buffer Solution II, pH 10.0. Dissolve 67.5 grams of C.P. ammonium chloride in 200 ml. of glass-distilled water, add 570 ml. of reagent grade concentrated ammonium hydroxide, and dilute to 1 liter.

Standard Calcium Solution, 0.1 and 0.0005%. Dissolve 2.4972 or 0.1249 gram of reagent grade calcium carbonate, previously dried at 110° C., in dilute hydrochloric acid. Make to 1 liter with glass-distilled water. This solution contains 1.00 mg. or 0.05 mg. of calcium, respectively, per ml.

Standard Magnesium Solution, 0.02 and 0.005%. Dissolve 0.2 or 0.05 gram of analytical reagent grade magnesium turnings in dilute hydrochloric acid and dilute to 1 liter with glass-distilled water. This solution contains 0.2 or 0.05 mg. of magnesium per ml.

Standard Solutions of (Ethylenedinitrilo)tetraacetic Acid Disodium Salt (Na_2EDTA), 1.5 and 0.06%. Dissolve 15 or 0.6 gram of the reagent in glass-distilled water and make to 1 liter. Standardize the solutions against the standard calcium and magnesium solutions. The titers for the two solutions are 1.607 or 0.0636 mg. of calcium per ml., and 0.968 or 0.0379 mg. of magnesium per ml., respectively.

Standard Phosphate Solution, 0.1%. Dissolve 4.3903 grams of C.P. potassium dihydrogen phosphate in glass-distilled water, and make to 1 liter. This solution contains 1.00 mg. of phosphorus per ml.

Calcium Indicator, calcein-charcoal–potassium chloride mixture manufactured by the G. Frederick Smith Chemical Co.

Magnesium Indicator. Dissolve 0.2 gram of Eriochrome Black T (Eastman Kodak P6361) in 50 ml. of analytical reagent grade methanol containing 2.0 grams of hydroxylamine hydrochloride.

Principle of Calcium and Magnesium Titration

Addition of excess Na_2EDTA to a neutral or slightly acidic solution containing calcium, magnesium, and phosphate ions causes chelation of calcium and magnesium with part of the Na_2EDTA, and thus prevents precipitation of calcium and magnesium as $Ca_3(PO_4)_2$ in alkaline medium. By back-titrating the excess Na_2EDTA with standard magnesium chloride solution at pH 10.0, and using Eriochrome Black T as indicator, the amount of Na_2EDTA chelated with the sum of calcium and magnesium ions can be calculated. On the other hand, adjusting another portion of the same solution to pH 12.0, after addition of excess Na_2EDTA, releases magnesium from its complex with Na_2EDTA, and the magnesium subsequently precipitates in the alkaline medium as magnesium hydroxide and/or phosphate. From the back-titration of the excess Na_2EDTA with standard calcium chloride solution at pH 12.0 to 14, and using calcein as indicator, one can calculate the amount of Na_2EDTA reacted with calcium. Subtracting the amount of Na_2EDTA reacted with calcium from that reacted with the sum of calcium and magnesium, one can obtain the amount of Na_2EDTA reacted with magnesium. By knowing the titer of Na_2EDTA for each of calcium and magnesium, the amount of calcium and magnesium in the solution can be determined.

Analytical Procedures

Calcium and Magnesium Determination in Milk. For calcium determination, 10 ml. of 1.5% Na_2EDTA are added to 5 ml. of milk in a 125-ml. Erlenmeyer flask and mixed thoroughly. Ten milli-

liters of buffer solution I are mixed with the sample. Then about 20 mg. of calcium indicator are added, which changes the color of solution from white to a light pink. The excess Na_2EDTA is back-titrated with 0.1% standard calcium chloride solution with gentle swirling, using a magnetic stirrer. At the end point, the pink color is changed to a green fluorescence. Dilution with water is avoided, and an artificial fluorescent light is used as background. The amount of Na_2EDTA reacted with calcium is calculated and multiplied by the titer for calcium to obtain the amount of calcium in the sample.

For magnesium determination, 10 ml. of 1.5% Na_2EDTA are added to 5 ml. of milk in a 125-ml. Erlenmeyer flask, and mixed thoroughly. Ten milliliters of buffer solution II are mixed with the sample, followed by 10 drops of Eriochrome Black T indicator just before the titration to minimize the loss of ammonia. The white color of the solution changes to a definite blue. The excess of Na_2EDTA is back-titrated with 0.02% standard magnesium solution, using a magnetic stirrer to obtain the amount of Na_2EDTA reached with the sum of calcium and magnesium. At the end point, the blue color is changed to a red-violet. Artificial fluorescent light is also used and dilution with water is avoided. The amount of Na_2EDTA reacted with calcium is subtracted from that reacted with the sum of calcium and magnesium. The result is multiplied by the titer for magnesium to obtain the amount of magnesium in the sample.

Calcium and Magnesium Determination in Blood Plasma. For calcium determination, 10 ml. of 0.06% of Na_2EDTA are added to 3 ml. of blood plasma in a 125-ml. Erlenmeyer flask, and mixed thoroughly. Five milliliters of buffer solution I are mixed with the sample and about 20 mg. of calcein indicator are added. The excess Na_2EDTA is back-titrated with 0.005% standard calcium chloride solution until the greenish yellow color of the sample is changed to a definite green fluorescence as an end point. Titration and stirring should be done slowly, to avoid foaming. An artificial light is used and dilution with water is avoided. The calculation is the same as that of milk calcium.

For magnesium determination 10 ml. of 0.06% Na_2EDTA are added to 3 ml. of blood plasma in a 125-ml. Erlenmeyer flask and mixed thoroughly. Five milliliters of buffer solution II are mixed with the sample, followed by 5 drops of Eriochrome Black T indicator, both added just before the titration. The excess Na_2EDTA is back-titrated with 0.005% standard magnesium chloride solution until the bluish color of the solution is changed to a blue-violet. Titration and stirring should be done slowly to avoid foaming. Precautions concerning the light and dilution as well as calculation are the same as in milk magnesium.

Results and Discussion

Ten-milliliter aliquots of 1.5% Na_2EDTA were pipetted into 125-ml. Erlenmeyer flasks, each containing 5.0 mg. of calcium and 1.0 mg. of magnesium. Increasing amounts of 0.1% standard phosphate solution were added to these flasks. Calcium and magnesium were analyzed by the procedures described above. Recovered amounts of calcium and magnesium are presented in Table I.

Recovery experiments for calcium and magnesium were also made using various milk and blood plasma samples. To each of the 5.0-ml. milk samples, previously analyzed for calcium and magnesium, 5.0 mg. of calcium, 2.0 mg. of magnesium, and 5.0 mg. of phosphorus were added. To each 3.0 ml. of blood plasma, 0.5 mg. of calcium, 0.5 of magnesium, and 0.5 mg. of phosphorus were added. The results of analyses, the amounts of calcium and magnesium recovered, and the standard deviations are presented in Table II for milk and blood plasma.

Table I shows that calcium and magnesium added either in the absence or presence of phosphate ions were recovered to a great extent by this method. Neither small nor large amounts of phosphate had any effect on the recov-

(*Continued on page 158*)

Table I. Recovery of Calcium and Magnesium in the Presence of Phosphorus

Phosphorus, Mg. Added	Calcium, Mg.			Magnesium, Mg.		
	Added	Found[a]	Diff.	Added	Found[a]	Diff.
0.0	5.0	4.98	−0.02	1.0	1.02	+0.02
2.0	5.0	5.00	0.00	1.0	1.01	+0.01
3.0	5.0	4.95	−0.05	1.0	1.02	+0.02
5.0	5.0	5.03	+0.03	1.0	0.99	−0.01
8.0	5.0	4.95	−0.05	1.0	1.02	+0.02
10.0	5.0	4.98	−0.02	1.0	1.00	0.00

[a] Each value is an average of two independent determinations. Standard deviation for calcium ±0.0366. Accuracy ±0.732%. Standard deviation for magnesium ±0.0173. Accuracy ±1.73%.

Table II. Recovery of Calcium and Magnesium Added to Milk and Blood Plasma Containing Additional Phosphorus

Calcium, Mg.						Magnesium, Mg.					
	Added						Added				
Sample[a]	Ca	Mg	P	Found[a]	Diff.	Sample[a]	Ca	Mg.	P	Found[a]	Diff.
					Milk Samples						
5.014	5.0	2.0	5.0	10.108	+0.094	0.455	5.0	2.0	5.0	2.517	+0.062
5.769	5.0	2.0	5.0	10.831	+0.062	0.484	5.0	2.0	5.0	2.575	+0.091
5.560	5.0	2.0	5.0	10.606	+0.046	0.465	5.0	2.0	5.0	2.526	+0.061
5.062	5.0	2.0	5.0	10.156	+0.094	0.455	5.0	2.0	5.0	2.507	+0.052
5.030	5.0	2.0	5.0	10.060	+0.030	0.513	5.0	2.0	5.0	2.565	+0.052
5.432	5.0	2.0	5.0	10.462	+0.030	0.523	5.0	2.0	5.0	2.536	+0.013
4.982	5.0	2.0	5.0	10.060	+0.080	0.629	5.0	2.0	5.0	2.691	+0.062
					Blood Samples						
0.332	0.5	0.5	0.5	0.810	−0.022	0.034	0.5	0.5	0.5	0.534	0.000
0.292	0.5	0.5	0.5	0.806	+0.014	0.024	0.5	0.5	0.5	0.515	−0.009
0.295	0.5	0.5	0.5	0.791	−0.004	0.025	0.5	0.5	0.5	0.517	−0.008
0.332	0.5	0.5	0.5	0.820	−0.012	0.022	0.5	0.5	0.5	0.522	0.000
0.318	0.5	0.5	0.5	0.800	−0.018	0.023	0.5	0.5	0.5	0.534	+0.011
0.306	0.5	0.5	0.5	0.823	+0.017	0.033	0.5	0.5	0.5	0.527	−0.006
0.305	0.5	0.5	0.5	0.797	−0.008	0.006	0.5	0.5	0.5	0.497	−0.009

[a] Each value is an average of two independent determinations.
Standard deviation and accuracy for calcium ±0.073, ±1.46% in milk; ±0.016, ±3.20% in blood plasma.
Standard deviation and accuracy for magnesium ±0.065, ±3.25% in milk; ±0.008, ±1.60% in blood plasma.

(Continued from page 157)

ery. Standard deviation was ± 0.0366 for calcium and ± 0.0173 for magnesium. In the presence of additional amounts of phosphate, the milk and plasma constituents do not seem to interfere with calcium and magnesium results (Table II). Calcium and magnesium in milk were accurate to ± 1.46 and ± 3.25%, respectively; to ± 3.20 and ± 1.60%, respectively, in blood plasma. The positive values of the recovery test shown in Table II for milk samples were a matter of chance in that set of determinations.

The color densities of end points are slightly different in milk than in plasma or blank solution because milk has a turbid background while plasma and blank solutions are transparent. However, at the end points a sharp change in color is observed.

Huditz, Flaschka, and Petzold (*3*) obtained reliable results by applying the principle of this investigation in the indirect determination of phosphorus. The excess of Na_2EDTA added to the acid solute of magnesium ammonium phosphate hexahydrate was back-titrated with magnesium standard solution. However, in calcium determination adding Na_2EDTA in excess to the solution and titrating the excess with a standard calcium chloride gave unreliable results (*5*). Malkki (*4*) later successfully used a similar method for calcium and magnesium determination in milk. However, with modifications in indicator, buffers, and reagent concentration, the method is applicable to both milk and blood plasma directly and with a high degree of accuracy.

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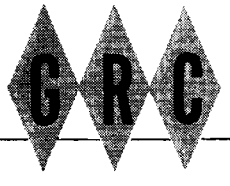

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FERTILIZER CALCULATIONS

The Calculation of Formulations for Granular Fertilizers

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The formulation of granular fertilizers is discussed with particular emphasis on the methods of calculating moisture, heat, and ammoniation requirements. The method is based on first defining the requirements for granulation and then selecting the raw materials to meet these requirements. The chemistry of ammoniation has been expanded to include a basis for calculating the ammonia retention of various mixtures of normal superphosphate, triple superphosphate, phosphoric acid, and diammonium phosphate. A comparison is shown between calculated results and actual plant tests.

THE PROBLEM of calculating formulations to be used in the manufacture of granular fertilizers is complex. The formulator is restricted to combinations of raw materials which give a product containing the required concentration of nutrients. Within this limitation, he must find combinations of raw materials which will granulate well in the plant. The granules produced must be in the proper size range, and simultaneously the required production level must be maintained. In addition, loss of nitrogen and reversion of available phosphate must be minimized. After considering all of the possible combinations, the formulator then must pick the combination which gives the lowest possible raw material costs.

The behavior toward granulation is the most difficult requirement to predict. The three main factors which control the formation of granules are moisture, heat, and soluble salt content. The problem is thus one of assigning numbers to these quantities which can be related to the optimum conditions for granulation. This paper aims to point out these relationships, to show how the conditions required for optimum granulation can be estimated, and to provide data for estimating the ammonia retention of complex phosphate mixtures in which phosphoric acid and diammonium phosphate are used along with superphosphates.

If the behavior of different formulations could be predicted with complete accuracy, there would be no need for running plant tests. Obviously, present knowledge of granulation is too incomplete to make these highly accurate predictions at the present time. However, even moderately accurate predictions can help the manufacturer reduce the loss of plant time and materials in attempting to make impossible formulations work, provide a sound basis for changes in the formulation to achieve the desired performance, assist in the selection of raw materials, and make cost comparisons possible between different formulations on a basis of approximately equivalent plant performance.

Requirements of a Formulation

There are many factors which influence the agglomeration of fertilizers, such as the size and porosity of the raw material particles, the equipment used, and the operating conditions. These factors are not considered in the present discussion because they cannot be controlled by specifying the pounds of raw materials to be used.

There are seven major requirements (Table I) which are controlled by the formulation of a complete (NPK) fertilizer. Consequently, seven raw materials will be required. Water and filler are considered as raw materials when used in a formulation. A raw material may be eliminated each time a requirement is removed, as in the omission of potassium chloride from a grade or not requiring a specified ammoniation level for the phosphates. At other times, the amount of a given raw material may be so small that for practical purposes it can be eliminated. However, the latter circumstance must be regarded as a special case and not the general rule. Table I gives the most common methods (not a complete list) of the ways of adjusting formulations to meet these requirements. The seven requirements are expressed mathematically

Table I. Common Raw Materials Generally Used to Meet Requirements of Granulation

Requirement	*Raw Materials Adjusted*
Units of N	Nitrogen solution, basic source[a]
Units of P_2O_5	Normal superphosphate, basic source[a]
Units of K_2O	Potassium chloride, basic source[a]
Total weight, dried	Add filler, low analysis. Replace normal with triple superphosphate, high analysis.
Ammoniation balance	Replace solution with anhydrous ammonia, to use extra ammonia retention. Add sulfuric acid, increase ammonia retention.
Water balance	Add water to increase moisture. Increase recycle or replace solution with ammonium sulfate to decrease moisture.
Heat balance	Add sulfuric acid, increase heat. Increase recycle or replace sulfuric acid and anhydrous ammonia or solution with ammonium sulfate, reduce heat.

[a] By "basic source" is meant that as much as possible of these raw materials are generally used and that other raw materials replace the basic source for some specific reason.

as functions of the variables involved in Table II.

Nutrient and Material Balances. The first four requirements are concerned only with achieving the proper analysis of the finished product. These requirements present no real problem in calculations. The ammoniation, water, and heat balances require further consideration, because they are less easily calculated and are directly related to the actual granulation process.

Ammoniation Balance. The term "degree of ammoniation" will be used here to express per cent of maximum ammonia retention by phosphates and acid which are utilized to the absorbed, uncombined ammonia added as anhydrous ammonia and as "free" ammonia in a nitrogen solution. The maximum (practical) ammonia retention for normal and triple superphosphates (*3*, *9*) is generally assumed to be 6 and 4 pounds per unit of phosphorus pentoxide, respectively, although there is by no means complete agreement among the various investigators. There are few or no experimental data on the ammonia retention of phosphoric acid in phosphate mixtures (*6*). Interactions occur with the gypsum present in normal superphosphate, as discussed in a later section on the chemistry of ammoniation. As a result of interactions, the ammonia retention of mixtures of phosphoric acid with superphosphate cannot be accurately calculated by adding up the ammonia retention of the different phosphates. Figure 1 translates this chemistry into a usable form for calculating the ammonia retention of mixed phosphates. Formulations based on this chart have been run successfully in a number of different plant tests; however, additional testing is being carried out under more thoroughly controlled conditions.

The total ammonia retention of the formulation may be calculated as follows. The units of phosphorus pentoxide added as normal superphosphate divided by the total units of phosphorus pentoxide in the formulation give the per cent of phosphorus pentoxide added as normal superphosphate and which may be readily determined. Similarly, the per cent of the phosphorus pentoxide added as triple superphosphate and as phosphoric acid may be determined. By locating a point on the three-component diagram (Figure 1), the average ammonia retention of the mixture in pounds of ammonia per unit of phosphorus pentoxide in the mixture can be read from the contour lines of constant ammonia retention. Thus, by multiplying the average ammonia retention per unit of phosphorus pentoxide by the total units of phosphorus pentoxide in the formulation, the total ammonia retention of the phosphate mixture is obtained (Equation R-5). The ammonia retention of sulfuric acid is based on the conversion of the sulfuric acid to ammonium sulfate. The weight of 66° Bé. acid divided by 3.1, or the weight of 60° Bé. acid divided by 3.72, gives the pounds of ammonia which can be held. The sum of the ammonia retention of the total phosphates added to that of the sulfuric acid gives total ammonia retention. The per cent of maximum ammoniation is the per cent of total ammonia retention neutralized by the total uncombined ammonia.

Water Balance. The moisture re-

Table II. Equations Expressing Requirements

Units of N = $(a_1A + b_1B + c_1C + d_1D)/2000$ — R-1

Units of P_2O_5 = $(d_2D + e_2E + f_2F + g_2G)/2000$ — R-2

Units of K_2O = $h_3H/2000$ — R-3

Total weight (dried) = 2000 lb. = $A + B + C + D + E + F + G + H + I + J + 20(M_p - M_f)$ — R-4a

M_p = moisture in product as % H_2O

M_f = moisture in feed as % H_2O = $(b_6B + e_6E + f_6F + g_6G + i_6I + j_6J)/(A + B + C + D + E + F + G + H + I + J)$ — R-4b

Per cent of maximum practical ammoniation utilized = $(a_4A + b_4B + d_4D)/[f(d_2D, e_2E, f_2F, g_2G) + i_8I]$ — R-5

$f(d_2D, e_2E, f_2F, g_2G)$ = (av. lb. NH_3 per unit of P_2O_5) × $(d_2D + e_2E + f_2F + g_2G)/20$ (total units of P_2O_5)
(from Figure 1)

Recycle required = R = lb. of recycle per lb. of feed = $(M_f - M_0)/(M_0 - M_r)$ — R-6

M_f = moisture in feed, see Equation R-4b

M_r = moisture in recycle as % H_2O

M_0 = moisture required for granulation = $f(b_5B)$ see Figure 2

Heat required = $H_0(1 + xR) = e_7E + f_7F + g_7G + i_7I$ — R-7

$H_0 \approx 3500$ cal./100 g., $x \approx 0.6$ } see "Heat Balance"

Symbols

Material	Lb./Ton	Per cent N	P_2O_5	K_2O	Free NH_3	NH_4NO_3	H_2O	Heat
Ammonia, anhydrous	A	a_1	..	..	a_4	..	..	..
Nitrogen solutions	B	b_1	..	..	b_4	b_5	b_6	..
Ammonium sulfate	C	c_1	..	..	..	..	..	..
Diammonium phosphate	D	d_1	d_2	..	d_4	..	..	..
Phosphoric acid	E	..	e_2	..	..	..	e_6	e_7
Normal superphosphate	F	..	b_2	..	..	..	f_6	f_7
Triple superphosphate	G	..	g_2	..	..	..	g_6	g_7
Potassium carbonate	H	..	..	h_3	..	..	..	..
Sulfuric acid	I	..	..	..	..	..	i_6	i_7
Water	J	..	..	..	..	..	j_6	..

i_8 = lb. of ammonia absorbed per pound of sulfuric acid.

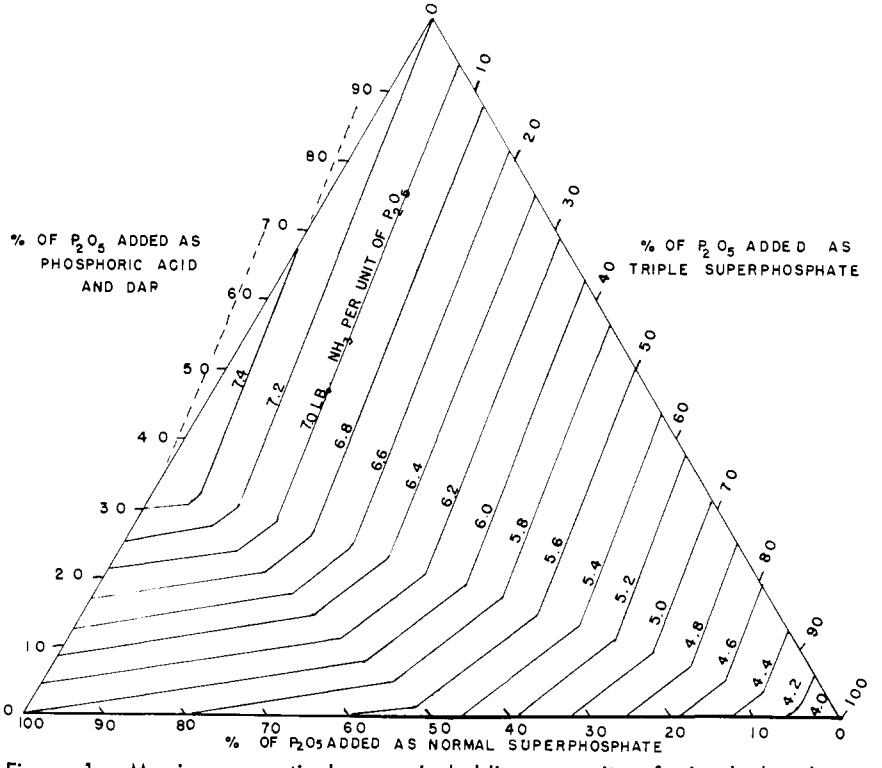

Figure 1. Maximum practical ammonia-holding capacity of mixed phosphates

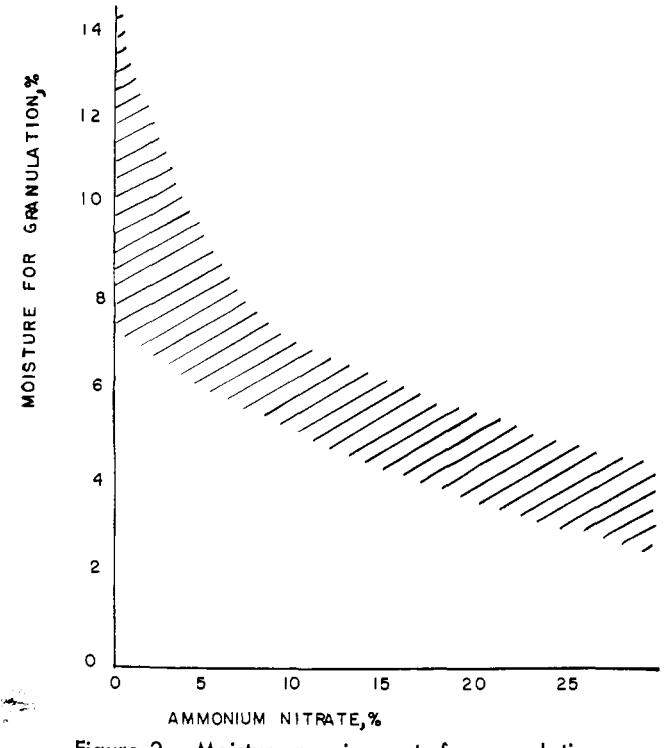

Figure 2. Moisture requirements for granulation

quirements for optimum granulation will depend upon the temperature of granulation and the amounts of the various soluble salts which are present (*2*, *5*, *7*, *11*, *13*), and to some extent, upon the equipment which is used in the plant. The solubility of ammonium nitrate is considerably greater than that of other salts used in fertilizers with the exception of urea. Consequently, as a first approximation, it may be assumed that the ammonium nitrate concentration will be the governing factor with respect to the amount of water required for granulation. Furthermore, if it is assumed that the granulation will be taking place around 200° F., it is possible to establish a general relationship between the moisture requirements for granulation and the amount of ammonium nitrate present in the formulation. At higher temperatures, the greater solubility of the salts decreases the amount of water required. However, this effect is partially compensated for by the increased evaporation of water in the ammoniator.

The moisture content in the granulator will depend upon the relative amounts and moisture contents of both the feed materials and the recycle added to the ammoniator-granulator system. Because recycle is used as a means of reworking of fines (including those produced from milling oversized particles), the amount of recycle will depend upon how closely the product is sized. As a result, the formulator must have some knowledge of what level of recycle to expect for the particular product being prepared and the capabilities of the plant to handle different amounts of recycle. Because the formulation used will automatically balance out at a characteristic recycle ratio in the plant, it is up to the formulator to adjust the formulation to give approximately the desired recycle ratio.

The calculation of water balance may be considered, by assuming that a new formulation has been obtained and that it is to be checked for water balance. From the amount of nitrogen solution used and the per cent of ammonium nitrate present, the per cent of nitrate in the final product may be calculated as ammonium nitrate, although part of the nitrate will be present as other salts. By using the calculated per cent of ammonium nitrate, the approximate moisture requirements for granulation may be obtained from Figure 2. This moisture content is that expected in the ammoniator after the feed and recycle have been blended together. To estimate how much recycle will be required, it is necessary to know how much water will be introduced with the raw materials and how dry the recycle will be. By use of Equation R-6, the recycle may be calculated.

If, for example, it is assumed that a recycle of 0.5 pound per pound of product is generally required (from experience) to give the desired plant operation and maximum plant production and a recycle of 3.5 is calculated, it is fairly certain that the formulation will be too wet, unless higher recycles than 0.5 are used. If the recycle calculated is negative, the formulation will probably require water which can easily be added as required. However, in this case, it may be cheaper to reformulate and use more nitrogen solution. In general, one would like to have at least three similar formulations, before making an actual plant test, which differed in the amounts of raw materials to give recycle ratios of 0, 0.5, and 1.0. The chances would then be very good that one of these would give close to the desired performance in the plant and little time would be wasted in the final selection of the formulation.

Moisture, of course, is only one aspect of the formulation problem and, for the moment, it has been assumed that other requirements, such as heat, have been fulfilled.

The data presented in Figure 2, at best, can predict moisture requirements for granulation to within 1%. Larger deviations will be encountered frequently, because of the failure to consider the effects of the other soluble salts, temperature variations, type of equipment used, and solution time for the various salts. However, the results are in rough agreement with the studies of Hardesty and coworkers (*5*) who have studied the effects of various salt concentrations on moisture requirements for granulation. In spite of these limitations, the use of moisture relationships is extremely useful in discarding formulations which are completely out of line with respect to the proper moisture balance. The greatest deviations from the curve (Figure 2) occur at low ammonium nitrate concentrations. Under these conditions, the effects of other soluble salts are of greater importance, because they are not obscured by the more soluble ammonium nitrate. Fortunately, formulations employing low concentrations of nitrogen generally require additional water. Under these conditions, the amount of water can be determined in the plant test and need not be predicted with accuracy in computing the formulation.

Little or no information is available on the effects of urea on moisture requirements for granulation. Because the solubility of urea is similar to ammonium nitrate, it may be expected that the effect on granulation will be similar. On this basis, formulations employing urea can be calculated by assuming that its effect on granulation will be approximately equivalent to that of the same weight of ammonium nitrate. Because urea and ammonium nitrate form a eutectic of very high solubility, moisture requirements for granulation of mixed urea–ammonium nitrate formulations would be expected to be much less than the value predicted on the preceding basis (*3*). No plant studies have been made yet which would allow moisture requirement correlation. However, limited pilot plant and plant experience with mixed urea–ammonium nitrate solutions indicates that more

water is required when urea replaces part of the nitrate, all other conditions being equal.

Heat Balance. The major aim in calculating a heat balance for the formulation is to ensure that the materials are brought to a suitable temperature range for good granulation. The heat produced must raise the temperature to the granulation temperature. In addition, a certain amount of heat will be lost, the amount depending upon the size and type of equipment employed. The evaporation of water in the ammoniator acts as a temperature modifier because, if excessive heat is produced, the amount of cooling achieved by evaporation of water will be greater.

A method has been developed by which a comparison can be made between the amount of heat which is required and the amount of chemical heat produced by reactions in the ammoniator. The units in which the heat balance is expressed are unimportant as long as a consistent system is employed. The calculations are based on small calories per hundred grams of fertilizer. By multiplying these numbers by 1.8, the British thermal units per 100 pounds of fertilizer are obtained, or by multiplying by 36, the British thermal units per ton may be calculated. The specific heat of the fertilizer mixes can be calculated by totaling the individual specific heat of each raw material. However, a check on formulations for a 5–20–20, 10–20–10, and 12–12–12 grade indicated that the variation in specific heat from grade to grade in most cases does not justify the time spent in making these calculations. For practical purposes, it may be assumed that the fertilizer materials have a specific heat of approximately 0.35. A typical value for the heat required to bring the raw materials being fed to the plant at 60° F. up to the granulating temperature would be 3500 calories. Of these, approximately 2700 calories are consumed in heating the feed from 60° to 200° F. and the remaining 800 calories are lost in water evaporation and by heat transfer through the equipment. With raw materials at 0° F., an additional 1200 calories might be required, whereas at 100° F., the heat requirements might be 800 calories less. Heat will be absorbed also in heating the recycle from the temperature at which it is fed into the system up to the temperature of granulation. The amount of heat absorbed will depend upon the amount and the temperature of the recycle. If the recycle is entering the system at 110° F., the temperature of the recycle must be increased by only 85° F. to bring it to a temperature of 200° F. for granulation, as compared with the temperature of the raw materials which must be increased by 140° F. Consequently, in this case, only 0.6 as much heat will be absorbed in heating 1 pound of recycle than is absorbed in heating 1 pound of feed. The over-all heat requirements can thus be estimated by an equation of the type

$$H = H_0(1 + xR)$$

in which

$H_0 \approx 3500$; R = pounds of recycle per pound of product

The value of x is determined by the recycle temperature and can be calculated by the equation

$$x = \frac{T_g - T_r}{T_g - T_f} \approx 0.6$$

in which

T_g = temperature in the granulator
T_r = temperature of the recycle
T_f = temperature of the raw materials

Steam is sometimes used as a source of heat in granulation, although the more common practice is to depend exclusively upon chemical heat. When using steam as a heat source, 1 pound of 100% quality steam per ton of fertilizer is equivalent to 27 calories per 100 grams of fertilizer. The over-all heat balance may then be used to estimate the steam requirements.

The chemical heat is produced almost exclusively by reactions of the free ammonia present in the nitrogen solutions or of the anhydrous ammonia with the phosphates or other acidic materials in the formulation. Because the amount of heat produced per pound of ammonia absorbed depends to a much larger extent upon the material reacting with the ammonia than upon whether the ammonia is added in the form of a nitrogen solution or anhydrous ammonia, it is logical to calculate the amount of heat produced on the basis of the different types of ammonia-absorbing material rather than on the form in which the ammonia is introduced. The heat produced when anhydrous ammonia is absorbed by various types of superphosphates has been determined in the laboratory by Hardesty and Ross (*4*). These experimental results have been replotted (Figure 3) in terms of the heat produced per unit of phosphorus pentoxide when different levels of ammoniation are employed. When presented in this way, the results show that the heat produced in absorbing a given amount of ammonia by one unit of phosphorus pentoxide is essentially the same for both normal superphosphate and triple superphosphate whether manufactured from Tennessee or Florida rock. Although the amount of heat per unit of phosphorus pentoxide is independent of the type of superphosphate used, the amount of heat is influenced to a large extent by the amount of ammonia absorbed. Thus, normal superphosphate ammoniated to

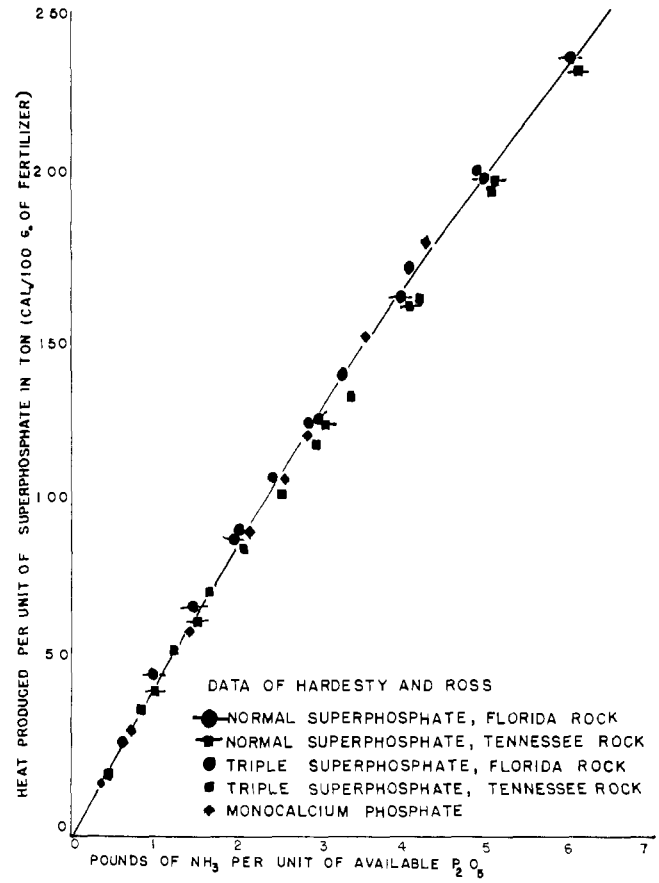

Figure 3. Heat of ammoniation of superphosphates

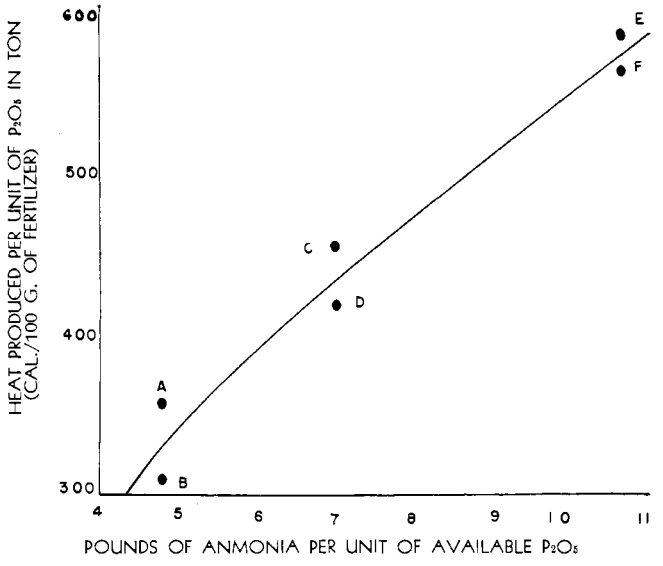

Figure 4. Heat of ammoniation of 75% phosphoric acid

6 pounds of ammonia per unit of phosphorus pentoxide will produce 233 calories, whereas triple superphosphate ammoniated to 4 pounds of ammonia per unit of phosphorus pentoxide will produce 160 calories (Figure 3). Because the concentration of phosphorus pentoxide in the fertilizer is the same whether expressed in units per ton or grams per hundred grams of fertilizer, the total heat produced by ammoniation of the superphosphate may be estimated by multiplying the units of normal superphosphate in the grade by 233 and the units of triple superphosphate by 160 or whatever numbers correspond to the degree of ammoniation used in the formulation.

The amount of heat produced by the ammoniation of phosphoric acid has been calculated from the heats of formation (*12*) of the raw materials and products formed (see Chemistry of Ammoniation). The amount of heat produced depends to some extent on whether the ammonium salts are produced as solids or as solutions and how much gypsum (from normal superphosphate) is present to increase ammonia retention. However, these effects (Figure 4) are of less importance than the amount of ammonia absorbed. Thus, the heat produced per unit of phosphorus pentoxide when phosphoric acid reacts with ammonia depends primarily on the amount of ammonia absorbed. The heat produced may be estimated to within 10% by assuming that 500 calories are produced per unit of phosphoric acid in formulations which are fully ammoniated.

One pound of 66° Bé. sulfuric acid in the formulation liberates 24.6 calories on ammoniation of 100 grams of fertilizer, whereas 1 pound of 60° Bé. acid releases 18.4 calories per 100 grams of fertilizer. By summing the various sources of chemical heat, the total may then be compared with the estimated requirements based on operating conditions, amount, and temperature of the recycle employed. If the heat balances calculated in these two ways agree within 1000 calories, satisfactory granulation temperatures can generally be expected.

Calculating New Formulations

The discussion so far has been concerned with the analysis of existing formulations to find out what factors render them good or poor, and the evaluation of untried formulations. There is no easy way of preparing a new formulation based on a given set of raw materials. Because correcting for one of the requirements may upset some of the other requirements previously established, a completely satisfactory formulation generally requires a series of approximations. One may start out using only nitrogen solution, normal superphosphate, potassium chloride, and a filler or triple superphosphate. A check of the ammoniation balance will show whether sulfuric acid is required or whether anhydrous ammonia may be used. After suitable adjustment has been made for the ammoniation balance, a check of the moisture balance will determine whether some of the nitrogen solution must be replaced by ammonium sulfate, or if additional water will be required.

After establishing the proper water balance, the heat balance is next checked to determine whether additional acid must be added, or if acid must be removed and replaced by an equivalent amount of ammonium sulfate. In the meantime, these changes have upset the material balance, necessitating readjustment of the amount of filler or triple superphosphate to end up with the proper weight which, in turn, changes the ammoniation balance which must then be recalculated, etc. Eventually, however, a consistent set of results should be obtained.

In order to find the optimum formulation for good granulation, one may calculate a series of such formulations based on different recycle rates. If one calculates a formulation to be used with a relatively high recycle rate, and then uses less, overgranulation will result because of too much heat and moisture. On the other hand, if one calculates a formulation to be used with very little recycle, and then uses more recycle, insufficient granulation will result because the mixture is too cold and too dry. Somewhere in between these extremes must lie an optimum formulation. This optimum formulation is best found in the plant by testing a series of formulations calculated to use different recycle rates.

If one is primarily interested in formulations for the study of the economics of selecting different raw materials, one then need be certain only that the formulation is sufficiently close to the optimum and that the final test in the plant will not result in significant changes in the raw material cost. The method of formulation which has been described should be sufficiently accurate for good cost comparisons. By this method, it may be determined which nitrogen solution would be most economical in use, whether the use of phosphoric acid or diammonium phosphate can lead to over-all cost savings, and at which recycle rate the plant should operate to give the best balance between raw material cost and production capacity.

This method of calculating formulations is not a simple one. Considerable man-hours can be spent in solving the various formulation problems which arise daily. For this reason, the Monsanto Chemical Co. has spent considerable time and effort in developing a program by which an electronic computer can carry out these computations rapidly. Thus, in a matter of seconds, a formulation can be completely solved (*10*).

Formulating with Phosphoric Acid and Diammonium Phosphate

The problems of formulating with phosphoric acid are essentially the same as those with triple superphosphate. Formulations may be calculated in which either part or all of the triple superphosphate is replaced with phosphoric acid. In making these calculations, it is necessary to take into consideration the higher concentration of phosphorus pentoxide in the phosphoric acid, the greater ammonia retention, the higher water content, and the greater amount of heat produced on ammoniation of phosphoric acid as compared with triple superphosphate.

In contrast to phosphoric acid, diammonium phosphate provides an equally high nutrient raw material which contains no water and produces essentially no heat in the formulation. The ammonia present in diammonium phosphate is held much less tightly than that present in ammonium sulfate. Failure to recognize this fact can result in improper formulation causing ammonia losses. This problem, however, may be easily corrected by providing sufficient extra ammonia retention to hold that part of the ammonia which otherwise would be lost from the diammonium phosphate. A simple method for determining a correct degree of ammoniation in formulations employing diammonium phosphate is to calculate the diammonium phosphate as if it were a mixture of anhydrous ammonia and 100% phosphoric acid. On this basis, the combined units of phosphorus pentoxide from diammonium phosphate (and phosphoric acid, if any) are regarded as coming from phosphoric acid. The ammonia in the diammonium phosphate is added to the total free ammonia (anhydrous ammonia and the free ammonia in the nitrogen solution).

Diammonium phosphate, as purchased, contains approximately 10.4 pounds of ammonia per unit of phosphorus pentoxide which corresponds to 0.256 pound of ammonia per pound of diammonium phosphate used.

Chemistry of Ammoniation of Phosphates

The conditions for ammoniation in a granulation plant are generally held within limits: 4 to 10% moisture, 160° to 240° F., and a contact period of a few minutes. Consequently, the chemistry is concerned primarily with what happens under these conditions. The resulting mixture is rather complex and the question of how the various elements are chemically combined requires more information than is obtained by analysis of the elements present. Starting with the work of Bassett (*7*), numerous investigators have studied the problem. Keenen (*8*) made certain conclusions on the basis of the chemical composition of the water-soluble and citrate-soluble fractions. White, Hardesty, and Ross (*14*) based their results primarily on the amount of ammonia lost on heating air-dried samples previously ammoniated with different amounts of ammonia. Other investigations have been based on the amount of water-soluble and citrate-insoluble phosphorus pentoxide present (*9*, *15*). While the reactions occurring with individual phosphates have been studied extensively, little consideration has been given to complex mixtures. To be able to predict the behavior of phosphoric acid and diammonium phosphate as raw materials, required more quantitative knowledge of the chemistry of the ammoniation of mixtures of different phosphate raw materials.

The same chemistry must explain the ammoniation of both normal and triple superphosphate. The only major difference in the reactions must result from the large amount of gypsum present in the normal superphosphate. Unless essentially complete conversion of gypsum to ammonium sulfate and dicalcium phosphate is assumed, the higher ammonia retention of normal superphosphat (on a phosphorus pentoxide unit basis) cannot be readily explained. The chemical reactions (1 to 6) are consistent with the observed water-soluble phosphorus pentoxide content of ammoniated triple superphosphate, but do not account for the presence of 20 to 35% of water-soluble phosphorus pentoxide in normal superphosphate ammoniated to 6 pounds of ammonia per unit of phosphorus pentoxide. The water extraction might cause the ammonium sulfate formed in situ to solubilize some of the phosphate

$$(NH_4)_2SO_4 + CaHPO_4 \rightarrow (NH_4)_2HPO_4 + CaSO_4$$

In any event, the proposed chemistry has been able to provide a basis for formulations with phosphoric acid and diammonium phosphate which has been successful in plant tests.

The basic chemistry has been placed on a semiquantitative basis by calculating the degree to which the various reactions go to completion. The calculations were made in such a manner that the basic chemistry was brought into agreement with the accepted practices on ammoniation rates. The ammoniation rate of phosphoric acid was next calculated by assuming that the same basic chemistry applies, and that the individual reactions occur to the same extent. Finally, firsthand experience was obtained on a series of plant demonstrations at the Ark-Mo Plant Food Co. and other plants to ensure that theory and practice were in general agreement.

The study of the basic chemistry relating to ammoniation practice assuming optimum conditions of temperature and moisture gives the following results.

Reaction	Completed, %	
$Ca(H_2PO_4)_2 + NH_3 \rightarrow CaHPO_4 + NH_4H_2PO_4$	100	(1)
$NH_4H_2PO_4 + CaSO_4 + NH_3 \rightarrow CaHPO_4 + (NH_4)_2SO_4$	100	(2)
$H_3PO_4 + NH_3 \rightarrow NH_4H_2PO_4$	100	(3)
$NH_4H_2PO_4 + NH_3 \rightleftharpoons (NH_4)_2HPO_4$	50	(4)
$3CaHPO_4 + NH_3 \rightleftharpoons Ca_3(PO_4)_2 + NH_4H_2PO_4$	33	(5)
$2CaHPO_4 + CaSO_4 + 2NH_3 \rightleftharpoons Ca_3(PO_4)_2 + (NH_4)_2SO_4$	25	(6)

Equations 5 and 6 are similar in that both involve the conversion of dicalcium phosphate to a more basic form, which has been represented as tricalcium phosphate. Thirty-three and 25% reaction, respectively, for these equations results in the same final mole ratio of tricalcium to monocalcium phosphate of 1 to 6.

The differences in ammonia retention (pounds of ammonia absorbed per unit of phosphorus pentoxide) of mixtures of phosphates can be explained in terms of Equations 1 to 6, and the quantity of gypsum added by use of normal superphosphate. The gypsum reacts with phosphoric acid or triple superphosphate to increase the ammonia capacity. Because of this interaction, the ammonia retention of mixtures of phosphate sources is not simply the sum of the capacities of the individual components.

The amount of gypsum formed by acidulation of pure phosphate rock may be represented by the equation:

$$Ca_{10}(PO_4)_6F_2 + 7H_2SO_4 \rightarrow 3Ca(H_2PO_4)_2 + 7CaSO_4 + 2HF \quad (7)$$

Commercial phosphate rock generally contains a higher proportion of calcium, as well as other impurities, and fluorine is incompletely removed during acidulation. In actual practice, approximately 7.5 moles of gypsum are produced for every 3 moles of monocalcium phosphate. On the other hand, triple superphosphate resulting from acidulation with phosphoric acid may be pictured as an impure monocalcium phosphate with essentially no gypsum present.

One mole of monocalcium phosphate contains one mole of phosphorus pentoxide and as the first step in ammoniation absorbs one mole of ammonia (Equation 1). Expressed in fertilizer terminology, this reaction corresponds to an ammoniation rate of 2.4 pounds of ammonia per unit of phosphorus pentoxide in forming dicalcium phosphate and monoammonium phosphate. However, the gypsum present in normal superphosphate can react further with ammonia and monoammonium phosphate to produce more dicalcium phosphate and ammonium sulfate (Equation 2), absorbing an additional 2.4 pounds of ammonia per unit of phosphorus pentoxide or a total of 4.8 pounds. Further ammoniation to a maximum total of 6.0 pounds requires that a still more basic phosphate be formed (Equation 6) which is represented as tricalcium phosphate, although it might be a form of hydroxyl apatite. Thus, about one fourth of the dicalcium phosphate can be converted to an equivalent amount of tricalcium phosphate with reasonably efficient absorption of an additional 1.2 pounds of ammonia and without serious reversion to citrate-insoluble phosphorus pentoxide.

The initial step in the ammoniation of triple superphosphate also follows Equation 1. The ratio of tricalcium to di-

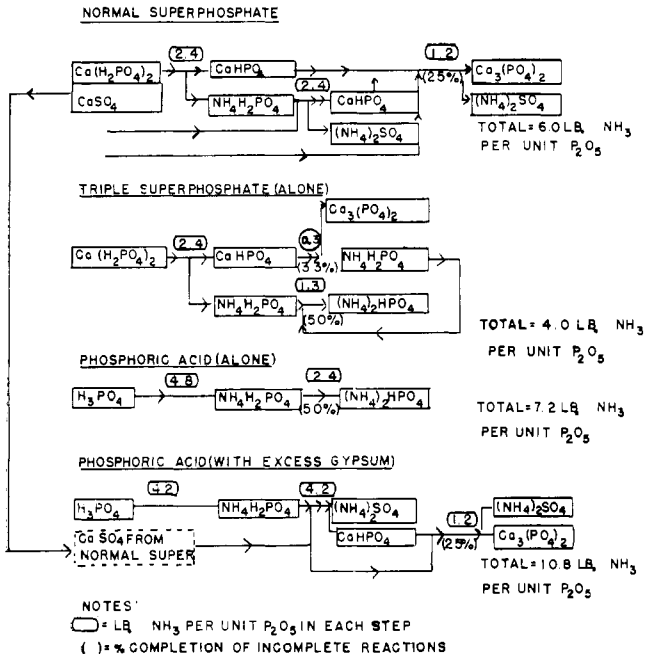

Figure 5. Outline of ammoniation reactions

calcium phosphate will be about the same as for normal superphosphate, if the triple superphosphate particles are sufficiently soft and porous to permit penetration of ammonia which reacts according to Equation 5. However, because only 1 mole of monocalcium phosphate is produced (Equation 1) and the reaction is only one third complete, only an additional 0.3 pound of ammonia can be absorbed. Consequently, the additional ammonia absorption which can be realized must result from the conversion of monoammonium phosphate to diammonium phosphate (Equation 4) which, in going halfway to completion, absorbs about 1.3 pounds of ammonia ($^1/_9$ mole of monoammonium phosphate is produced according to Equation 5, in addition to the 1 mole produced according to Equation 1). Thus, the total ammonia absorption is 4.0 pounds of ammonia per unit of phosphorus pentoxide. With phosphoric acid alone, ammoniation results first in the formation of monoammonium phosphate on absorption of 4.8 pounds of ammonia per unit of phosphorus pentoxide (Equation 3) 50% of which is then converted to diammonium phosphate with absorption of an additional 2.4 pounds of ammonia (Equation 4) for a total of 7.2 pounds of ammonia per unit of phosphorus pentoxide.

When phosphoric acid is used in conjunction with normal superphosphate, which is the usual case, the excess gypsum present in the normal superphosphate acts to increase further the ammonia retention of the phosphoric acid. Assuming adequate gypsum present, the monoammonium phosphate produced, Equation 3, can react with gypsum as in Equation 2 to absorb 4.8 pounds more of ammonia (2.4 pounds per $^1/_2$ unit of acid) and the resulting dicalcium phosphate can react, according to Equation 6, to absorb an additional 1.2 pounds for a total of 10.8 pounds per unit of phosphorus pentoxide as phosphoric acid.

Theoretically, the gypsum in normal superphosphate also can increase the ammonia retention of triple superphosphate to equal that of the normal superphosphate itself. However, because the diammonium phosphate formed in the triple superphosphate, particles first must diffuse out of the triple superphosphate and then diffuse into the normal superphosphate particles to reach the gypsum—complete reaction does not take place. For lack of more complete information concerning this reaction, it is assumed that the reaction goes only about halfway. In other words, the maximum rate for triple superphosphate with excess normal superphosphate present is assumed to be 5 pounds of ammonia per unit instead of the theoretical 6 pounds per unit. In contrast to triple superphosphate, the phosphoric acid being applied in liquid form and producing only soluble salts on ammoniation can react more completely with the gypsum in the normal superphosphate to increase its ammoniation capacity.

The foregoing chemical reactions have been outlined in schematic form (Figure 5). The contribution of each reaction to the total absorption of ammonia is given.

Finally, to complete the information on ammoniation rates, it is necessary to determine the amount of excess gypsum present in normal superphosphate which is available for increasing the ammonia capacity of phosphoric acid or triple superphosphate. In actual practice, about 7.5 moles of gypsum are formed with each 3 moles of monocalcium phosphate (rather than the theoretical 7.0 moles according to Equation 1) or 2.5 moles of gypsum per mole of phosphorus pentoxide. It appears that about half of the gypsum ($1^1/_4$ moles) is used up in ammoniating the normal superphosphate and the other half is available for reaction with phosphoric acid or triple superphosphate. The excess gypsum from one unit of normal superphosphate is sufficient to give the maximum ammoniation rate per one half unit of phosphoric acid or one unit of triple superphosphate. As already mentioned, the phosphoric acid is capable of reacting with gypsum, because it is added in liquid form and is initially converted to soluble ammonium salts which can penetrate the normal superphosphate. Consequently, in calculating the maximum practical ammoniation rates, it is assumed that:

Normal superphosphate always has an ammonia capacity of 6 pounds per unit of phosphorus pentoxide.

Phosphoric acid has a capacity of 10.8 pounds per unit in amounts up to one half the units of normal superphosphate present and 7.2 pounds per unit in excess of this amount.

Triple superphosphate has a capacity of 5 pounds per unit in amounts up to equal the units of normal superphosphate present and 4 pounds per unit in excess of this amount.

In addition, sulfuric acid will react completely with ammonia to form ammonium sulfate

$$H_2SO_4 + 2NH_3 \rightarrow (NH_4)_2SO_4$$

In order to provide a convenient method for the determination of the ammonia capacity of a mixture of phosphates in a formulation, a triangular diagram (Figure 1) has been prepared which represents all possible combinations of phosphoric acid, normal superphosphate, and triple superphosphate. This chart provides compactly the summation of all the theoretical considerations which have been presented. To use the chart, it is necessary to calculate the per cent of the total units of phosphorus pentoxide added in each of the three forms. The percentages thus calculated locate a single point which (by interpolation between lines) gives the average ammonia capacity for the phosphate mixture in pounds of ammonia per unit of phosphorus pentoxide. Thus, by multiplying the average ammonia capacity by the total units of available phosphorus pentoxide the total capacity for ammonia is obtained.

The diagram (Figure 1) is divided into three areas (the ammoniation-rate lines being parallel in each area). The lower left area represents compositions having enough gypsum from normal superphosphate for the maximum ammoniation of

Table III. Formulations, in Pounds, Used in Plant Tests

Material	1 12-12-12		2 10-20-10		3 8-24-8		4 16-20-0		5 5-20-20		6 10-20-0		7 11-48-0		8 5-20-20		9 12-12-12	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Ammonia, anhydrous	25	25	78	78	110	110	49	49	73	73	...	...	...	...	...	...	...	...
Nitrogen solution	432[a]	360	367[a]	306	189[a]	158	540[a]	450	108[a]	90	455[b]	428	228[b]	214	94[c]	94	335[b]	315
Ammonium sulfate	300	300	...	...	...	...	400	400	...	...	...	...	...	...	77	77	278	277
Diammonium phosphate	...	...	...	...	...	...	...	...	...	...	...	...	610	610	...	...	225	214
Phosphoric acid	160	125	361	270	331	248	368	276	405	304	...	...	405	304	...	...	...	...
Normal superphosphate	756	694	1020	969	1030	979	700	665	850	808	1170	1077	...	...	353	325	710	652
Triple superphosphate	...	...	...	...	204	194	130	124	17	16	386	363	880	835	725	700	...	...
Potassium chloride	400	400	333	333	267	267	...	...	667	667	...	...	...	...	667	667	410	408
Sulfuric acid	43	40[d]	...	...	...	...	...	...	...	...	...	...	...	...	144[e]	111	165	128
Total weight	2116	1944	2159	1956	2131	1956	2187	1964	2120	1958	2011	1868	2123	1963	2060	1974	2123	1994

Nitrogen Solution Composition

NH_3, %	NH_4NO_3, %	H_2O, %
[a] 16.6	66.8	16.6
[b] 25.0	69.6	6.0
[c] 23.8	69.8	6.4

[d] 66° Bé. sulfuric acid. [e] 60° Bé. sulfuric acid.

Note: Dry weight = wet or "as is" weight minus weight of water in component.

Table IV. Moisture Balance

Formulation No.	NH_4NO_3, % = b_5 B/2000	Moisture in Recycle, % = M_R	Moisture in Feed, % = M_F, Eq. R-4b	Moisture for Granulation, % = M_G from Figure 2			Recycle Calculated, Lb. Recycle/Lb. Product, $R = (M_F - M_G)/(M_G - M_R)$			Recycle Found, Lb. Recycle/Lb. Product
				High	Av.	Low	Low	Av.	High	
1	14.4	2.5	8.6	6.3	5.4	4.5	0.6	1.1	2.0	1.45
2	12.2	2.5	11.0	6.6	5.8	4.9	1.1	1.6	2.5	1.50
3	6.3	1.8	15.4	8.6	7.4	6.3	1.0	1.4	2.0	0.94
4	18.0	2.7	11.0	5.8	4.8	3.8	1.7	3.0	6.6	2.48
5	3.6	2.3	11.8	10.3	8.5	6.7	0.2	0.6	1.3	1.20
6	15.7	2.0[a]	6.8	6.1	5.1	4.2	0.2	0.5	1.2	0.20
7	15.7	2.0[a]	8.9	6.1	5.1	4.2	0.2	0.5	1.2	0.25–0.50[a]
8	2.6	2.0	11.3	11.2	9.4	7.2	0.1	0.4	0.8	0.75
9	11.3	2.5	6.5	7.6	6.3	5.0	0.1	<0.1	<0.1	0.00[b]

[a] Estimated. [b] Fines not removed. Product yield = 95% of 6/20 mesh.

Table V. Ammoniation Balance

Formulation No.	Phosphate Units: Superphosphate Normal	Superphosphate Triple	Phosphoric Acid	DAP	Maximum Ammoniation: Lb. NH_3/unit P_2O_5 = Y, Figure 1	Lb. Ammonia/Ton: All phosphates = Y (units of P_2O_5)	Sulfuric acid	Total	Free Ammonia Added, Lb.: As solution = b_4 B/100	As anhydrous	As DAP = d_4 D/100	Total	Maximum Ammoniation, %
1	7.5	..	4.5	..	7.57	91	14	105	72	25	..	97	92
2	10.2	..	9.8	..	7.50	150	..	150	61	78	..	139	93
3	10.3	4.7	9.0	..	6.84	164	..	164	31	110	..	141	86
4	7.0	3.0	10.0	..	6.94	139	..	139	90	49	..	139	100
5	8.5	0.5	11.0	..	7.38	148	..	148	18	73	..	91	62
6	8.9	11.1	..	..	5.36	107	..	107	114	..	..	114	107
7	..	20.7	11.0	16.3	5.83	280	..	280	57	..	156	213	76
8	3.4	16.6	..	..	4.50	90	38	128	18	94	..	112	88
9	6.9	..	..	6.0	7.55	91	63	154	84	..	58	142	92

Table VI. Heat Balance

	1	2	3	4	5	6	7	8	9
	Heat Produced, Cal./100 G. Fertilizer								
Normal superphosphate, units × 233	1750	2380	2400	1630	1980	2590	..	793	1610
Triple superphosphate, units × 160	..	..	750	480	60	1440	3310	2660	..
Phosphoric acid, units × 500	2260	4920	4500	5000	5500	..	5500	..	..
Sulfuric acid, lb. × 24.6 for 66° Bé. lb. × 18.4 for 60° Bé.	1060	..	..	..	..	..	..	2650	3040
Total heat added, cal./100 g. fertilizer	5070	7300	7650	7110	7440	4030	8810	6103	4650
Recycle, lb./lb. product	1.45	1.50	0.94	2.48	1.20	0.20	0.5	0.75	0
Ammoniator temperature, °F., T_0	190	193	193	180	200	170	210 est.	195	220
X, $(T_0 - T_r)/(T_0 - T_f)$	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.6	0.6
H_0	3500	3500	3500	3500	3500	4200[a]	4200[a]	3500	3500
Est., heat required, cal./100 g. fertilizer	6540	6650	5450	8700	6010	4700	6000	5400	3500

[a] Raw materials were at 33° to 38° F.

both phosphoric acid and triple superphosphate. The area covering the lower central portion has enough gypsum for all of the phosphoric acid and part of the triple superphosphate. The upper area contains enough gypsum for only part of the phosphoric acid and none for the triple superphosphate to have its maximum ammoniation rate. Thus, it is assumed in making the calculations that because phosphoric acid is in liquid form, it will preferentially react with gypsum. However, the chemistry would be the same in either case.

Plant Tests

The data summarized in Tables III, IV, V, and VI were obtained in five different plants, all of which employed continuous drum-type ammoniators with nominal capacity in excess of 15 tons per hour of product. All but formulation No. 9 were run in plants employing controlled recycle systems. Emphasis has been placed on the more unusual types of formulation in the selection of examples, because these generally present more of a problem to the formulator.

The data indicate the precision with which predictions can be made when formulating on the systematic basis outlined in the preceding sections.

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FERTILIZER CALCULATIONS

Granular Fertilizer Formulation with the IBM 702 Computer

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A granular fertilizer formulation system is used to evaluate economics and operating characteristics of a series of 1–1–1 ratio granular fertilizers. A method of calculating heat requirements for granulation is illustrated. As an example, the effects of varying amounts of diammonium phosphate and phosphoric acid on 12–12–12, 13–13–13, and 14–14–14 grade granular fertilizer formulations are used. The relationship of the effect of variation in these raw materials upon raw material cost and recycle to yield a workable formulation is shown. By using the manufacturer's raw material costs and specifications, it is possible to evaluate the use of specific raw materials in manufacturing operations.

THE MANUFACTURER of granular mixed fertilizers can benefit from the combination of a practical granular fertilizer formulation system and the speed of an electronic computer. The formulation system described by Payne and Webber (*2*) is flexible enough to be adapted to any mixed fertilizer granulation process. By adapting a formulation system such as this to an electronic computer program one can rapidly evaluate the effects of variations in operating conditions, equipment, and raw materials on economic and operational aspects of manufacture.

To produce a granular fertilizer formulation with the formulation system mentioned above, it is necessary to solve discontinuous and nonlinear functions. For this reason, a nonlinear computer program is used which is an iterative process comparable to the trial-and-error approach to the solution of a problem. Where it might take hours for a human to solve a problem, it is a matter of seconds with an electronic computer (*1*).

It is possible to study how certain factors affect granular fertilizer formulations. For example, one can obtain a series of formulations at several recycle levels, using various raw materials and raw material costs. It is possible to limit the level of use of certain raw materials. The heat balance equation can be adjusted to compensate for heat losses in certain equipment or to compensate for extreme ambient temperature conditions.

The purpose of this paper is to show how the fertilizer manufacturer can use such a formulation system to study thoroughly the effects of many variables through the speed of an electronic computer. In evaluating and using a series of fertilizer formulations one can most profitably study the economic relationships and the heat requirements of these formulations. A study of the depend-

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To produce a granular fertilizer formulation with the formulation system mentioned above, it is necessary to solve discontinuous and nonlinear functions. For this reason, a nonlinear computer program is used which is an iterative process comparable to the trial-and-error approach to the solution of a problem. Where it might take hours for a human to solve a problem, it is a matter of seconds with an electronic computer (*1*).

It is possible to study how certain factors affect granular fertilizer formulations. For example, one can obtain a series of formulations at several recycle levels, using various raw materials and raw material costs. It is possible to limit the level of use of certain raw materials. The heat balance equation can be adjusted to compensate for heat losses in certain equipment or to compensate for extreme ambient temperature conditions.

The purpose of this paper is to show how the fertilizer manufacturer can use such a formulation system to study thoroughly the effects of many variables through the speed of an electronic computer. In evaluating and using a series of fertilizer formulations one can most profitably study the economic relationships and the heat requirements of these formulations. A study of the depend-

ence of raw material cost upon other factors can be made when it is possible to obtain a large number of formulations. By evaluating the heat requirements of his granulation unit under varying ambient operating conditions, the manufacturer can select a fertilizer formulation which will ensure a good granular product.

The heat requirements for granulation are dependent upon raw material temperatures, ambient temperature, production rate, amount and temperature of recycle, and heat loss from the granulation equipment. The formulation system being used here (*2*) is based upon the assumption that a fairly constant temperature is maintained in the granulating mass. Control of other factors which affect formulation (moisture and soluble salt content) have been discussed previously (*1*). The control of heat necessary to maintain the proper granulating temperature will be discussed here.

The heat requirement for proper granulation is given by the equation

$$H = H_0(1 + aR) + k' \qquad (1)$$

where

H = heat required for granulation, in calories per 100 grams of product
H_0 = heat required to raise 100 grams of fertilizer material to granulating temperature exclusive of heat losses from granulating equipment
R = recycle, in pounds of recycled material per pound of product
k' = constant for a particular manufacturing unit under certain operating conditions

and $a = \dfrac{T_g - T_r}{T_g - T_f}$

where T_g = temperature in granulator
T_r = temperature of recycled material
T_f = temperature of raw materials

The previous publication by Payne and Webber (*2*) expresses this heat requirement by the equation

$$H = (H_0 + k')(1 + aR)$$

where k' under nominal operating conditions is estimated to be 800 calories to account for heat loss through vaporization of water and radiation. If the value for H as produced by the chemical reactions in the manufacturing process is within ± 1000 calories of the value required by the recycle level, it is assumed that this will give the proper amount of heat. Although not rigorously correct, this expression was chosen because it is more convenient and gives a good approximation to the desired value. It has been shown that Equation 1 provides much better flexibility for this formulation method in adapting the system to specific manufacturing sites and operating conditions. Further, it has been found that by using Equation 1 for calculating heat requirements, the value of H can be determined to a better precision than the 1000 calories previously used.

In adapting the heat equation to his particular plant operation, the manufacturer must consider two things: the temperatures of the raw materials and the ambient temperature at which he is operating, and the value of k' for his equipment. In adapting the heat equation to existing operating temperatures, the values of H_0 and the constant a must be recalculated. This will be illustrated in an example. The value of k' can be estimated by an analysis of formulations which are known to work in the equipment being used. By analyzing these workable formulations for heat requirements at various recycle levels, a value of k' can be calculated and its average value used in selecting subsequent formulations.

To illustrate the use of heat calculations a specific case will be considered in which this method was used to select formulations for a particular manufacturing unit. The case in question involved the start-up of a new granulation unit. As no fertilizer had ever been made with the equipment, there was no way to estimate heat requirements from previously used formulations. A series of formulations was taken from computer calculations in which the heat produced varied over a wide range. The start-up was conducted under extreme weather conditions with subzero temperatures persisting for some time. It was decided to produce a 12–12–12 fertilizer at a rate of about 5 tons per hour in the plant which was rated at a maximum of 20 tons per hour. The plant used a rotary drum ammoniator-granulater and had facilities for controlling recycle.

To be safe, in case excessive fines were produced, the material was recycled at a rate of 1 pound of recycle per pound of product. This would also allow for more flexible control by use of recycle.

In calculating the heat requirement for operation under these conditions the following assumptions were made:

T_f = 0° F.
T_g = 210° F.
T_r = 40° F.

The value of T_r was low, but was assumed to be justified because of the low production rate used.

Then, to calculate the value of H_0 as outlined by Payne and Webber (*2*):

$$H_0 = (210/1.8 \times 0.35 \times 100) + 800$$
$$H_0 = 4900$$

This value is determined by multiplying the temperature change of raw materials to the temperature of the granulater in degrees centigrade times the specific heat times the weight of material in grams. This yields the calories required to raise the temperature of 100 grams of raw material to the granulating temperature.

Table I. Heat Requirements for Granulation Unit

$H = H_0(1 + aR) + k'$

	Case 1	Case 2
T_g, ° F.	210	210
T_f, ° F.	10	60
T_r, ° F.	80	100
k'	1600	1600
H_0	3900	2900
a	0.65	0.73
R^a	0.5	1.0
H	6800	6600

[a] Values of recycle, R, taken from operating data.

To calculate the value a,

$$a = \frac{210 - 40}{210 - 0}$$

$$a = 0.81$$

Hence,

$$H = 4900\ (1 - 0.81R)$$
$$H = 8800$$

When this 8800-calorie formulation was run at the start-up, it was found that too much chemical heat was being produced, giving excessive temperatures in the ammoniator. The next coolest formulation (the one with less chemical heat produced) was then selected. This formulation produced chemical heat at the rate of 6600 calories per 100 grams of product. The recycle necessary to produce a good granular product was about 0.5. Thus, by use of this formulation system and intelligent estimation of operating conditions, a good granular product was produced on the second formulation tried.

When this information is applied to Equation 1, the more rigorous means of expressing heat requirements, this particular manufacturing unit is seen to require a value of k' of about 1600. Table I summarizes two cases of different operating conditions for the above-mentioned 6600-calorie formulation. These are actual operating data. The estimate of T_r of 40° F. for the above example proved to be too low, the actual value being about 80° F.

Both Case 1 and Case 2 used the same formulation, thus requiring a higher recycle under warmer operating conditions (Case 2). It is here indicated, then, that a higher production rate could be obtained by using a formulation with a lower H value for warm weather operation. However, the raw material cost at this lower value of H could possibly be higher, thus offsetting the advantage of higher production capacity. This again depends upon the particular plant site.

Economic Studies

Probably the most valuable application of a formulation-computer program of

Table II. Raw Materials

	Analysis, $N-P_2O_5-K_2O$	Cost, Delivered, Dollars
Nitrogen solution[a]	41.0–0–0	1.35/unit
$(NH_4)_2SO_4$	21–0–0	1.69/unit
Normal superphosphate[b]	0–20–0	0.95/unit
Triple superphosphate[c]	0–46–0	1.40/unit
KCl	0–0–60	0.57/unit
H_2SO_4, 66° Bé.	...	21.20/ton
H_3PO_4, 75%	0–54.2–0	1.45/unit
Diammonium phosphate	21–53–0	115.00/ton

[a] 72.5% NH_4NO_3, 19.0% NH_3, 8.5% H_2O.
[b] 8.0% H_2O.
[c] 5.0% H_2O.

this type is the study of the factors which influence raw material costs. A hypothetical study will be shown to illustrate how this method can be used and the conclusions which can be drawn from such a study.

An electronic computer is capable of producing a large number of fertilizer formulations in a very short time. The effects of such factors as recycle rate, raw materials, and individual raw material costs upon total raw material costs can be thoroughly studied.

As an example, a series of 1–1–1 ratio fertilizers in the range of analysis from 12–12–12 to 15–15–15 was studied. In this hypothetical case a manufacturer is considering the use of phosphoric acid and/or diammonium phosphate as raw materials in his plant. A series of formulations is requested in which various recycle rates and various levels of use of diammonium phosphate and phosphoric acid are stipulated. By making comparisons of the raw material costs with these variables the manufacturer can decide whether to use these raw materials and how to use them.

In Table II are listed the raw materials, their analyses, and the delivered costs which were used in this study. No attempt was made to select raw material prices which would apply to a specific geographic location—those selected were simply chosen to provide some basis for running a series of formulations which could be used to illustrate a method.

The two factors to be considered in choosing a formulation or raw material are the total raw material cost per ton of product and the production rate which can be attained with the formulation being used. For this reason the interrelationships of raw material cost, recycle level, and amount of raw material being used in the formulation must be evaluated. Figures 1 through 4 are graphical representations of this interdependence of the three factors.

Figure 1 shows a plot of raw material cost in dollars per ton of product *vs.* pounds of 75% phosphoric acid per ton of formulation at different recycle levels. In all cases in this paper, recycle is defined in units of pounds of recycle per pound of product. For making a 12–12–12 at the particular manufacturing site being evaluated, the raw material costs generally decrease as more phosphoric acid is incorporated in the formulation. Further, as higher recycle levels are allowed, raw material costs drop. In the cases of low recycle, the raw material costs rapidly reach a minimum value and then increase as more phosphoric acid is added. The reason for the drop in raw material cost with increasing phosphoric acid is that the more concentrated phosphoric acid allows room in the formulation for the cheaper, less concentrated raw materials. Further, phosphoric acid with its higher ammonia-holding capacity allows the use of more low-cost ammonia. The manufacturer must decide for himself whether the decrease in raw material cost with increasing recycle and the accompanying decrease in production rate is acceptable to him.

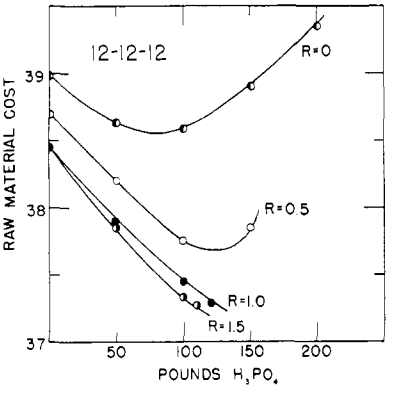

Figure 1. Effect of phosphoric acid on raw material cost at various recycle levels for 12–12–12

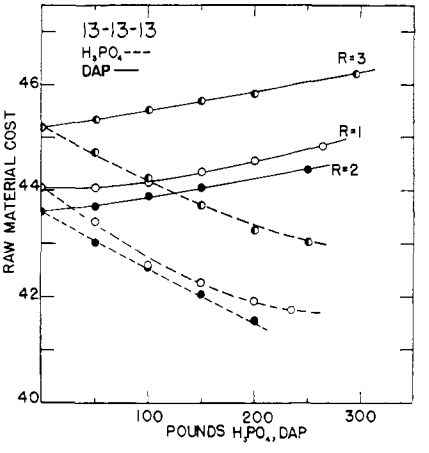

Figure 2. Effects of phosphoric acid and diammonium phosphate on raw material cost at various recycle levels for 13–13–13

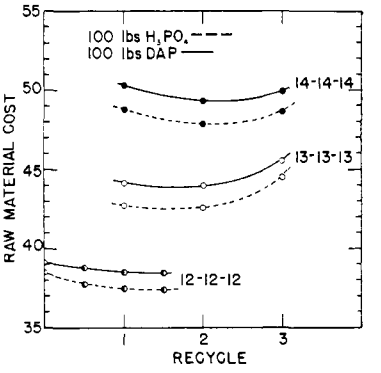

Figure 3. Effect of recycle on raw material cost at 100 pounds of phosphoric acid or diammonium phosphate for various 1–1–1 grades

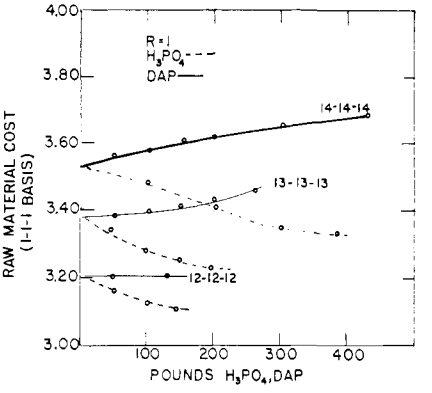

Figure 4. Relative raw material cost as a function of use of phosphoric acid and diammonium phosphate for various 1–1–1 grades at constant recycle level

In Figure 2 the effects of increasing levels of phosphoric acid and diammonium phosphate at different recycle levels are shown. This graph indicates that it is unprofitable to use diammonium phosphate and that the minimum raw material cost is attained under conditions of a recycle value around 2 and at about 200 pounds of phosphoric acid per ton of formulation.

Raw material cost *vs.* recycle level has been plotted for three different 1–1–1 grades at a constant level of 100 pounds of phosphoric acid and diammonium phosphate in Figure 3. Here it is shown that for all three grades it is cheaper to use phosphoric acid rather than diammonium phosphate, the minimum cost being in the neighborhood of 1 to 2 pounds of recycle per pound of product.

Figure 4 illustrates an interesting comparison of the different grades being studied. Plotted on the ordinate of the graph is the raw material cost of fertilizer formulations reduced to a 1–1–1 basis to equate the costs of raw materials for 12–12–12, 13–13–13, and 14–14–14 grades. These values were obtained by dividing formulation raw material costs

by 12, 13, and 14, depending upon the fertilizer analysis. In the case where no phosphoric acid or diammonium phosphate is used, the unit cost increases as over-all analysis increases. However, in some cases, by changing raw materials, the unit cost of making a higher analysis material becomes less. For instance, at a recycle value of 1, a 14–14–14 grade fertilizer can be produced at a lower unit cost than 13–13–13 where no phosphoric acid is used by including 400 pounds of phosphoric acid in the formulation. In addition, this will produce an increase in production rate and plant nutrient of about 8%. This graph illustrates that another factor to be considered is the cost of changing to higher analysis products in terms of changes in raw materials and the increased production rate in terms of plant nutrient that can be realized.

The various ways in which a series of formulations can be evaluated from the standpoint of economics of raw material costs gives the manufacturer a sound basis from which to decide on changes in his operations. He will be able to decide upon a formulation and make changes after estimating the effect of changes in production rate with changes in recycle. He can evaluate the advantages of adding new raw materials in terms of increased profits as opposed to the cost of installing new equipment.

It should be emphasized that the foregoing example is a study of a particular location and cannot be used to generalize the economic effects of diammonium phosphate and phosphoric acid.

Acknowledgment

The authors thank Howard Greiner, Ke-Wash Fertilizer Co., Keota, Iowa, for granting permission to use data from his plant operation.

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(2) Payne, J. H., Webber, R. T., *Ibid.*, **8,** 164 (1960).

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FERTILIZER SOLUTIONS ANALYSIS

Determination of Total Nitrogen and Nitrogen Distribution in Fertilizer Solutions

J. D. SLATER, J. S. HILL, and D. C. SESSO

Grand River Chemical Division, Deere and Co., Pryor, Okla.

A fairly rapid yet accurate procedure was developed for the analysis and sampling of nitrogen fertilizer solutions. The Kjeldahl distillation for ammonia content and the use of Devarda alloy treatment, with Kjeldahl distillation, for nitrate content were found satisfactory. For urea content, modifications of the urease hydrolysis technique were made. A convenient and reproducible sampling procedure was tested.

DURING THE FORMULATION of high nitrogen content fertilizer solutions, containing more than one compound as a source of nitrogen, it is necessary to know the concentration of each nitrogen compound in the solution. This information is desirable for estimation of vapor pressure and salting-out properties and for assurance that the total nitrogen content is acceptable.

High nitrogen fertilizer solutions may contain urea, ammonium nitrate, and ammonia in any combination, covering a fairly wide range of total nitrogen content. For characterization of a solution containing all three compounds, three analyses on some combination of either three components or two components and a total is required in general. To devise the most accurate and rapid sampling and analytical technique, a number of methods (*1, 2, 5, 10*) were considered, evaluated, and investigated.

The determination of total nitrogen by the familiar Kjeldahl technique, as given in the standard methods of the Association of Official Agricultural Chemists including the most recent revisions (*1, 6, 7*), could not be used, because of the time required and the poor accuracy and precision obtained when prepared mixtures were analyzed. Although excellent results could be obtained, by various modifications of the recommended Kjeldahl procedures for the analyses of the pure components of the mixture alone, the determination of the total nitrogen was discarded as too time-consuming. For consistent results, however, work completed during the course of this study indicated that urea samples might require a fairly dilute digestion and nitrate samples would require almost anhydrous conditions for best recovery (Table I).

For rapid estimation of urea alone, the urease enzymatic hydrolysis method of Fox and Geldard (*3*) as modified by Smith, Dock, and Rich (*9*) appeared very promising if analysis time could be reduced and/or if accuracy could be improved. The latest method (*7*) using the enzymatic hydrolysis of urea to ammonium carbonate is a variation of the method by Smith, Dock, and Rich but the back-titration employed represents no basic improvement. However, with considerable reliance upon the literature cited, a method satisfactory with regard to both speed and precision was developed.

For the determination of ammonia and the ammonia from the urea, if present, a distillation of the ammonia from the caustic solution into a boric acid solution was adopted. For samples containing only ammonia and urea, a single acid titration of the solution followed by a urease hydrolysis and analysis of the neutralized solution appeared to be excellent.

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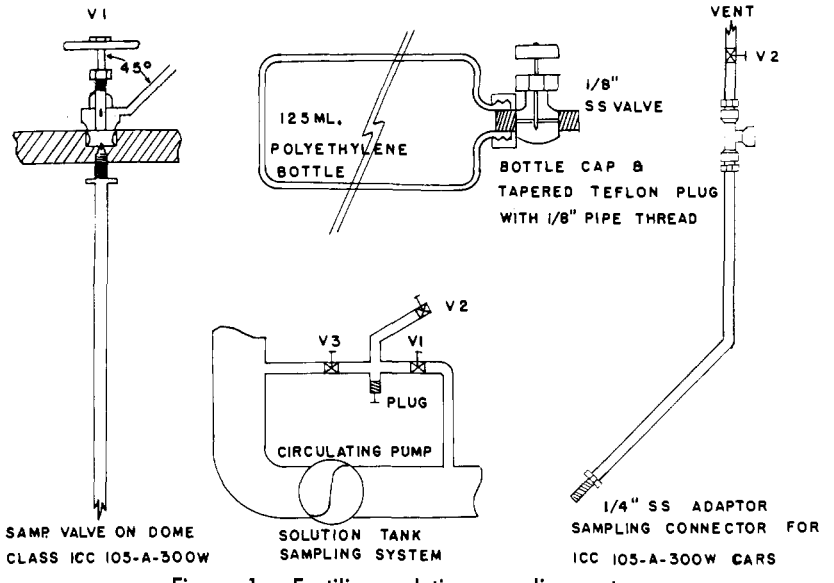

Figure 1. Fertilizer solution sampling system

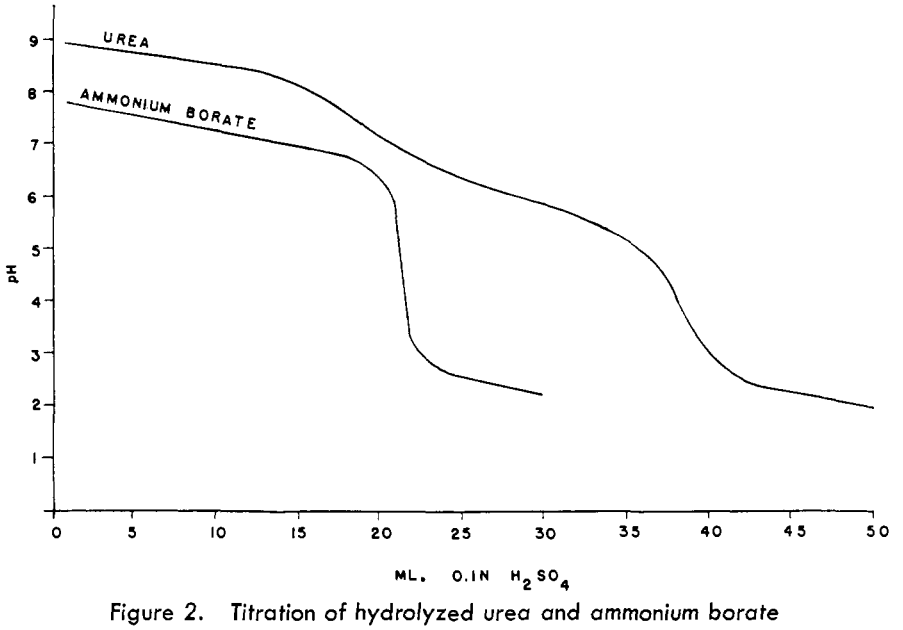

Figure 2. *Titration of hydrolyzed urea and ammonium borate*

For the determination of nitrate content, when present, a satisfactory analysis would be obtained by treating the sample solution, after removal of ammonia, with Devarda alloy and again distilling into a boric acid solution (*1*).

Experimental

Apparatus and Special Reagents. Sulfuric acid solution, 0.1*N*, standardized.

Urease powder, Fisher Scientific Co., U-21.

Devarda alloy, Mallinckrodt G-234.

Kjeldahl distillation apparatus (Precision Scientific), two and six place.

pH Meter, Beckman Model H-2.

Procedure. Carefully weigh a sample containing not more than 1.0 gram of ammonia into a 500-ml. volumetric flask. Dilute to the mark with distilled water.

Transfer a 25-ml. aliquot of the solution to a 250-ml. iodine flask and add 15 ml. of distilled water. Neutralize the solution to pH 4.0 using a recently buffered pH meter. (If only urea and ammonia are present in the sample, this titration could be used for ammonia estimation.) Then add 50 ± 2 mg. of the urease powder from a calibrated dipper. Swirl the solution in the flask to effect suspension of the powder, then add 50 ml. of boiling, distilled water. Stopper the flask and allow it to stand for 30 minutes. Add 1 drop of capryl or octyl alcohol and titrate again to pH 4.0 using standard acid solution. During titration do not use mechanical stirring. Calculate the urea nitrogen content by:

$$\% \text{ urea nitrogen} = \frac{[(A)(B) - F] \times 1.401 \times 500}{(\text{wt. of sample})(25)}$$

where A = ml. of standard acid used for titration
B = normality of standard acid used
F = blank factor, $(A)(B) = F$, for titration of 50 mg. of urease powder

After determination of urea, add 3 ml. of standard acid to the solution titrated above. Transfer the solution completely to an 800-ml. Kjeldahl flask and add sufficient distilled water to obtain a total volume of 300 ml. Carefully pour 50 ml. of a 50% sodium hydroxide solution down the neck of the flask so that the caustic solution forms a layer underlying the solution in the flask. Connect the flask to the Kjeldahl distillation assembly, swirl the flask, and heat to distill approximately 125 ml. of distillate into a 50-ml. boric acid solution (5% by weight). Titrate the boric acid solution with standard acid to pH 4.7. Calculate the distillable nitrogen by:

$$\% \text{ distillable nitrogen} = \frac{(A - B)N \times 1.401 \times 500}{(\text{wt. of sample})(25)}$$

where A = ml. of acid used for titration of sample
B = ml. of acid used for titration of blank (blank solution is water and 50 mg. of urease powder treated as a sample)
N = normality of standard acid

Immediately after the determination of the distillable nitrogen, add to the Kjeldahl flask approximately 125 ml. of distilled water and 5.0 grams of Devarda alloy, carefully wrapped in a nitrogen-free filter paper. As soon as the initial reaction in the flask starts to subside, apply heat, distill, and collect 125 ml. of distillate in a 50-ml. boric acid solution, titrate, and calculate nitrate nitrogen as above for distillable nitrogen, by using an appropriate blank correction.

Ammonia nitrogen =

(distillable nitrogen) − (urea nitrogen)

Total nitrogen =

(distillable nitrogen) + (nitrate nitrogen)

Sampling. For the analysis of fertilizer solution, during or following blending, a sample must be taken of a material which can have a considerable vapor pressure. To obtain reproducible samples from mixing or blending tanks, modifications were made of the sampling method of Greene (*4*) as presented in Figure 1.

To sample, first fill the polyethylene bottle about one third with distilled

water. Squeeze it to force the water to just below the base of the valve. Close it and weigh the bottle and its content. Open valves V_1 and V_3 in the pump bypass, with the plug in place, and close V_2. When circulating solution is at the proper temperature, close valves V_3 and V_1, open valve V_2, and remove the plug. Insert the sample bottle in place of the plug, close valve V_2, and open valves V_3 and V_1. After about 2 minutes, carefully open the valve on the sample bottle, just for the period required to allow the bottle to return to normal bottle shape. Close valves V_3 and V_1, open valve V_2, remove the sample bottle, and replace the plug. Clean the sample bottle valve and reweigh the bottle and contents. Follow a similar procedure for sampling tank cars. For 49 samples of high vapor pressure urea-ammonia solutions analyzed during April of 1959, the average difference between duplicate samples and the analyses for urea was 0.09% and for ammonia 0.08%.

Table I. Total Nitrogen Recovery vs. Method Used for Analysis

	Nitrogen, %			
Sample	Theoretical	1958[a]	1959[a]	Present work
Ammonium sulfate	21.18	21.20	21.08	21.08
	21.18	21.13	21.24	21.14
Urea	46.55	46.47	46.21	46.43
	46.55	46.58	46.26	46.51
Potassium nitrate	13.86	13.33	13.67	13.71
	13.86	13.35	13.73	13.94

[a] AOAC revised method for the year indicated.

Table II. Urea Recovery Data vs. Sample and Operator

	Urea, %			
Operator	Crude Urea, Considered 97.71%		Recrystallized Urea, Considered 100%	
1	97.75	97.71	99.96	99.93
2	97.65	97.54	99.92	100.08
3	97.51	97.50	100.28	100.07
4	97.66	97.63	100.00	100.10
5	97.53	97.35	99.89	99.94
6	97.93	97.91	100.02	99.91
7	97.73	97.57	...	...
8	97.74	97.82	...	...
9	97.71	97.73	...	...
Av.	97.66		100.00	
Std. error	±0.15		±0.11	

Discussion

The accuracy and precision of the urea determination for urea are demonstrated in Table II for a series of samples taken from a lot of recrystallized urea and samples taken from a crude urea where urea was determined by both Kjeldahl analysis and analyses for impurities. The analyses were made by nine different analysts with no more than two analyses made at any one time. The accuracy and precision of the procedure for a urea and ammonia mixture are indicated in Table III, while Table IV gives the results for two urea-ammonia-nitrate mixtures. The mixtures used in this study were prepared by blending, by weight, recrystallized urea, ammonium sulfate (Fisher Scientific Co. primary standard A-938), and recrystallized potassium nitrate.

Early in the study it was realized that a small blank correction was required for each titration. However, this observed blank could be considered constant for any one given lot of urease and any one given lot of Devarda alloy, if the same amounts of each reagent were used in every test.

Direct addition of the urease as a powder to the solution eliminated the need for preparation of usual urease suspensions which change effectiveness with age and require almost constant attention for blank value. Under the conditions given for the enzymatic hydrolysis, 50 mg. of the urease powder could easily convert up to 0.35 gram of urea in 30 minutes.

The end points of the titrations were selected on the basis of curves prepared by plotting pH *vs.* acid used during the titration of numerous samples of varying size and composition (Figure 2). To supplement the pH meter, suitable indicators were also tested and evaluated. For the titration of ammonia in boric acid solution, the mixed indicator solution of Sher (*8*) seemed the most satisfactory with regard to sharpness of color change, change at desired pH, and additional warning color change before the actual end point. For titration of the hydrolyzed urea, a comparable indicator was desired. After trial of several indicators, a mixed indicator solution prepared as follows gave fairly good results.

Table III. Nitrogen Recovery from a Prepared Blend Containing Ammonia and Urea

	Nitrogen, %		
	Urea	Ammonia	Total
	19.95	13.73	33.68
	19.85	13.52	33.37
	19.96	13.56	33.49
	19.86	13.43	33.40
	19.87	13.41	33.28
Av.	19.90	13.53	33.44
Std. error	±0.053	±0.13	±0.15

Table IV. Nitrogen Recovery from Prepared Blends Containing Urea, Ammonia, and Nitrate

	Blend to Contain 28.0% N				Blend to Contain 32.0% N			
	Nitrogen, % in							
	Urea	Ammonia	Nitrate	Total	Urea	Ammonia	Nitrate	Total
	14.62	6.76	6.69	28.07	16.46	7.84	7.70	32.00
	14.64	6.66	6.69	27.99	16.48	7.59	7.62	31.69
	14.58	6.70	6.70	27.98	16.47	7.73	7.49	31.69
	14.61	6.54	6.64	27.79	16.61	7.75	7.91	32.27
	14.65	6.64	6.95	28.24	16.63	7.56	7.68	31.87
Av.	14.62	6.66	6.73	28.01	16.53	7.69	7.68	31.90
Std. error	±0.02	±0.06	±0.12	±0.16	±0.08	±0.12	±0.15	±0.17

Carefully weigh out 0.20 gram of 4 - (4 - dimethylamino - 1 - naphthylazo) - 3-methoxybenzenesulfonic acid powder (Eastman) into a small beaker. Add exactly 5.18 ml. of 0.100*N* sodium hydroxide solution and 0.22 gram of indigo carmine powder (Fisher Scientific Co.), and then dilute to 100 ml. with distilled water. Use four drops of indicator for each analysis and take the end point of the titration as the color change from green through gray to a faint purple.

The data in the tables, with the exception of Table II, were obtained with the pH meter. For Table II, pH meter and indicator were used indiscriminately.

Acknowledgment

The authors thank the personnel of the Analytical Section, for the analyses of the many samples prepared or collected during this study.

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FERTILIZER TECHNOLOGY

Fineness of Commercial Florida Land Pebble and Other Phosphates Used in Superphosphate Manufacture

W. M. HOFFMAN

Soil and Water Conservation Research Division

E. J. KOCH

Biometrical Services, Agricultural Research Service

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Published collaborative studies were examined and screen analyses made on 16 samples of ground Florida land pebble to answer these questions: How reliable are routine screen analyses? How fine is the −200-mesh fraction? How coarse is the +100-mesh fraction? The precision of the −200-mesh determination, slightly less than half that of −100-mesh determination, corresponds with somewhat more than ±2% of the sample at 95% confidence. The average diameter of the −200-mesh (−74-micron) fraction is reduced by about one sixth in grinding from 50 to 85% finer than 200 mesh; the −50-micron fraction is not noticeably altered. The mean diameter of the +100-mesh fraction, excluding +32-mesh material, increases with the percentage of whole sample remaining on the 100-mesh sieve. The mean diameter of the −32-mesh fraction of the whole sample can be inferred from the percentage through the 200-mesh sieve to about 10 microns.

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These five rocks are considered as a set typifying the ranges in physical character and analytical difficulty encountered in domestic ground phosphate rock. Grade and variety were not considered. This is desirable because the emphasis is on variability of determining two common screen fractions rather than one fineness per se. This course seems permissible because grade and variety can be factors only in so far as they influence the physical homogeneity and screenability of the rock.

Standard deviations of individual laboratory means were calculated and classified into frequency distributions, in order to evaluate laboratory performance on the basis of precision. Differences among laboratories were handled in a similar manner to show laboratory performance with respect to accuracy.

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FERTILIZER TECHNOLOGY

Fineness of Commercial Florida Land Pebble and Other Phosphates Used in Superphosphate Manufacture

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Table I. Florida Land Pebble Phosphates Used in Physical Composition Study

Lot No.	Grade BPL, %	Grade P_2O_5, %	Amount Passing 200-Mesh Sieve[a], %
3372	68	31.0	50
3373	68	31.1	85
3374	70	32.1	70
3375	72	33.0	50
3378	72	33.1	55–60
3379	72.5	33.2	70–75
3156	73	33.4	[b]
3157	73	33.5	[b]
3155	73.5	33.7	80–85
3301[c]	75	34.5	50
3376	75	34.3	60
3381	75	34.5	70–75
3377	75	34.3	72
3380	76	34.6	55–60
3302[d]	76	34.7	85
3359-b	77.5	35.5	60–65

[a] Producer's specification.
[b] Special preparation used by Fox *et al.* (*4*).
[c] Association of Florida Phosphate Mining Chemists' check sample 30; used by Haven and Jacob (*5*).
[d] Association of Florida Phosphate Mining Chemists' check sample 31; used by Haven and Jacob (*5*) and by Jacob and Hoffman (*6*).

Table II. Mechanical Composition of Florida Land Pebble Phosphates by Soil Survey Method of Analysis

Lot No.	Percentage Smaller Than 2 μ	20 μ	50 μ	105 μ	177 μ	250 μ	500 μ	1000 μ
3375	6.4	18.8	40.0	65.2	82.8	90.4	96.6	99.8
3378	7.8	20.8	41.3	66.3	80.7	88.3	94.6	98.6
3301	7.3	20.2	42.1	65.7	83.8	95.3	99.6	100.0
3372	6.2	20.0	43.2	70.8	84.6	92.1	97.5	99.7
3380	6.4	19.9	43.0	73.5	90.4	97.1	99.6	100.0
3359-b	6.9	26.4	47.7	72.5	90.1	96.4	99.3	100.0
3376	5.9	20.2	44.7	72.2	89.6	96.0	99.2	99.9
3374	6.5	25.4	52.4	79.3	91.1	96.2	99.0	99.9
3381	6.7	22.4	49.7	82.7	95.6	98.7	99.8	100.0
3157	7.6	31.3	55.1	83.3	98.4	99.9	100.0	...
3377	6.5	24.1	55.6	85.9	96.5	99.0	99.8	100.0
3379	8.3	26.1	53.0	83.6	95.6	98.7	99.8	100.0
3156	10.1	36.1	68.5	92.8	98.4	99.5	99.9	100.0
3373	10.0	35.3	61.7	88.6	99.6	99.9	100.0	...
3155	9.9	33.1	68.1	92.0	97.4	99.1	99.7	99.9
3302	7.8	38.7	71.2	95.0	99.4	99.9	100.0	...

Table III. Range of Fineness and Experimental Precision for Ground Phosphate Rock[a]

Rock Variety	Range of % Passing Sieve	Av. Std. Dev. of Means (within Laboratories)	Av. Differences between Single- and 7-Laboratory Means (among Laboratories)
	100-Mesh Sieve		
Pebble, 23 obs.	76.3–98.4	0.11	0.40
Other, 27 obs.	95.5–99.5	0.10	0.31
	200-Mesh Sieve		
Pebble, 23 obs.	54.4–87.2	0.30	0.58
Other, 27 obs.	80.1–93.5	0.24	1.01

[a] 2 lots of Florida land pebble and 1 each of Florida soft phosphate, Tennessee brown rock, and Wyoming phosphate.

Combined analyses of variance were made to obtain estimates of the within-laboratory and laboratory-to-laboratory components of variance within the four varieties of rock. These components were calculated for both the −100- and −200-mesh fractions. Estimates were obtained of the expected variance of the means for various schemes of collaborative analyses differing in both the number of determinations and the number of laboratories by combining the two components of variance in the following formula:

$$\sigma_x^2 = \frac{\sigma_l^2}{l} + \frac{\sigma_d^2}{ld}$$

where σ_x is the expected variance, σ_l^2 is the estimated component for laboratories on a single lot, σ_d^2 the estimated component for determinations in a single laboratory, and l and d, respectively, are the number of laboratories and the number of determinations per laboratory. Confidence limits were calculated by multiplying the expected variance by the appropriate t value.

Physical Composition. The 16 lots of Florida land pebble described in Table I were analyzed by the official method of the Association of Official Agricultural Chemists for mechanical analysis of phosphate rock (*6*) and also by the Soil Survey Laboratory procedure for the mechanical analysis of soil (*7*). The first procedure, used in the collaborative study, involves wet-sieving a sample of the lot on a 200-mesh sieve followed by dry-sieving the retained fraction with the use of the 100- and 200-mesh sieves. In the second procedure the sample is first split at the 50-micron size by dispersing it in water with the use of agitation and sodium hexametaphosphate as a dispersant, pouring the suspension through the sieve, and washing the retained fraction thoroughly with water. The coarse material is fractionated by dry-sieving, whereas the filtered suspension is analyzed by sedimentation and pipetting. The particle size distributions of Florida land pebble rocks from single determinations of the respective size classes by the Soil Survey method are given in Table II. Plots (not shown in figures) of these accumulative results were used to interpolate on the chord the percentages smaller than 149 and 74 microns, corresponding with the openings of standard 100- and 200-mesh sieves, respectively.

Particle Size of Screen Separates. The fineness, or coarseness, of a screen fraction is gaged by the diameter of the particles in it. Values for this index of fineness were calculated from the particle size distributions given in Table II. Accordingly, the mean weight diameters of screen fractions having special interest were obtained by summing the products of the average diameters and weight fraction of the various size classes and dividing by the sum of the weight fractions.

Table IV. Precision of Screen Analyses in Successive Years on One Lot of Florida Land Pebble

Lab. No.	Standard Deviation of Laboratory Mean: −100-Mesh Fraction 1955	−100-Mesh Fraction 1956	−200-Mesh Fraction 1955	−200-Mesh Fraction 1956
1	0.09	0.09	0.58	0.54
2	0.08	0.12	0.12	0.05
3	0.08	0.08	0.08	0.24
4	0.05	0.03	0.66	0.21
5	0.05	0.00	0.12	0.04
6	0.05	0.16	0.57	0.17
7	0.13	0.03	0.12	0.40

Collaborative Study Results

Variability in Collaborative Study. The over-all variability of results on Florida land pebble and that for the other three rock varieties are shown in Table III. The range of fineness is considerably greater for the Florida land pebble than for the other phosphates. However, the variations within a laboratory determination are approximately the same for a given screen fraction. The differences between single- and seven-laboratory means show much more variation among laboratories than between determinations within a laboratory.

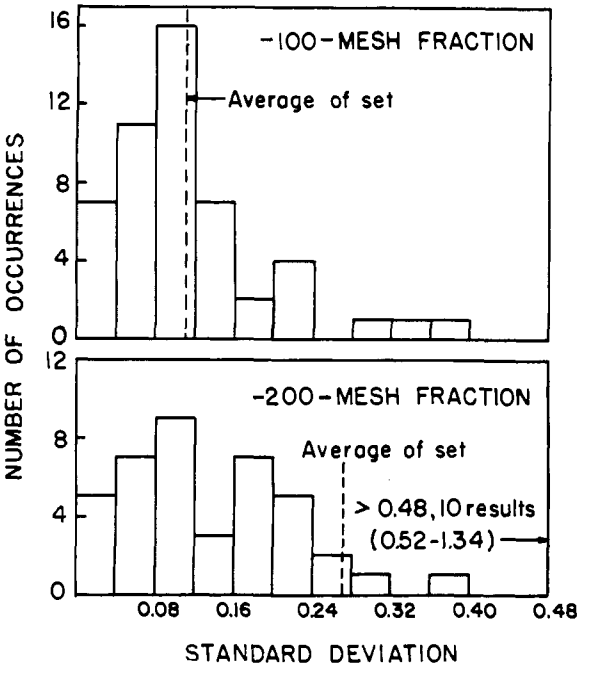

Figure 1. Frequency distribution of standard deviations of 50 laboratory means of triplicate determinations of −100- and −200-mesh fractions

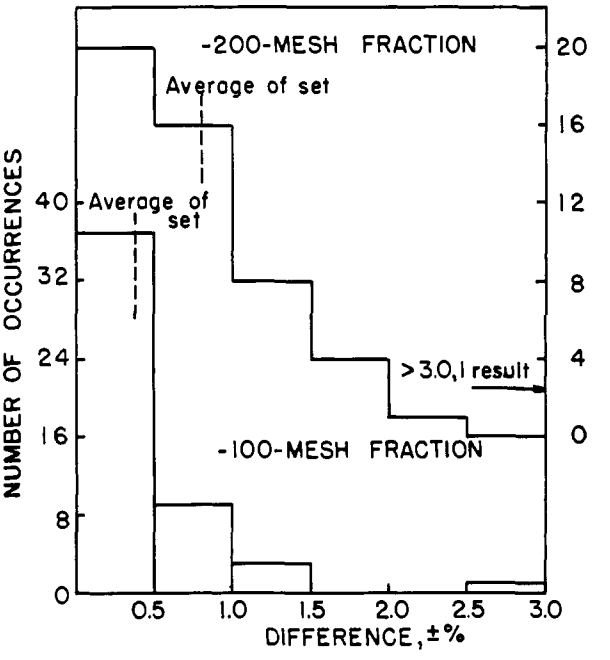

Figure 2. Frequency distribution of 50 differences between single- and seven-laboratory means of triplicate determinations of −100- and −200-mesh fractions

Precision of Determinations. Consistency of laboratory performance as regards agreement of triplicate determinations is illustrated in Table IV by standard deviations of laboratory means obtained in successive years on a lot of Florida land pebble (No. 3302, Table I). The precision is noticeably lower for the −200-mesh fraction and varies among laboratories and between years.

The observed frequency of standard deviations of laboratory means for all lots of rock used in the collaborative study that lie within class intervals of 0.04 is depicted in Figure 1. In the case of the −200-mesh fraction 10 of the 50 results lie off the figure—a circumstance responsible for the average of the set falling well above the range of highest frequency.

Class intervals, D, of 0.5% confidence limits in terms of fraction of sample passing a given screen were established. The ranges of standard deviations that would result in determinations of the specified precision were calculated. The 50 standard deviations for both the −100-mesh and −200-mesh fractions were then tabulated according to these calculated ranges (Table V). All 50 triplicate determinations of the −100-mesh fraction had standard deviations that would give confidence limits of less than ±1%. Similarly, 44 of the determinations of the −200-mesh fraction lie in the first two classes. The six larger standard deviations were distributed among laboratories and rocks as follows: Laboratory 1, one each to pebble and Wyoming; Laboratory 4, two to pebble; Laboratory 6, one each to Tennessee and Wyoming.

Table V. Frequency Distributions of Standard Deviations by Classes of Precision

Range of Precision, D, ± %	Calcd. Range of Std. Dev.,[a]	Obsd. Frequency of Std. Dev. −100-mesh fraction	Obsd. Frequency of Std. Dev. −200-mesh fraction
0.5	0.295	47	38
0.5 to 1.0	0.295 to 0.590	3	6
1.0 to 1.5	0.590 to 0.886	0	4
1.5 to 2.0	0.886 to 1.181	0	0
2.0	1.181	0	2

[a] Range that would result in range of precision (95% confidence) specified in 1st column; derived from relationship, $D = t(2s^2/n)^{1/2}$, in form $s = 0.5904\ D$, in which $n = 3$ and $t = 4.303$ from Student's t distribution for 2 degrees of freedom.

Table VI. Accuracy of Screen Analyses in Successive Years on One Lot of Florida Land Pebble

Lab. No.	Difference between Single- and 7-Laboratory Means: −100-Mesh Fraction 1955	−100-Mesh Fraction 1956	−200-Mesh Fraction 1955	−200-Mesh Fraction 1956
1	0.00	0.11	0.12	0.92
2	0.03	0.05	−0.48	−0.15
3	−0.07	0.21	0.59	−0.15
4	−0.14	0.15	−0.84	−1.81
5	0.36	−0.42	1.02	−0.68
6	0.00	0.08	0.29	1.19
7	−0.20	−0.19	−0.71	0.69
Mean of 7 laboratories	98.47	98.42	87.21	87.18
Std. error of mean	0.18	0.22	0.70	1.04

Accuracy of Determinations. Consistency of laboratory performance as regards agreement with the all-collaborator mean is illustrated in Table VI by differences between single-laboratory and seven-laboratory means obtained in successive years in Florida land rock No. 3302 (Table I). The differences for the −200-mesh fraction are not only the larger but also show the wider variation.

The observed frequency of differences for all lots of rock used in the collaborative study that lie within class intervals of 0.5% is depicted in Figure 2. In the case of the −100-mesh fraction 46 of the 50 differences were within ±1%. The corresponding figure for the −200-

mesh fraction is 36. These frequencies, though somewhat lower, follow the order exhibited by the standard deviations of laboratory means. The 14 differences that were greater than ±1% were divided nearly equally among laboratories 2 to 9—four pebble, six Wyoming, two Tennessee, and two Florida soft phosphate. With the exception of one very high value, the differences range up to 2.5%, which is something less than the desirable accuracy of triplicate determinations. Perhaps the results of phosphate rock analyses by different laboratories could be brought into closer agreement by the use of methods developed in bone char studies for sieve calibration (*3*) and determination of the sieving end point (*2*).

Expected Reliability of Screen Analyses. Expected confidence limits for screen analysis are given in Table VII. Values are shown for the limits of means of one to three determinations per laboratory in one to seven laboratories. As would be anticipated, the expected confidence limits are much less for the −100-mesh fraction than the corresponding limits for the −200-mesh fraction.

Table VII. Expected Confidence Limits (95%) for Screen Analysis of Phosphate Rock

No. of Detns. in Laboratory	Confidence Limits, ±% of Sample			
	1 lab.	2 lab.	3 lab.	7 lab.
	−100-Mesh Fraction			
1	1.30	0.918	0.751	0.491
2	1.27	0.900	0.735	0.481
3	1.26	0.894	0.731	0.477
	−200-Mesh Fraction			
1	2.60	1.84	1.50	0.984
2	2.51	1.78	1.45	0.948
3	2.48	1.75	1.43	0.936

However, the −200-mesh fraction, which the trade considers the critical index of fineness of grind, possesses the principal interest at the moment. According to the table, a single determination of this fraction in one laboratory can be expected to lie within ±2.60% of the true mean 95% of the time. With triplicate determinations in one laboratory this figure can be lowered to ±2.48%—a trifling reduction. On the other hand, the mean of single determinations in three laboratories would be expected to lie within ±1.50% of the true mean. If it be desired to attain a confidence limit of less than ±1% for the −200-mesh fraction, single determinations in at least seven laboratories will be required. These findings illustrate the large laboratory-to-laboratory variation in comparison with the rather small variation among determinations within a laboratory.

Physical Composition

Comparison of Results by Two Procedures. The interpolated results present an opportunity for an interesting comparison of the performances of the AOAC and Soil Survey procedures. The results are given in Table VIII, the arrangement being in increasing order of the −200-mesh fraction found by the official AOAC procedure. The differences between corresponding results occur with both signs, and the absolute values are generally smaller for the −100-mesh fraction, where they range from 0.1 to 4.2, mostly all below the level of statistical significance. The differences for the −200-mesh fraction range from 0.3 to 5.3 with the exception of the one significant difference of 7.8. Triplicate determinations of the two size fractions made days apart by the two methods on Florida land pebble rocks 3372 and 3302 (Table I) mark the Soil Survey results with the higher precision. The standard deviations of the four procedure means ranged from 0.05 to 0.12 by the Soil Survey method in comparison with 0.05 to 0.40 for the official AOAC method. However, the differences between paired procedure means followed the pattern for single determinations (Table VIII), being 0.03 and 0.30 for the coarse fractions and 2.47 and −1.70 for the fine fractions.

Average Particle Size of Screen Separates. Answers to the last two of the three major questions raised in the introductory paragraph are provided by the average particle sizes of the appropriate screen fractions of rocks ground to different degrees of fineness.

Fraction Finer than 200 Mesh. The dependence of the mean particle size of the −200-mesh fraction on its weight per cent of the lot is depicted in Figure 3,*A*. The mean diameters range from about 36 to 28 microns and fall along a downward sloping line, drawn by inspection, which indicates a reduction in mean particle size as the rock is ground to show larger and larger percentages finer than 200 mesh. For example, milling a rock from 40 to 80% finer than this sieve would be expected to lower the average particle size of this separate about 7 microns. On the other hand, the mean size of the −50-micron fraction removed from lots having the fineness shown was not appreciably affected by fine grinding (Figure 3,*B*). Hence, it appears that in current mill practice the grinding process operates only on particles coarser than 50 microns.

Fraction Coarser Than 100 Mesh. Necessary definition of the size range +100-mesh fraction requires a stated upper limit of the range. It happens that a few of the test rocks were 100% finer than 500 microns (Table II), whereas the others contained 0.1 to 5.4% of coarser material. A reasonable and practical upper limit would thus appear to be 500 microns, or 32 mesh. Accordingly, the mean weight diameters of the 32- to 100-mesh fractions are plotted against the percentage of the lot retained on the 100-mesh sieve in Figure 4. The scatter of the experimental points is markedly greater here than in the case of the −200-mesh fraction. Nevertheless, the results show an obvious trend to larger average particle diameters with increase in the amount of material retained on the 100-mesh sieve. The average particle size ranges from about 180 to nearly 260 microns.

An opinion voiced in the past, and presumably based on the extreme hardness of the quartz constituent, regards the +100-mesh fraction as being silica for the most part. This view is not valid for ground rock marketed at

Table VIII. Single Determinations of −100- and −200-Mesh Fractions

Lot No.	Percentage Finer Than 100 Mesh: Soil Survey proc.	Official screen proc.	Diff.[a]	Percentage Finer Than 200 Mesh: Soil Survey proc.	Official screen proc.	Diff.[a]
3375	76.5	74.5	2.0	53.1	51.9	1.7
3378	75.5	74.7	0.8	54.5	52.7	1.8
3301	77.2	75.2	2.0	54.5	52.8	1.7
3372	79.8	78.6	1.2	57.7	57.0	0.7
3380	84.2	83.3	0.9	59.2	58.0	1.2
3359-b	84.1	79.9	4.2	60.7	59.8	0.9
3376	83.5	80.5	3.0	59.0	61.4	−2.4
3374	87.0	84.7	2.3	66.6	66.3	0.3
3381	91.1	91.3	−0.2	67.4	68.6	−1.2
3157	93.0	90.2	2.8	69.1	68.6	0.5
3377	92.6	89.8	2.8	70.6	68.7	1.9
3379	91.5	89.7	1.8	69.5	69.5	0.0
3156	96.5	97.0	−0.5	81.4	73.6	7.8
3373	96.0	92.6	3.4	75.9	81.2	−5.3
3155	95.5	95.3	0.2	80.7	83.5	−2.8
3302	97.9	98.2	−0.3	83.9	86.8	−2.9

[a] Minus sign indicates result by Soil Survey procedure was smaller. Use of interaction between methods and samples as experimental variance yields 3.00 and 6.17 as least significant differences (with 95% confidence) between results by two procedures for two size fractions, respectively.

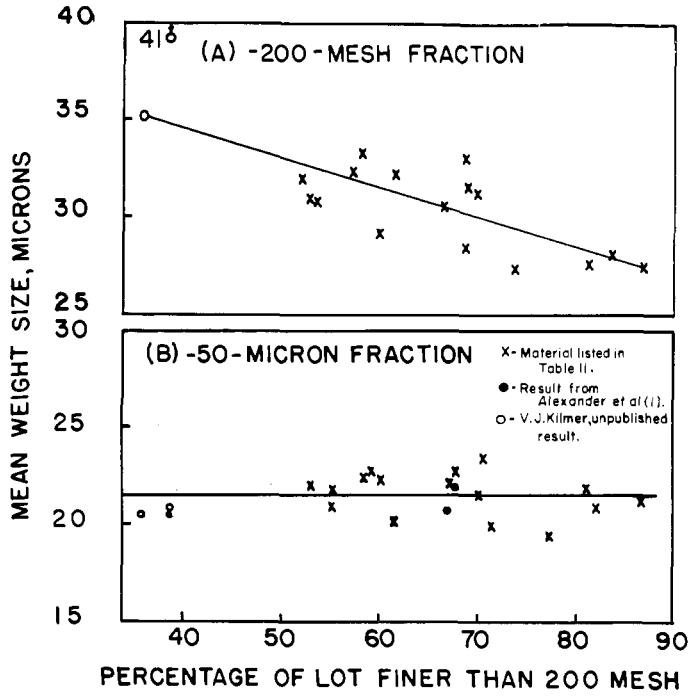

Figure 3. Average particle diameter of −200-mesh and −50-micron fractions of Florida land pebble

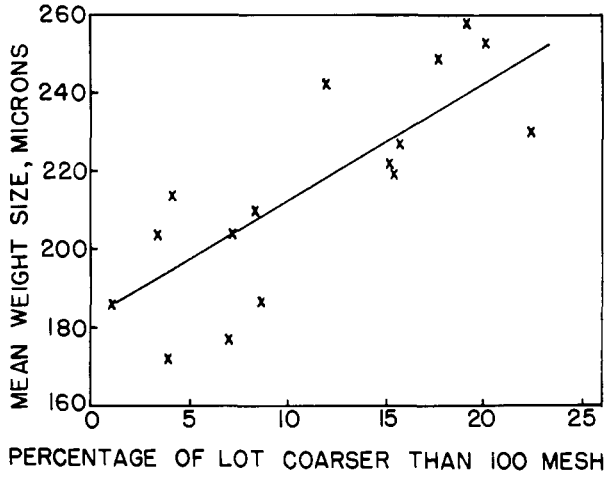

Figure 4. Average particle diameter of 32- to 100-mesh fractions of Florida land pebble

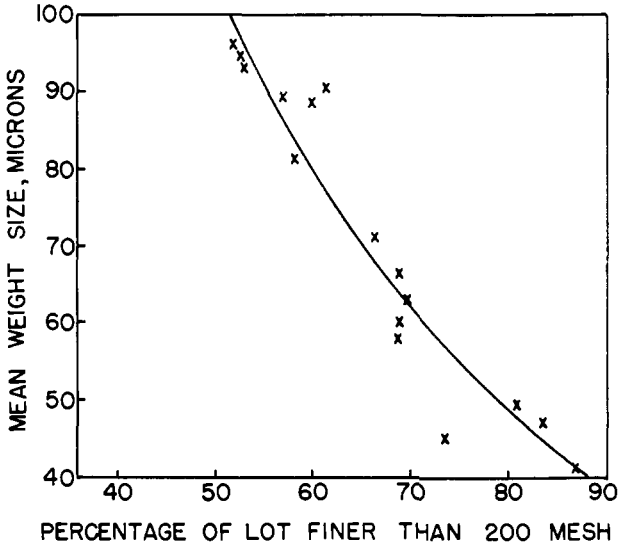

Figure 5. Average particle diameter of −32-mesh lots of Florida land pebble

the present time. The +100-mesh fractions of the test rocks showed P_2O_5 contents that ranged from 28.4% for a rock containing 68% BPL to 34.5% for one containing 77.5% BPL.

Average Particle Size of Lot. The mean weight diameters of the test rocks, excluding from the calculation any +32-mesh particles, are plotted in Figure 5 with respect to the percentage of the lot finer than 200 mesh. According to a least squares treatment, the results follow a curvilinear relationship significantly better than a linear one at 90% confidence. The mean size decreases with finer grinding and ranges from about 100 microns for 50% finer than 200 mesh down to about 48 microns for 80% finer than 200 mesh. Thus, with milling techniques currently used in the phosphate rock industry in Florida, grinding from the coarse extreme of the usual fineness range to the other extreme approximately halves the particle size.

Applications

The data on the expected reliability of screen analyses and the average particle size of screen separates can be applied by the producers of ground phosphate rock toward improving their process control. This information can also be used by the superphosphate producers for improved control of their acidulation processes.

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Determination of Heptachlor Epoxide in Fat and Milk

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Earlier work by Davidow and others has shown that the insecticide heptachlor is altered, when ingested by dairy animals, to its epoxide (1,4,5,6,7,8,8-heptachloro-2,3-epoxy-2,3,-3a,4,7,7a-hexahydro-4,7-methanoindene). As heptachlor epoxide is found primarily in the fat, methods were developed for the determination of microgram quantities in butterfat and beef fat. The Polen-Silverman reagent which was originally developed for the determination of heptachlor is also a sensitive color-forming reagent for heptachlor epoxide. Methods are presented for the separation of heptachlor epoxide from butterfat and beef fat by chemical and chromatographic techniques and for the subsequent spectrophotometric analysis of the epoxide.

THE INSECTICIDE, heptachlor, which is used extensively for the control of certain forage insects, is metabolized by animals to an expoxide (*1, 7*). This metabolite has been identified as 1,4,5,6,7,8,8 - heptachloro - 2,3, - epoxy-2,3,3*a*,4,7,7*a* - hexahydro - 4,7 - methanoindene, more commonly known as heptachlor epoxide (Figure 1). Dairy animals fed heptachlor metabolize it to the epoxide which is stored in the fatty tissues of these animals and is excreted in their milk (*2, 3*). Therefore, to evaluate properly the safe level at which heptachlor can be applied to forage crops, sensitive and accurate methods were developed for the determination of microgram quantities of heptachlor and heptachlor epoxide in animal fat and milk. The basis for these methods is the procedure described for the analysis of heptachlor epoxide on alfalfa (*9*).

Reagents

PENTANE, colorimetric grade (Phillips Petroleum Co.). Check the residue from 2 liters of pentane evaporated from a 50° C. bath not to develop more absorbance at 410 mμ, when treated with Polen-Silverman reagent, than 2.5 γ of heptachlor epoxide.

DIETHYL ETHER, colorimetric grade (Eastman Kodak Co.). Same specification as for pentane.

FLOREX XXS, 60/90-mesh (Floridin Co.) activated and standardized for chromatography. Determine optimum activation experimentally for each commercial batch. Agitate the Florex for 15 minutes with excess pentane; allow it to settle. Decant the excess pentane and remove the residual solvent by application of suction from a water aspirator. Perform a trial activation on pre-treated Florex by heating it in an oven for 24 hours at 180° C. Check the activation by performing a complete analysis for heptachlor epoxide, measuring the recovery and interference upon fat fortified with known amounts of reference heptachlor epoxide. High interference means that the temperature of activation is too low. Low recovery means that the activation temperature is too high. Repeat the pretreatment and activation of portions of a particular batch of Florex until maximum recovery and minimum interference occur upon analysis of a synthetic sample of heptachlor epoxide in fat.

HEPTACHLOR EPOXIDE reference standard, melting point 160–161.5° C., minimum assay 99% (Velsicol Chemical Corp.)

POLEN-SILVERMAN REAGENT. Dissolve 33 grams of 85% potassium hydroxide (reagent grade) in 28 grams of water. Add an equal volume of butyl Cellulose (distilled from potassium hydroxide pellets) and 30.5 grams of ethanolamine (freshly distilled). Dilute to 1 liter with butyl Cellosolve. The reagent is stable if stored in tightly stoppered bottles.

MODIFIED POLEN-SILVERMAN REAGENT. Dilution (1 to 1) of Polen-Silverman reagent with benzene (reagent grade) renders it stable for about 1 month. Fresh reagent should be prepared frequently and calibration curves should be adjusted if required.

BENZENE-ISOPROPYL ALCOHOL solution, 4 to 1 by volume.

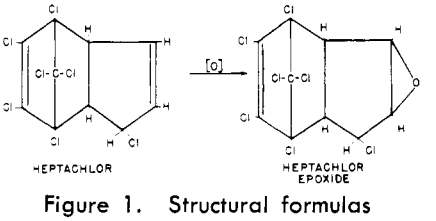

Figure 1. Structural formulas

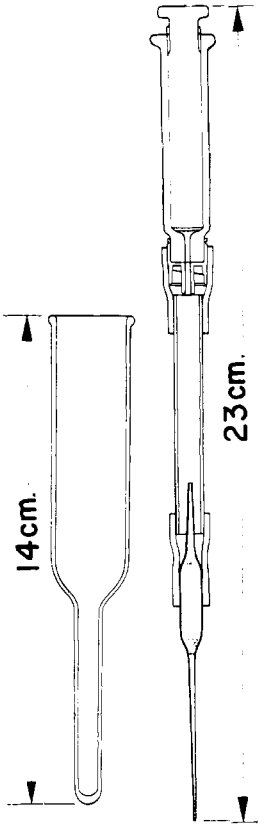

Figure 2. Special micro-equipment

Left. Reaction tube
Right. Filter-pipet assembly

Special Apparatus

A more complete description of the apparatus listed is given by Ordas, Smith, and Meyer (*5*) in their method for the determination of heptachlor and chlordan residues.

Cream Separator. DeLaval No. 9.

Microreaction Tubes, microtransfer pipet, and syringe (Figure 2). Draw out a piece of 6-mm. glass tubing to a capillary with a 1-mm. bore. The pipet has a length of 10 cm. with a volume of approximately 1 ml. Attach the pipet to a 1-ml. hypodermic syringe with a piece of Tygon tubing.

Microcuvettes, quartz microcells, 1.5 × 10 × 25 mm., 0.375 ml. (Pyrocell Manufacturing Co., New York).

Treatment of Samples

Milk (Separation of Butterfat). Remove most of the aqueous phase from the milk sample with the cream separator. Mix the cream with anhydrous sodium sulfate (reagent grade), and grind thoroughly in the food grinder. Enough anhydrous sodium sulfate should be used so that the mixture will be friable. Store the cream-sulfate mixture in polyethylene bags for at least 24 hours at 18° F. or lower.

Extract the mass with pentane (colorimetric grade) for 4 hours in a Soxhlet extractor fitted with a glass wool plug. Strip off the pentane from the extract on a steam bath. Avoid excessive heating to prevent loss of heptachlor epoxide. Use a stream of dry air or nitrogen to remove the last traces of solvent.

Animal Fat. Slice samples of animal fat into small pieces and mix with anhydrous sodium sulfate. Grind the mixture with a food grinder. Transfer the mass to a polyethylene bag, and store at 18° F. or lower for at least 24 hours. Use the same procedure for the extraction of animal fat samples as described for butterfat.

Removal of Interfering Substances

Chemical Treatment. If samples are to be analyzed for heptachlor epoxide only, an alkaline saponification removes a major portion of the fat. When analyses for both heptachlor and heptachlor epoxide are required, the alkaline treatment will destroy much of the heptachlor, if present. An acid treatment which gives high recovery of heptachlor is used in this case.

Saponification (Heptachlor Epoxide Only). Melt the fat sample on a steam bath and weigh up to 20 grams (10 grams are preferred) of the melted fat into a 250-ml. Erlenmeyer flask. Add to the flask 15 ml. of 95% ethyl alcohol and 10 ml. of 50% aqueous potassium hydroxide, and heat at reflux for 5 minutes. Wash down the condenser with 15 ml. of distilled water. Cool the solution to room temperature and transfer to a 500-ml. separatory funnel. Wash the flask with three 5-ml. portions of pentane, and transfer the washings to the separatory funnel.

Add 200 ml. of pentane to the funnel. Shake the funnel thoroughly for 1 minute. Allow the layers to separate, and remove the alcohol (lower) layer. Save the alcohol layer, as it will be re-extracted. Wash the pentane extract with 100 ml. of distilled water acidified with 1 to 2 ml. of 1*N* hydrochloric acid. The acid is used to prevent the formation of emulsions. Discard the water layer and rewash the pentane with another 100 ml. of water. Discard the second water wash and transfer the pentane extract to a 500-ml. Erlenmeyer flask.

Return the alcohol layer to the separatory funnel and extract with 100 ml. of pentane. Discard the alcohol and wash the pentane layer with water as previously described.

Combine the pentane extracts in a 500-ml. Erlenmeyer flask and dry with anhydrous sodium sulfate. Filter the dried pentane extract through coarse filter paper or a glass wool plug into a 500-ml. Erlenmeyer flask fitted with a ground-glass joint. Attach a distillation column to the flask, and concentrate the extract on a 50° C. water bath to about 10 ml.

Acid Treatment (Both Heptachlor and Heptachlor Epoxide). If fat samples are to be analyzed for both heptachlor and heptachlor epoxide, the alkaline treatment described above may not be used as it will result in a substantial loss of heptachlor. Substitute the following treatment with strong sulfuric acid:

Melt the fat on a steam bath and weigh up to 20 grams (10 grams are preferred) into a 500-ml. separatory funnel containing 350 ml. of pentane. Add to the solution 35 ml. of a 1 to 1 mixture of 15% fuming sulfuric acid and concentrated sulfuric acid (reagent grade). Shake the funnel for about 1 minute, and allow 10 minutes for the layers to separate. Discard the acid (lower) layer.

Wash the pentane layer twice with 100-ml. portions of distilled water and discard the water layers. Dry the pentane solution with anhydrous sodium sulfate. Filter the pentane extract into an Erlenmeyer flask, wash the sulfate, and filter with additional small portions of pentane to complete the transfer. Concentrate the combined extracts to 10 ml. by heating on a 50° C. water bath, and distilling the excess pentane through a column.

Chromatography. Prepare a chromatographic column by inserting a small plug of glass wool into a 50-ml. Mohr buret. Pour 10 grams of Florex (activated and standardized) into the column with gentle tamping to pack the adsorbent properly. Wet the column with pentane. Carefully wash the concentrated extract from the alkaline treatment or acid treatment into the chromatographic column with a few milliliters of pentane. Elute the column with 200 ml. of pentane. Discard the first 50 ml. of elutriate. Collect the next 150 ml. which contain heptachlor, if any. Heptachlor may be determined in this fraction by the method of Ordas, Smith, and Meyer (*5*).

Now elute the column with 200 ml. of 2% ethyl ether (colorimetric specifications) in pentane. Be careful not to allow the column to dry between additions of eluent. Collect the ether-pentane elutriate in a 500-ml. Erlenmeyer flask fitted with a ground-glass joint. Attach a distilling column to the flask and concentrate the contents to about 15 ml. on a 50° C. water bath.

Transfer the concentrate to a microreaction tube (Figure 2) and evaporate to dryness on a 40° C. water bath.

Spectrophotometric Determination of Heptachlor Epoxide

Add 0.5 ml. of the modified Polen-Silverman reagent to the contents of the microreaction tube and heat in a 100° C. bath for exactly 15 minutes. Remove the reaction tube from the bath, and cool immediately to room temperature by immersing the tube in a beaker of cold water. Dilute the colored reaction product formed to 0.5 ml. with the benzene–isopropyl alcohol solution, and transfer the solution to a microcuvette by use of the special microtransfer pipet (Figure 2). Determine the absorbance of the solution at 410 mμ (Beckman Model DK-1 spectrophotometer or equivalent), using the benzene–isopropyl alcohol solution as reference. The heptachlor epoxide concentration is determined from a calibration curve derived from measurements made upon fat containing known amounts of heptachlor epoxide.

Detection of Interference

The shape of the heptachlor epoxide absorption curves is used to establish the presence of interference. The following criterion has been established to determine if a valid measurement for heptachlor epoxide has been obtained.

For absorbance readings greater than those obtained for uncontaminated fat (about 0.3 absorbance), the absorption curve must have a peak at 410 mμ to be a true measure of heptachlor epoxide. If a recording spectrophotometer is not available, the presence of a peak is determined by taking absorbance readings at 390, 410, and 430 mμ. The absorbance reading at 410 mμ must be greater than the average of the readings at 390 and 430 mμ. If no peak is found,

either no heptachlor epoxide is present or else interference is predominant and no valid quantitative estimation of heptachlor epoxide can be made on the solution; the sample must be reanalyzed.

Calibration

Fat with Zero Heptachlor Epoxide (Blanks). Analyze by the complete procedure (treatment of samples, removal of interfering substances, and spectrophotometric determination) at least five samples of uncontaminated fat from different sources. Estimate the mean absorbance (410 mμ) and the 95% confidence absorbance range for the blank samples (zero heptachlor epoxide) using standard statistical methods for analysis of variance (*8*, *10*).

Fat Containing Heptachlor Epoxide. Accurately prepare a standard solution of approximately 5 γ of heptachlor epoxide per milliliter of colorimetric grade pentane. Pipet aliquots of from 1 to 6 ml. of the standard solution into separate Erlenmeyer flasks. Add to each a constant weight (10 grams are suggested) of control sample fat and analyze by the complete procedure from removal of interfering substances up to and including spectrophotometric determination.

Working Curve. Construct a smooth curve through plotted points of micrograms of heptachlor epoxide added *vs.* absorbance of the reaction product at 410 mμ. The curve is not necessarily a straight line over its entire length nor does it pass through the origin. It may be used to determine heptachlor epoxide at absorbance values higher than the upper 95% confidence limit determined for the blanks.

Extended Range. For determination of heptachlor epoxide in higher concentrations, the range may be extended. When absorbance of the color complex exceeds about 1.0, one may use smaller samples or greater dilution in the final solution upon which absorbance is read. Whichever course is chosen, the technique used for calibration and for measurement of unknowns must be the same. The conditions suggested give an absorbance value of roughly 1.0 at a heptachlor epoxide concentration of 3 to 3.5 p.p.m. in fat.

Minimum Range. Where an inadequate number of fat blanks are available to establish good statistical information on measurements at or near zero heptachlor epoxide content, a base-line technique and calibration is suggested. The principle described under detection of interference may be utilized.

Discussion

Reagent Sensitivity. The Polen-Silverman reagent, although developed for the colorimetric determination of heptachlor (*6*) (maximum absorption peak at 560 mμ), was also found to be a sensitive reagent for heptachlor epoxide. When reacted with Polen-Silverman reagent, heptachlor epoxide produces a bright yellow product with maximum absorption at 410 mμ. The reaction product of heptachlor epoxide and the Davidow reagent has the same qualitative absorption curve, but, as can be seen from Figure 3, the sensitivity of the Polen-Silverman reagent is about 2.5 times that of the Davidow reagent. Because of this greater sensitivity, and also because heptachlor residue procedures are standardized with Polen-Silverman reagent, this reagent was used for the determination of heptachlor epoxide.

Background Absorption. Interferences. The present method is complicated by rather high blanks—i.e., development of absorption at the wave length of the analysis even in the absence of heptachlor epoxide. The apparent measurement of heptachlor epoxide in blanks varies materially between the fat of different animals and also between specimens taken from the same animal at different times. This effect is too large to disregard or to treat by the conventional method of averaging the values determined on a small number of reference uncontaminated controls and then subtracting the mean value from all subsequent determinations. To illustrate the magnitude of variation in the blank absorption, statistical data from typical studies are cited in Table I. From these data, the least significant differences (*8*) at the 95% confidence level may be estimated. They are ca. 0.33 absorbance unit for butterfat and ca. 0.16 unit for beef fat. These statistical parameters must be applied to measurements intended to discriminate between the presence or absence of heptachlor epoxide in animal fat or in milk.

The response of the reagent to materials other than heptachlor epoxide may give rise to systematic errors for which statistical treatment of data will not correct. This situation may arise when changing batches of solvent or chromatographic sorbant, or as a result of changes in procedures. Two well-known brands of diethyl ether, each marked Reagent Grade, gave absorbance values (at 410 mμ) of 0.1 and 0.5 when treated directly with the reagent under identical conditions. Experimental verification of solvent and reagent conformance to specifications must be regarded as essential to the valid application of the present method.

Recovery. The interaction of a relatively large background absorption and varying recovery efficiencies through the range of measurements is illustrated in Figure 4. The comparison is made between absorbance values derived from reagent reacted directly with heptachlor epoxide (in pentane) and the values obtained upon complete analyses of fat samples to which known amounts of heptachlor epoxide have been added. The experimental curve for fats is neither linear over its range nor does it pass through zero absorbance for pure fat. The systematic discrepancy between the theoretical and experimental curves is eliminated by the calibration described in the present method. The data taken from this type of calibration may, therefore, be considered to be corrected to 100% recovery and corrected for background absorption (reagent blank).

Influence of Heptachlor. If hepta-

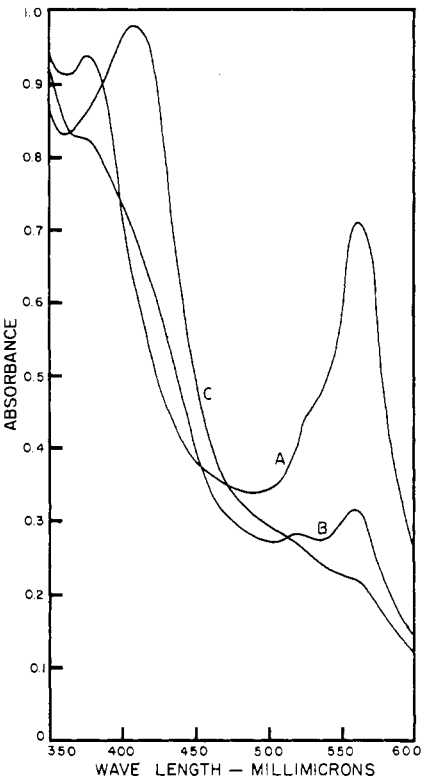

Figure 3. Absorption curves of color complexes (each with 20 γ of reactant)

A. 20 γ of heptachlor and 5 γ of heptachlor epoxide
B. 10 γ of heptachlor and 10 γ of heptachlor epoxide
C. 5 γ of heptachlor and 20 γ of heptachlor epoxide

Table I. Analysis of Variance

Background absorbance of fat plus reagent (zero heptachlor epoxide)

Basis: 10 grams fat—analysis by total method

	Butterfat	Beef Fat
	No. of Samples	
Absorbance	12	32
Mean value	0.239	0.171
Std. dev., single determination	0.107	0.074
Upper limit for zero heptachlor epoxide (95% confidence level)	0.475	0.321
Least significant difference (95% level)	0.333	0.155

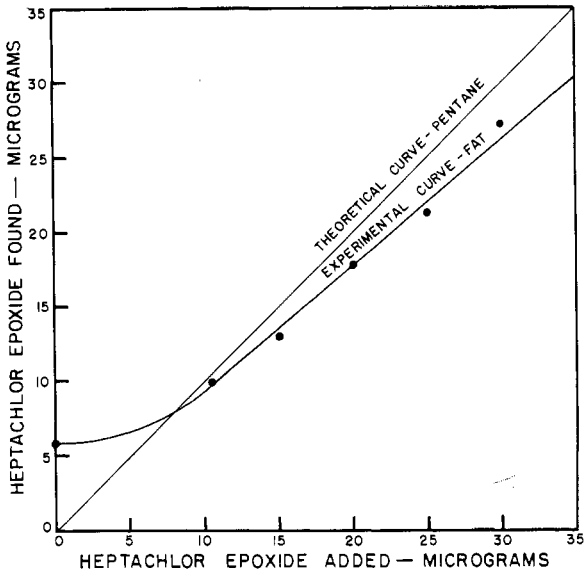

Figure 4. Recovery curve, uncorrected data

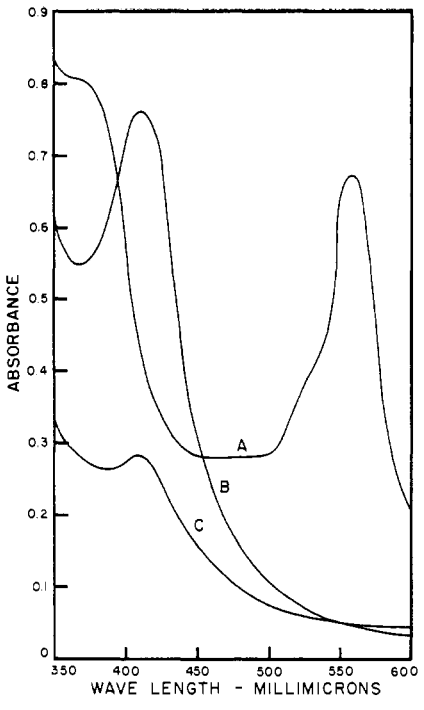

Figure 5. Absorption curves of heptachlor and heptachlor epoxide mixtures

A. Heptachlor (Polen-Silverman reagent)
B. Heptachlor epoxide (Polen-Silverman reagent)
C. Heptachlor epoxide (Davidow reagent)

chlor is present in samples to be analyzed for heptachlor epoxide, an enhancing effect will be exerted. This is illustrated in Figure 5. It is possible to analyze these mixtures in a single color development by applying calculations to measurements at the two characteristic peak wave lengths. A procedure involving chromatographic separation and separate color development is more reproducible and, hence, is preferred.

If a large amount of heptachlor is present in the sample, part of it may appear in the epoxide fraction. Figure 5 shows that the influence of heptachlor upon the heptachlor epoxide determination is significant, and erroneous results will be obtained if the proper corrections are not applied. A standard two-wave-length method for treating a two-component system must be used to make this correction (*4*).

As a general rule, heptachlor is absent under normal circumstances. When this fact has been indicated for a particular set of determinations, it will usually be more convenient to use saponification in preference to acid treatment in the cleanup. Slightly lower absorbance values result from acid treatment. By using acid treatment in the calibration, however, the appropriate correction to 100% recovery will yield the final analytical results. All things considered, the saponification step appears more convenient where a choice is available.

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INSECTICIDE RESIDUES

Procedure for Cleanup of Plant Extracts Prior to Analyses for DDT and Related Pesticides

CONSTANCE ANGLIN and W. P. McKINLEY

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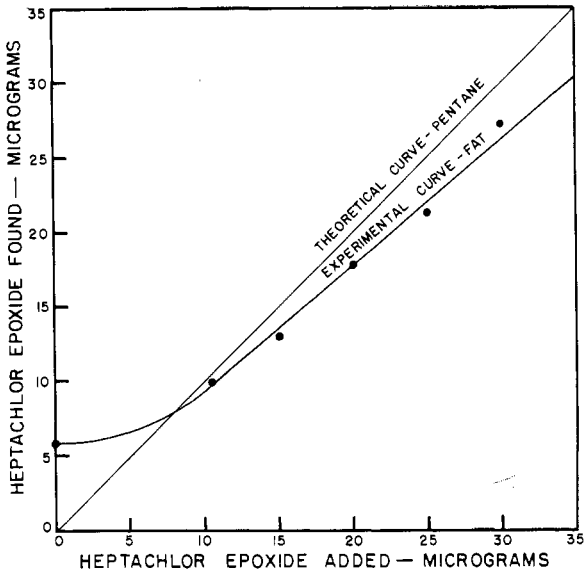
Pigments and waxes are extracted with pesticide residues from plant material, and interfere in quantitative and qualitative methods of analysis. Therefore, a cleanup procedure is needed, which will efficiently remove pigments and waxes from the organic solvent without removing or destroying the pesticides. Recently (*8*) stress has been placed on more efficient techniques for extracting pesticide residues from fruits and vegetables. These more vigorous extraction methods require more efficient cleanup procedures, because more pigments and waxes, as well as pesticides, are present in the extracts. Many cleanup procedures described in the literature are applicable in respect to one type of crop or one specific pesticide. A cleanup procedure was sought which would remove essentially all



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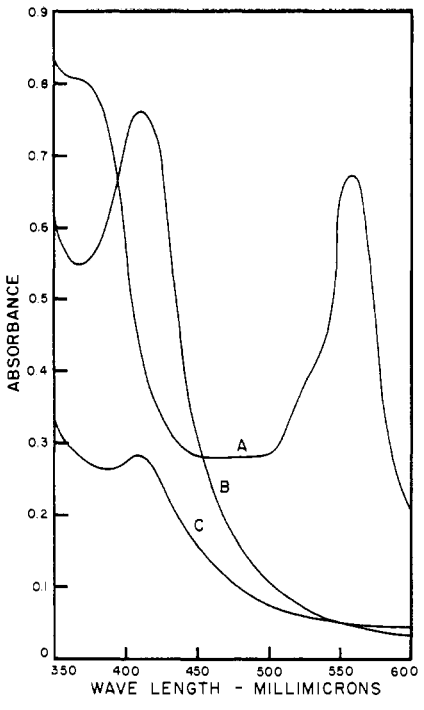

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A method is presented for the removal of pigments and waxes from plant extracts prior to determining residues of the DDT group of pesticides. The waxes are precipitated from an acetone solution of the extract at −70° C. and the pigments are removed by passing a benzene solution of the extract through a Florisil column. The method is applicable to a variety of plant materials, including leafy vegetables, brassic crops, and citrus and waxy fruits. Data show the recovery of DDT and other related chlorinated hydrocarbon pesticides following this cleanup procedure. The procedure is compared with others mentioned in the literature.

pigments and waxes from a heavily contaminated extract from any type of plant material and yet provide a good recovery of a wide range of pesticides.

The Jones-Riddick (*7*) and the Burchfield-Storrs (*1*) methods efficiently remove waxes from extracts but, with the more efficient extraction techniques, they do not remove the pigments completely.

Hoskins *et al.* (*6*) have described a polyethylene-alumina column for the removal of waxes. Unfortunately, however, the brand of polyethylene suggested was not available and a substitute had to be used. Under these conditions the method was unsatisfactory, as the polyethylene was slightly soluble in the eluting solvent.

McKinley and Mahon (*9*) have used a Florisil column with petroleum ether–ethyl ether as the eluting solvent for the isolation of pesticides from extracts of fat. A Florisil column with benzene as eluting solvent was found efficient for the removal of pigments but not waxes from plant extracts.

Fairing and Warrington (*4*) used acetone at − 15° C. to separate methoxychlor from the unsaponifiable portion of fats and waxes.

The present paper describes a cleanup procedure in which the waxes are removed by precipitation from an acetone solution of the extractives at − 70° C. and the pigments are removed by Florisil column chromatography. This procedure is compared for efficiency of removal of extraneous material and recovery of chlorinated hydrocarbon pesticides with the Jones-Riddick and Burchfield-Storrs procedures when combined with Florisil chromatography.

Method

Apparatus. Cooling Bath. A 1-quart Mason jar filled with dry ice and acetone serves as a cooling bath. It may be wrapped in cork sheeting for insulation. On top of the jar is placed a sheet of cork 11 × 11 × 0.4 cm. This cork sheet has two holes, each 2.5 cm. in diameter, through which are inserted 20 × 2.4 cm. test tubes. Through another hole, 0.7 cm. in diameter, is inserted a thermometer calibrated to −70° C. and kept upright by two elastic bands wrapped around the stem.

Büchner funnels with medium porosity fritted disks 4.0 cm. in diameter.

Chromatographic columns, 2.5 cm. in inside diameter, with coarse fritted-glass disks and stopcocks to regulate the rate of flow.

Reagents. Florisil, 60/100 mesh, a synthetic magnesium silicate prepared by the Floridin Co., Tallahassee, Fla. The Florisil is activated by heating at 300° C. for 2 hours; then it is stored in a desiccator.

Anhydrous sodium sulfate.

Procedure. Evaporate the extract of the plant material to approximately 10 ml. in a water bath at 60° C. under a stream of oil-free air. Transfer the entire residue to a 20 × 2.4 cm. test tube, using benzene to rinse the beaker. Continue the evaporation until 3 ml. of solvent remains in the test tube. Add 20 ml. of acetone and mix thoroughly. Place the test tube in the cooling bath at −70° C. and fill the other test tube in the bath with acetone to be used for rinsing. Immerse the Büchner funnel in another bath of dry ice and acetone. After the solutions and funnel have cooled for 15 minutes, remove the funnel, place it under suction, and rinse with 10 ml. of cold acetone. Pour the sample into the funnel and filter with suction. Proceed as quickly as possible, once filter and sample are removed from the dry ice–acetone bath. Rinse the test tube with two 3-ml. portions of cold acetone and replace the sample test tube in the bath between washings. Filter the rinsings through the residue in the funnel. Wash the residue with another 3 ml. of cold acetone. Pour the filtrate into a 100-ml. beaker; rinse the filter flask with two 5-ml. portions of acetone and add rinsings to beaker. Add 5 grams of anhydrous sodium sulfate and stir. Place the beaker under a stream of air and evaporate with occasional stirring until a dry powder remains.

Make a slurry of activated Florisil and benzene and add to the chromatographic columns until the Florisil column is 8 inches high. Adjust the stopcock so that the column runs at approximately 80 to 100 drops per minute. Add 5 grams of anhydrous sodium sulfate to the top of the column. When the benzene has just entered the sodium sulfate layer, add the dry, powdered sample. Place a 200-ml. volumetric flask under the column to collect the eluate. Rinse the 100-ml. beaker four times with 5-ml. portions of benzene. Add the rinsings to the column while allowing benzene to enter the sodium sulfate layer between each addition. Rinse the sides of the column with 5 ml. of benzene and then add 150 ml. of benzene. Allow the column to run to dryness and make eluate up to 200 ml.

This eluate represents the extract, free from pigments and waxes. Aliquots may be used for quantitative and qualitative analyses.

Results and Discussion

To test and compare the efficiency of cleanup, plant extracts containing large amounts of extractives other than pesticides were prepared by the method of Gunther and Blinn (*5*). This is a vigorous extraction method, and any procedure which would clean this extract would be likely to clean extracts prepared in other ways.

Procedure for Extract Preparation. Blend 500 grams of plant material for 5 minutes in a large Waring Blendor with 500 ml. of isopropyl alcohol. Add 1000 ml. of benzene, and continue the blending for an additional 5 minutes. Filter the mixture through cheesecloth. Pour the benzene layer off and wash four times with a volume of warm water equal to the volume of benzene. The water wash removes isopropyl alcohol from the benzene layer, and the volume of benzene recovered is an aliquot of the benzene added. Any pesticide present on the plant material is considered to be evenly distributed in the total volume of benzene. To check for losses of pesticides during the cleanup procedure, add known quantities of the pesticides to aliquots of the washed extract.

A cleanup procedure applicable to a wide variety of crops was being sought; therefore it was decided to test the procedure on extracts of apples, cabbage, lettuce, and oranges. These crops represent the waxy fruits, brassic crops, leafy vegetables, and citrus fruits, respectively.

Waxes from plant extracts were much less soluble in acetone than in other solvents such as *N,N*-dimethylformamide, ethyl acetate, *n*-hexane, pentane, isooctane, acetonitrile, and methyl ethyl ketone. Cooling the acetone to − 70° C. decreases the solubility of the waxes still further. From 1000 ml. of an apple extract, 0.0234 gram of wax was precipitated at 10° and 0.0792 gram was precipitated at −70° C.

Price or brand stamps had to be cut from the surface of the plant material

Table I. Efficiency of Removal of Extractives

Cleanup Procedure	Weight Residue, Grams				% Removal of Extractives			
	Orange	Apple	Lettuce	Cabbage	Orange	Apple	Lettuce	Cabbage
No cleanup	1.3275	1.2199	0.8167	1.1398				
Florisil chromatography	0.0949	0.0745	0.0114	0.0751	92.8	93.9	98.6	93.4
Acetone precipitation	0.6920	0.3793	0.4351	0.4266	47.9	68.5	46.7	62.5
N,N-Dimethylformamide–*n*-hexane partitioning	0.0463	0.0664	0.0307	0.0623	96.5	94.6	96.2	94.5
Acetonitrile–*n*-hexane partitioning + Florisil chromatography	0.0193	0.0028	0.0016	0.0029	98.5	99.8	99.8	99.7
N,N-Dimethylformamide–*n*-hexane partitioning + Florisil chromatography	0.0021	0.0017	0.0001	0.0033	99.8	99.9	100.	99.7
Acetone precipitation + Florisil chromatography	0.0370	0.0078	0.0136	0.0084	97.2	99.4	98.3	99.3

prior to extraction. Some of the dyes used in these stamps were not removed during the cleanup procedure.

Comparison of Cleanup Procedures. The efficiency of cleanup of the acetone precipitation–Florisil chromatographic method was compared with the methods of Jones and Riddick (*7*) and Burchfield and Storrs (*1*).

A slight modification of the Jones and Riddick method (*7*) has been used in this laboratory for some time and was used in the work described here. The plant extractives were dissolved in 25 ml. of *n*-hexane and extracted with four 10-ml. portions of acetonitrile. The combined acetonitrile extracts were back-extracted with 5 ml. of *n*-hexane. The acetonitrile solution now contained the pesticides; some of the plant pigments and waxes had been removed.

In the *N,N*-dimethylformamide–*n*-hexane partitioning procedure, the plant extractives were dissolved in 30 ml. of *n*-hexane and extracted first with 30 ml. of *N,N*-dimethylformamide and then with 10 ml. of *N,N*-dimethylformamide. The combined dimethylformamide extracts were diluted with 40 ml. of water, and any *n*-hexane separating was removed under a stream of air. The aqueous dimethylformamide was extracted with 20 ml. of *n*-hexane. This hexane contained the pesticides partially freed from extraneous material.

Neither method (*1*, *7*) removed all the pigments from the extract. Accordingly, these procedures were combined with the Florisil chromatographic procedure.

To compare the methods, large volumes of extracts were prepared as described. One thousand milliliters of each extract was carried through each cleanup procedure and, after cleanup, the solvent was evaporated in a tared flask and the weight of residue was determined. Another 1000 ml. of each extract was evaporated to dryness and the weight of residue was taken as the weight of extractives present before cleanup. Table I shows the result of this comparison. All procedures—viz., acetonitrile–*n*-hexane partitioning plus Florisil chromatography, *N,N*-dimethylformamide-*n*-hexane partitioning plus Florisil chromatography, and acetone precipitation plus Florisil chromatography—cleaned the extracts very satisfactorily. Only very small amounts of colorless residue were obtained.

Table II. Recovery of DDT

Cleanup Procedure	% Recovery of Added DDT	
	Apple extract	Lettuce extract
Acetonitrile–*n*-hexane	80	92
+ Florisil chromatography	80	92
	73	95
	63	97
N,N-Dimethylformamide–*n*-hexane +	30	54
	40	55
Florisil chromatography	47	64
	51	68
Acetone precipitation +	91	92
Florisil chromatography	97	95
	97	96
	97	96

These three procedures were then compared with respect to recovery of technical DDT. Large volumes of lettuce and apple extracts were prepared for this purpose. Technical DDT was added to the extracts at the level of 7 p.p.m. on the plant material, or 3.5 mg. of DDT per 1000 ml. of extract. One thousand milliliters of each extract was carried through each of the three cleanup procedures and the DDT present in the cleaned extract was determined by a method essentially the Downing and Norton modification (*3*) of the Schechter-Haller (*10*) DDT procedure (Table II). These figures show that the best recovery of DDT was obtained from extracts cleaned by the acetone precipitation–Florisil chromatographic procedure. The low recovery of DDT from apple extract using the acetonitrile-*n*-hexane partitioning procedure may be due to DDT trapped in the waxy material, which did not dissolve in either of the organic solvents during partitioning. When no plant extractives were present, technical DDT was recovered satisfactorily following any of the three cleanup procedures.

In the acetone precipitation–Florisil chromatographic procedure, the acetone precipitation always precedes Florisil chromatography. In the other two cleanup procedures, the order varies with the type of extract. In the case of a heavily pigmented extract, such as lettuce, the Florisil column step must precede the partitioning in order that the interphase between the two solvents can be seen. With waxy fruits, such as apples, the partitioning step must precede the column chromatography; otherwise some undissolved waxy material will settle on top of the column and interfere with the flow rate of the column. Difficulty is encountered often with emulsion formation during partitioning. It is a distinct advantage to be able to follow an identical procedure with all types of extract.

Table III. Recovery of Chlorinated Hydrocarbon Pesticides Following Acetone Precipitation–Florisil Chromatography

Pesticide	Level of Pesticide Addition, P.P.M.	No. of Analyses	% Recovery (Range)
p,p'-DDT	7	2	100–101
o,p'-DDT	7	2	93–96
Technical DDT	7	2	91–97
Rhothane	7	4	92–102
Methoxychlor	14	2	90–92
Kelthane	3	2	93–94

Recovery of DDT and Related Chlorinated Hydrocarbon Pesticides Following Acetone Precipitation–Florisil Chromatographic Cleanup. The recoveries of *p,p*-DDT, *o,p*-DDT, methoxychlor, Rhothane, and Kelthane following acetone precipitation–Florisil chromatographic cleanup were checked. These pesticides react quantitatively in the Downing and Norton (*3*) modification of the Schechter-Haller (*10*) DDT procedure, as shown in Figures 1 and 2; so this modified procedure was used for the quantitative analyses following cleanup. Figure 1 represents the absorbances obtained when 112 γ of the pesticides are nitrated and the color is developed in a total volume of 6 ml.

A large volume of apple extract was prepared and to 1000-ml. aliquots of this extract the pesticides were added in amounts which would be found on 500 grams of plant material if the pesticides were present at the tolerance

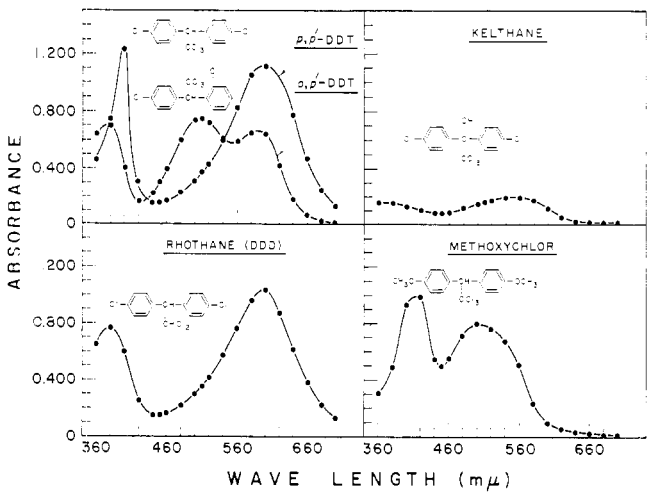

Figure 1. Absorption curves of nitrated chlorinated hydrocarbon pesticides

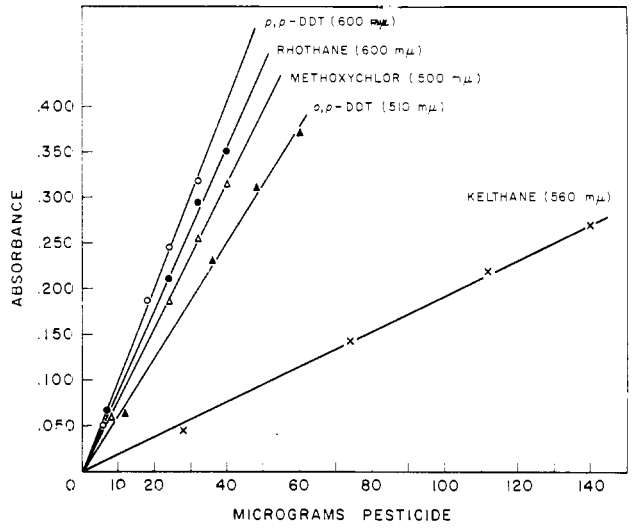

Figure 2. Calibration curves of nitrated chlorinated hydrocarbon pesticides

level permitted by the Canadian Food and Drug Regulations (*2*). The extracts were then carried through the acetone precipitation–Florisil chromatographic procedure and analyzed quantitatively. Colorimetric measurements were made at the following wave lengths: *p,p'*-DDT, 600 mμ; *o,p'*-DDT, 510 mμ; Kelthane, 560 mμ; methoxychlor, 500 mμ; Rhothane, 600 mμ.

As the extract had been prepared from apples purchased on the market, pesticides may have been present and therefore a "blank" determination was made. Two 1000-ml. aliquots of the extract were carried through the procedure without the addition of pesticides and absorbance measurements were made at each of the above wave lengths. These "blank" absorbance measurements were subtracted from the absorbance measurements of the fortified samples.

Data in Table III show that the recoveries for all this group of pesticides were 90% or better. It is planned to check the recoveries of other groups of pesticides following the acetone precipitation–Florisil chromatographic procedure.

Conclusion

The acetone precipitation–Florisil chromatographic procedure is recommended for routine use in the cleanup of plant extracts prior to determination of DDT and related pesticides. It is a general procedure, applicable to a wide variety of crops; it removes pigments and waxes efficiently from extracts prepared by even the most vigorous extraction techniques; the recoveries of DDT and related chlorinated hydrocarbon pesticides are good (much better than with the other methods investigated); and no extremely toxic solvents are involved. This efficient cleanup procedure permits the utilization of available chemical methods of analyses and may open the way for the use of methods not yet thoroughly explored for pesticide analyses, such as infrared and ultraviolet spectrophotometry and gas chromatography.

A routine procedure for determination of pesticide residues on plant material with unknown spray history might proceed as follows: extraction, acetone precipitation–Florisil chromatographic cleanup, qualitative analysis by the chromatographic procedure of McKinley and Mahon (*9*), and quantitative determination of the pesticides shown to be present. Many chlorinated hydrocarbon pesticides, when present singly in an extract, can be determined quantitatively by the method of Downing and Norton (*3*). This provides a further generalization in routine analyses.

Acknowledgment

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Quantitative Determination of Thiodan by Gas Chromatography

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The newly developed insecticide, Thiodan, may be determined indirectly by measuring evolved sulfur dioxide or by infrared spectrography. This paper deals with the quantitative determination of Thiodan isomers by gas chromatography and its adaptation to formulation analysis. No isomerization or decomposition of Thiodan occurs on the column at 250° C. as shown by infrared spectrography, melting point, and elemental analysis of the isolated products. With increased sensitivity the gas chromatographic technique may be adapted to residue analysis of Thiodan.

Analytical methods for the determination of the insecticide Thiodan (6,7,8,9,10,10 - hexachloro - 1,5,5a,6,9,-9a - hexahydro - 6,9 - methano - 2,4,3-benzodioxathiepin 3-oxide) are based on the hydrolysis of Thiodan to the corresponding alcohol and sulfur dioxide. The released sulfur dioxide is then determined by iodometric or colorimetric procedures (*4*, *6*).

Commercial Thiodan is a mixture of two geometric isomers (*3*), and the above chemical methods cannot distinguish between these isomers. These methods could also lead to low or erratic results due to the loss of sulfur dioxide, incomplete hydrolysis of Thiodan, and interferences from other oxidizable compounds. For these reasons a new method for the quantitative determination of Thiodan was investigated.

The technique of gas chromatography has recently been adapted to the analysis of various chlorinated and organic-phosphorus insecticides (*1*). The same technique has now been utilized for the quantitative determination of Thiodan and its isomers in commercial formulations.

Equipment and Materials

Gas Chromatograph Apparatus. Aerograph Model A-100-C, commercial gas chromatograph apparatus; Varian G-10 recorder, 1-mv. range selector switch; hot-wire tungsten filaments.

Column Packing. Dow-11 high-vacuum silicone grease (30% w./w.) on Chromosorb 35/80 using a 6-foot (1/4-inch-diameter) spiral stainless steel column.

Pipets. A 100- and a 10-μl. Hamilton syringe-type pipet were used for quantitative work. For preparative work a 1-ml. syringe was employed.

Infrared Spectrophotometer. A Perkin-Elmer Model 221 infrared spectrophotometer was used for the qualitative and quantitative determinations of Thiodan isomers.

Insecticide Standard Solutions. Analytical Thiodan and partially purified isomers A and B (obtained from Niagara Chemical Division, Middleport, N. Y.) were dissolved in *n*-hexane so that 1 ml. contained 10 mg.

Experimental Methods

Gas Chromatography. Appropriate volumes of insecticides, ranging from 2.5 to 50.0 μl. (25 to 500 γ), were injected into the rubber diaphragm of the heated injector block. The column had been previously equilibrated at 250° C. for 24 hours. The filament current was kept at 250 ma., and a helium gas flow of 52.6 ml. per minute was maintained.

Under these conditions a steady base line was obtained with no signal attenuation. The solvent, *n*-hexane, had a retention time of less than 1 minute, while Thiodan isomers A and B had retention times of 10.5 and 14.0 minutes, respectively. Resolution of the solvent and the two isomers was excellent (Figure 1).

For quantitative evaluation, the areas of the peaks were determined by half the product of the peak height and the width given by the inflection tangents at the base (*2*) (Figure 1). The ideal shape of the effluent curves permitted such an approximation. "Corrected concentration" was plotted against "area" and the same straight line re-

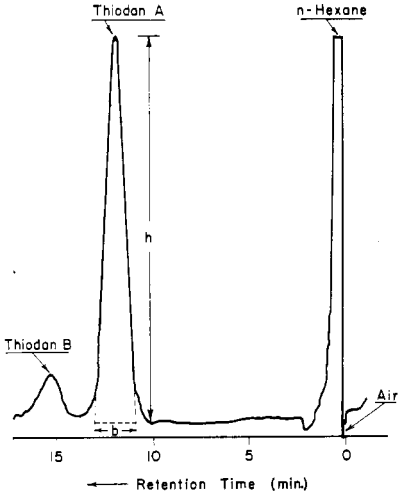

Figure 1. Gas chromatography effluent curve of impure Thiodan A

Area = $\frac{1}{2} b \times h$

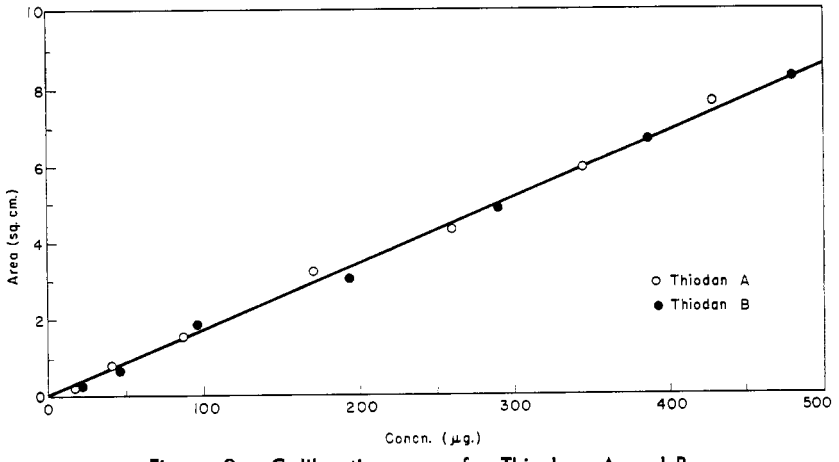

Figure 2. Calibration curve for Thiodans A and B

sulted over a range of 25 to 500 γ for each isomer, as is illustrated in Figure 2. The necessity for correction is discussed under Results and Discussion.

Miscible formulations of Thiodan were diluted appropriately with *n*-hexane, and peak areas due to each isomer were directly converted to concentration from the calibration curves. Relative isomer concentration was calculated by the simple equations:

$$\% \text{ isomer A} = \frac{\text{area}_{(A)}}{\text{area}_{(A)} + \text{area}_{(B)}} \times 100 \quad (1)$$

$$\% \text{ isomer B} = 100 - \% \text{ isomer A} \quad (2)$$

Preparation of Pure Thiodan Isomers. For the preparation of several milligrams of pure Thiodan isomers A and B, 1 ml. of the Thiodan solution, containing 10 mg. of mixed isomers, was injected into the column. As each isomer emerged from the column, as indicated by the recorder, it was condensed at room temperature in a U-shaped glass tube, 3 mm. in internal diameter, one end of which was inserted through the collector gasket at the effluent stream. Each isomer was examined by infrared spectroscopy in solution (carbon tetrachloride or carbon disulfide).

Melting points of these pure isomers were determined in an air bath with a glass capillary and were found to be 105–7° C. for A and 203–5° C. for B, in agreement with values reported in the literature (*3*).

Results and Discussion

Standard Curves. Thiodan isomers A and B (Niagara Chemical Division) were first examined by gas chromatography; Thiodan B contained a small amount of the lower melting isomer when 500-γ quantities were chromatographed. At lower concentrations this impurity was not detectable. Corrections for this impurity were made by the use of Equations 1 and 2. By this method it was determined that Thiodan B contained 3.0% of Thiodan A, and Thiodan A contained 14.1% of Thiodan B (Table I). The contamination of Thiodan A could be detected and measured quantitatively at the 300-γ level.

Gas chromatography was successfully employed to isolate small quantities of pure isomers, as shown by single effluent peaks. The column, however, was not suitable for the isolation of sufficiently large quantities of pure isomers to establish standard curves. Therefore, it was necessary to show that the peak areas, measured by the "half-peak-width" method, were additive. This was demonstrated by the fact that the standard curves, using the corrected values for Thiodan A and B, coincided, as may be seen in Figure 2.

Table I. Standard Curves for Thiodan Isomers

Concn., γ		Peak Areas, Sq. Cm.[a]		% Thiodan A
Apparent	Corrected	A_1	A_2	
		Thiodan A		
500	430	7.6[b]	1.2	86.2
400	344	6.0	0.9	86.4
300	258	4.4	0.7	85.2
200	172	3.3	0	...
100	86	1.5	0	...
50	43	0.7	0	...
25	22	0.2	0	...
				Av. 85.9
		Thiodan B		
500	484	0.2	7.9	2.5
		0.3	8.7	3.7
		0.2	8.4	2.7
				Av. 3.0
400	387	0	6.7	
			6.5	
			6.8	
300	291	0	5.0	
			4.8	
			4.9	
200	194	0	3.1	
			3.1	
100	97	0	2.0	
			1.9	
50	48.4	0	0.8	
			0.7	
25	24.2	0	0.4	
			0.4	

[a] A_1 = Thiodan A. A_2 = Thiodan B.
[b] Average of duplicates.

Table II. Analysis of Thiodan Formulation by Gas Chromatography

(2.288 mg. of formulated Thiodan in 50 μl. of *n*-hexane)

No. of Analysis	Peak Areas, Sq. Cm.[a]		
	A_1	A_2	A_3
1	5.6	2.9	0.2
2	6.3	3.1	0.3
3	6.1	3.4	0.3
4	6.4	3.4	0.3
Av.	6.1	3.2	0.3

γ Thiodan (Figure 2) corresponding to
6.1 sq. cm. = 358 γ
3.2 sq. cm. = 185 γ
543 γ (exptl.)

γ Thiodan (theoretical)
2,288 × 0.24 = 549.12 γ (theor.)

[a] A_3 = unidentified impurity.

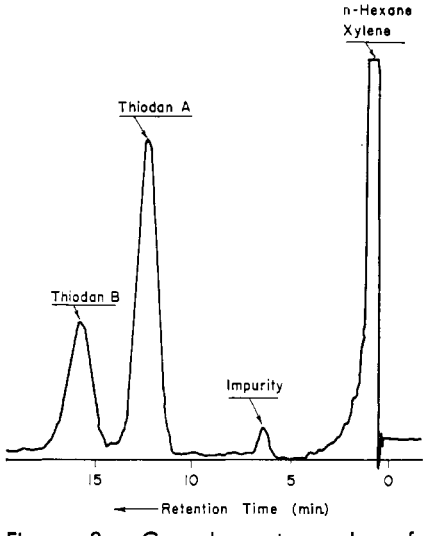

Figure 3. Gas chromatography effluent curve of Thiodan formulation

Qualitative runs were also made with chromatographically obtained isomers; single peaks resulted in each case. These experiments indicated that isomerization of the pure isomers did not occur at column temperatures of 250° C. This was further confirmed by an examination of the infrared spectra and other physical constants.

Analysis of variance of the data in Table I showed no significant difference between replicates at the 5% level and a high degree of reproducibility. One source of error might be the variability of pipetting due to the skill of the operator, but this error did not appear on comparing relative concentrations of isomers in a mixture. Excellent reproducibility of this ratio may be seen in Table I. This technique, therefore, may be successfully applied to the determination of isomer ratios, as in production control.

Analysis of Thiodan Formulation. To test the validity of the method described, a formulated preparation of Thiodan was analyzed. This formula-

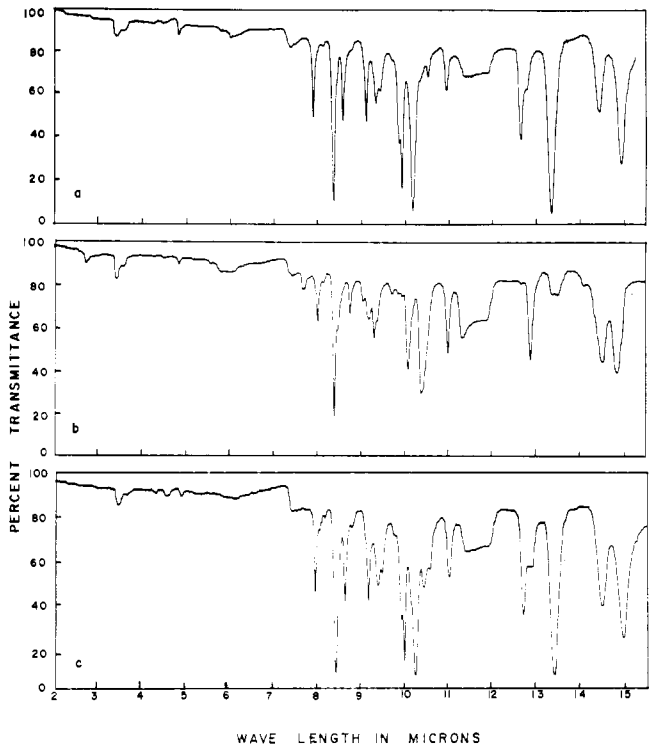

Figure 4. Infrared spectra of carbon disulfide solutions

a. Chromatographically pure Thiodan A (low melter) *b.* Chromatographically pure Thiodan B (high melter) *c.* Impure Thiodan A 0.03% solutions; 3-mm. liquid microcell

tion bore the following label: Thiodan 24.00% (by weight); xylene 69.00%; inert ingredients 7.00%. A weighed amount of this formulation was dissolved in 10.0 ml. of *n*-hexane, and an analysis was performed on 50-μl. aliquots with four replications. Table II summarizes the results of this analysis—Thiodan 23.69%; Thiodan A/Thiodan B = 1.92. The chromatographic pattern of this formulation shows the presence of a minor impurity which may be due to one of the inert ingredients (Figure 3). Xylene has a retention time similar to that of *n*-hexane (less than 1 minute).

Analysis of this formulation by infrared spectroscopy, measuring the absorption peak at 8.40 microns, gave a concentration of 23.43% of Thiodan (*5*). Thus, the two methods of analysis were shown to be in good agreement.

Infrared Spectroscopy. Chromatographically pure Thiodan B when dissolved in carbon tetrachloride yielded an infrared spectrum identical to that published in the literature (*3*).

The infrared spectrum of a solution of Thiodan B in carbon disulfide yielded two additional bands at 12.8 microns (strong) and 13.35 (weak) (see Figure 4, *b*). These bands may be associated with the carbon-chloride bond, as carbon tetrachloride absorbs strongly in the 12- to 14- micron region.

Figure 4 also illustrates the difference in infrared spectra of the chromatographically pure and contaminated samples of Thiodan A. The contaminated A shows an additional absorption band at 10.45 microns which is absent from the spectrum of pure A. As gas chromatographic analysis showed that the impurity was due to the B isomer, this band may correspond to a strong absorption peak of pure B in this region. This 10.45-micron band may also be present in the published spectrum of Thiodan A, indicating a possible contamination with B (*3*).

Figure 4, *a* also shows three additional absorption bands for Thiodan A when examined in carbon disulfide. These bands are at 12.63, 12.74 (weak), and 13.28 microns and may again be associated with the carbon-chloride bond.

Possible Isomerization or Decomposition by Gas Chromatography. The possibility existed that either Thiodan isomer could be interconverted at the temperature of the chromatographic column (250° C.). However, chromatographically isolated Thiodans A and B yielded single effluent peaks when rechromatographed at concentrations at which 2 to 3% conversion could have been detected.

Elemental analysis of chromatographically pure Thiodan B gave the following results: Found, % C 25.29; % H 1.55; % S 7.37. Calculated, % C 26.55; % H 1.47; % S 7.87. This was taken as evidence that Thiodan B was not decomposed on the chromatographic column. Furthermore, determination of the retention times of possible decomposition products gave: Thiodan A 10.5 minutes, Thiodan B 14.0 minutes, Thiodan alcohol (Hooker Chemical Corp., Niagara Falls, N. Y.) 9.5 minutes, and Thiodan ether (Hooker) 5.3 minutes. Mixtures of these four compounds were well resolved under the stated experimental conditions.

When pure Thiodan isomers were chromatographed, perfectly symmetrical retention curves were obtained, and no additional peaks due to Thiodan alcohol or ether were observed. These observations then precluded the possible isomerization or decomposition of Thiodan isomers during gas chromatography at elevated temperatures.

Future Research

Preliminary experiments with an ionization detector (Sr^{90}) and amplifiers indicate that a much higher sensitivity, on the order of 0.1 to 1.0 γ of Thiodan, may be achieved by gas chromatography.

Experiments are in progress in this laboratory combining gas chromatography and infrared spectroscopy for determination of Thiodan isomer residues in raw agricultural crops.

Acknowledgment

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Elemental analysis of chromatographically pure Thiodan B was performed by V. H. Tashinian, Department of Chemistry, University of California, Berkeley, Calif.

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ISOTOPE-LABELED INSECTICIDE

Thiodan-5a, 9a-C_2^{14}

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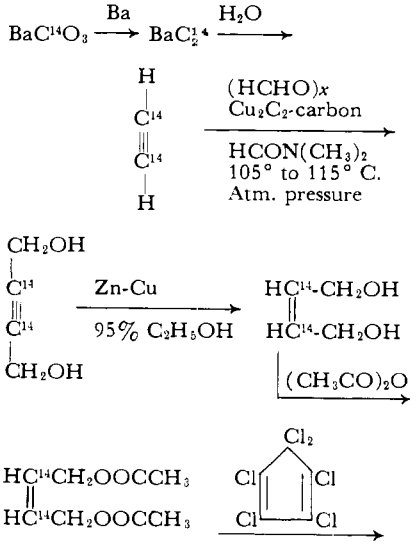
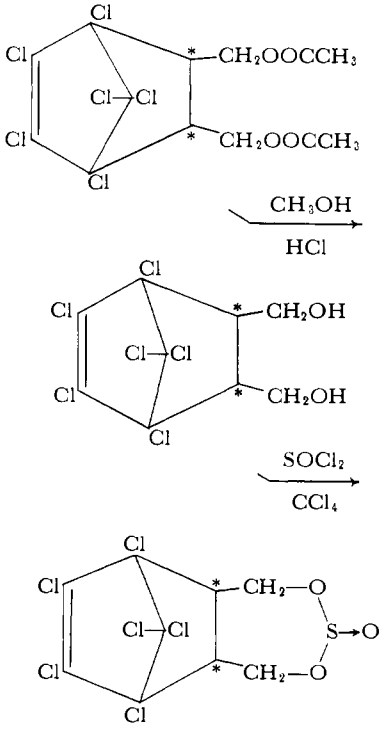
The insecticide 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide-5a,9a-C_2^{14} (Thiodan-5a,9a-C_2^{14}) was prepared for use in biological studies which required that quantities as low as 1 part per billion be detectable. Starting with a 411-mc. quantity of barium carbonate-C^{14}, the quantity of final product obtained was 210 mc., which represents a radiochemical yield of 51%.

THIODAN is a pesticide which is particularly effective on forage insects, potato insects, and most species of aphids. This synthesis of a radiotagged material was undertaken to permit the direction of as little as 1 part of Thiodan per billion in biological studies. Because feeding experiments with cows were contemplated, the positions of the tagged atoms in the molecule had to be located to minimize any possibility of exchange during ruminant metabolism. These considerations therefore precluded the use of tritium, sulfur-35, or chlorine-36 as a tracer atom. The introduction of carbon-14 into a ring position in Thiodan, however, met the requirements.

A minimum of 75 mc. of Thiodan-5a,9a-C_2^{14} with a minimum specific activity of 2 μc. per mg. was required for the biological studies.

The following scheme was adopted for the synthesis of Thiodan-5a,9a-C_2^{14}:





The first two steps had already been carried out on a small scale (*2*). The other steps had been carried out with radioactive chemicals (*1*, *3*, *4*, *7*, *8*).

Thiodan was first prepared with nonradioactive chemicals, starting from barium carbonate. The intermediates and the products were characterized to verify their identities. A pilot batch was made at a low level of radioactivity to be sure that the radioactivity could indeed be attached to the Thiodan. More analytical data were obtained in that run than in the subsequent ones because it was desirable to conserve radioactivity.

It was deemed prudent to prepare the highly active material in two batches of approximately 200 mc. each as a precaution against accidental loss. Subsequent runs were made for the purpose of scavenging radioactive material.

Experimental

Each batch was kept separate throughout the preparation.

A. Barium Carbide-C^{14}. The starting materials consisted of 1.78 grams of $BaC^{14}O_3$ containing 200 mc. (19.64% C^{14}) and 0.9 gram containing 210 mc. (75.5% C^{14}). Fourteen grams of barium metal and 1.78 grams of barium carbonate-C^{14} were placed in an 18 × 150 mm. test tube by alternating 2-gram portions of thin metal shavings with approximately 0.3-gram portions of barium carbonate-C^{14}. A stream of argon gas was directed into the tube while the charge was heated with a Fisher burner. The mixture momentarily became incandescent when it reacted.

B. Acetylene-1,2-C_2^{14}. Barium carbide-C_2^{14} was warmed while 135 ml. of water were added. After the reaction subsided, the mixture was refluxed for 30 minutes with a hydrogen sweep. The acetylene-1,2-C_2^{14} was condensed in a glass spiral cooled with liquid nitrogen and then transferred quantitatively to a gas-holding bulb. Mass spectrographic analysis indicated 97.8% acetylene, 1.2% ethylene, and 1.0% ethane.

Some unconverted material (19 mc.) remained as $BaC^{14}O_3$ and elemental carbon-14. These were recovered and converted to barium carbide-C^{14}.

C. 2-Butyne-1,4-diol-2,3-C_2^{14}. The catalyst was prepared by bubbling commercial acetylene into a mixture of 12 grams of Nuchar C-190-N activated

carbon suspended in a solution consisting of 200 ml. of water, 8 grams of cupric sulfate pentahydrate, 25 ml. of aqueous 28% ammonia, and 12.5 grams of hydroxylamine hydrochloride. The solids were filtered and washed thoroughly first with water and then with dimethylformamide. One gram of bismuth trioxide was added.

The catalyst, 6.3 grams of 95% paraformaldehyde (equivalent to 0.2 mole of formaldehyde), and 60 ml. of dimethylformamide were stirred in the reaction flask and the acetylene-1,2-C_2^{14} was added to the evacuated apparatus along with recovered acetylene from a previous run. The reaction mixture was heated in the presence of commercial acetylene at atmospheric pressure with only occasional stirring until the temperature reached 95° C. A reduced rate of stirring was used at first to avoid solution of an excessive quantity of acetylene which would desorb at a higher temperature. When desorption began, the stirrer was turned on full and after a short time the gas was reabsorbed. The reaction was maintained at 105° to 115° C. for 13 hours, while about 0.1 mole of commercial acetylene was absorbed. The absorption rate varied with the purity of the gas. The cooled reaction mixture was filtered onto a sintered glass disk built into the apparatus. The unreacted acetylene was condensed in a spiral with liquid nitrogen. The filtrate and dimethylformamide washings were vacuum-distilled through a small Vigreux column. The 2-butyne-1,4-diol-2,3-C_2^{14} distilled at 125° C. at 5 mm., and the last part was driven over with an electrically heated tape.

The same catalyst was used in the subsequent batches in order to conserve the radioactivity which remained on the catalyst. When the rate of absorption of gas fell off, addition of new catalyst and solvent did not increase the rate; nor did condensation of the gas with liquid nitrogen followed by evacuation of the system and evaporation of the gas back into the system.

When gas from the reaction mixture was condensed with liquid nitrogen and commercial acetylene was added to the reaction mixture, the absorption of gas was rapid. Analysis of the condensed gas by mass spectrometer showed the presence of 75% carbon dioxide, 1% allene or propyne, and 24% acetylene. The carbon dioxide was not radioactive, while the acetylene had a specific activity of 2.45 μc. per mg. The gas was shaken with potassium hydroxide solution to remove carbon dioxide and the remaining acetylene-1,2-C_2^{14} was returned to the reactor. The source of the carbon dioxide was not known.

The yields of 2-butyne-1,4-diol-2,3-C_2^{14} from the principal runs were, respectively, 7.6 and 5.2 grams, specific activity 15.0 and 24.8 μc. per mg., total activity 114 and 129 mc., radiochemical yield from $BaC^{14}O_3$, 57 and 61.5%. Some additional activity was obtained by using the catalyst in a subsequent run, so that a total of 289 mc. of radioactivity was obtained in the form of butynediol from 411 mc. of $BaC^{14}O_3$ for a radio yield of 70%.

Commercial 2-butyne-1,4-diol (8.6 grams) was added to the extract along with the rest of the recovered dimethylformamide, and the mixture was distilled. This batch was carried along in the synthesis to scavenge radioactivity from other places in the synthesis.

D. *cis* - 2 - Butene-1,4-diol-2,3-C_2^{14}. Cupric sulfate pentahydrate (0.1 mole), dissolved in 600 ml. of water, was stirred with 0.5 gram atom of zinc dust until the color of the solution was no longer blue, and a zinc-copper couple had formed. The solids were filtered off and washed thoroughly with water.

Each batch of 2-butyne-1,4-diol-2,3-C_2^{14} dissolved in 600 ml. of 95% ethyl alcohol was stirred with freshly prepared zinc-copper couple and refluxed for 1 hour. The mixture was filtered and the product was concentrated by distillation. The residue which remained at 100° C. at 20 mm. gave the same infrared spectrum as *cis*-2-butene-1,4-diol.

E. *cis*-2-Butene-1,4-diol-2,3-C_2^{14} Diacetate. *cis*-2-Butene-1,4-diol-2,3-C_2^{14} was added to 0.2 mole of refluxing acetic anhydride during 1/3 hour. After 2 additional hours of refluxing, the product was distilled at 114° C. at 14 mm. The average material yield was 75%.

F. 1,4,5,6,7,7 - Hexachlorobicyclo-[2,2,1] - hept - 5 - ene - 2,3 - dimethanol-2,3-C_2^{14} Diacetate. Hexachlorocyclopentadiene (36 grams) and a batch of butenediol diacetate were stirred and heated under nitrogen at 165° C. for 8 hours. The unreacted portion was removed by distillation up to a kettle temperature of 165° C. at 1 mm. The first principal batch gave 24.5 grams of product and 17.8 grams of distillate.

The distillate of each previous batch was used in the subsequent batch, and the quantity of hexachlorocyclopentadiene was reduced by a corresponding amount.

In the scavenging batch, the distillate was subjected to second and third heating periods and additional radioactive product resulted.

G. 1,4,5,6,7,7 - Hexachlorobicyclo-[2,2,1] - hept - 5 - ene - 2,3 - dimethanol-2,3-C_2^{14}. Each batch of Diels-Alder adduct was alcoholyzed with 2 volumes of methanol per gram in the presence of 0.025 volume of concentrated hydrochloric acid per ml. of methanol by refluxing for 2.5 hours. The product was recrystallized with the aid of additional methanol and Darco G60 activated carbon. The yields of the principal batches were 13.4 and 12.7 grams.

After the scavenging batch had been crystallized from the mother liquors left from the preceding batches, evaporation of the mother liquor left a residue of 23 grams containing 32 mc. of radioactivity. This residue was mixed with nonradioactive 1,4,5,6,7,7-hexachlorobicyclo- [2,2,1]-hept-5-ene-2,3-dimethanol and the mixture was crystallized from methanol. The addition of nonradioactive material and crystallization were repeated. The final mother liquor had an activity of 24.8 mc. which was evidently not part of the desired compound.

H. 6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a - hexahydro - 6,9 - methano-2,4,3 - benzodioxathiepin 3 - oxide-5a,9a-C_2^{14} (Thiodan-5a,9a-C_2^{14}). Each product from G was refluxed with 1 ml. of carbon tetrachloride per gram while 0.27 ml. of thionyl chloride per gram was added during 0.5 hour. After the mixture had refluxed for 5 hours longer, the excess reagent and solvent were removed by distillation in vacuum up to a kettle temperature of 100° C. The product was sparged with nitrogen for a short time at elevated temperature in vacuum. The infrared spectrum of each product served to verify its identity (Figure 1).

The yield of the first principal batch was 14.6 grams, 3.7 μc. per mg., 54 mc. total; and of the second principal batch, 14.4 grams, 5.91 μc. per mg, 85 mc. total, 93.2% Thiodan. Other batches yielded additional quantities of Thiodan with less radioactivity. The total radiochemical yield for all of the batches combined was 210 mc., 51% of theoretical, from 411 mc. of barium carbonate-C^{14}.

I. Analytical Procedures for Radiochemical Assay of C^{14}-Tagged Intermediates and Thiodan. ACETYLENE-1,2-C_2^{14}. The sample was transferred by vacuum techniques to the combustion apparatus in which copper oxide at 300° C. oxidized the gas to carbon dioxide. The latter was absorbed in a magnetically stirred solution consisting of 5 ml. of standard 0.5*N* sodium hydroxide containing 117.7 mg. of sodium carbonate to dilute the activity. Following the addition of 5 ml. of 0.5*M* barium chloride solution, the excess alkali was titrated with standard 0.1*N* hydrochloric acid to a phenolphthalein end point. Subtraction of the latter titration from a blank titration gave the amount of alkali consumed by the carbon dioxide and corresponded to the quantity of acetylene.

Small aliquots of the barium carbonate were transferred to tared Millipore membranes mounted on a Tracerlab filter tower apparatus, filtered, washed with hot water, and vacuum-dried. The membranes were weighed, then mounted on Tracerlab ring and disk assemblies, and counted on the

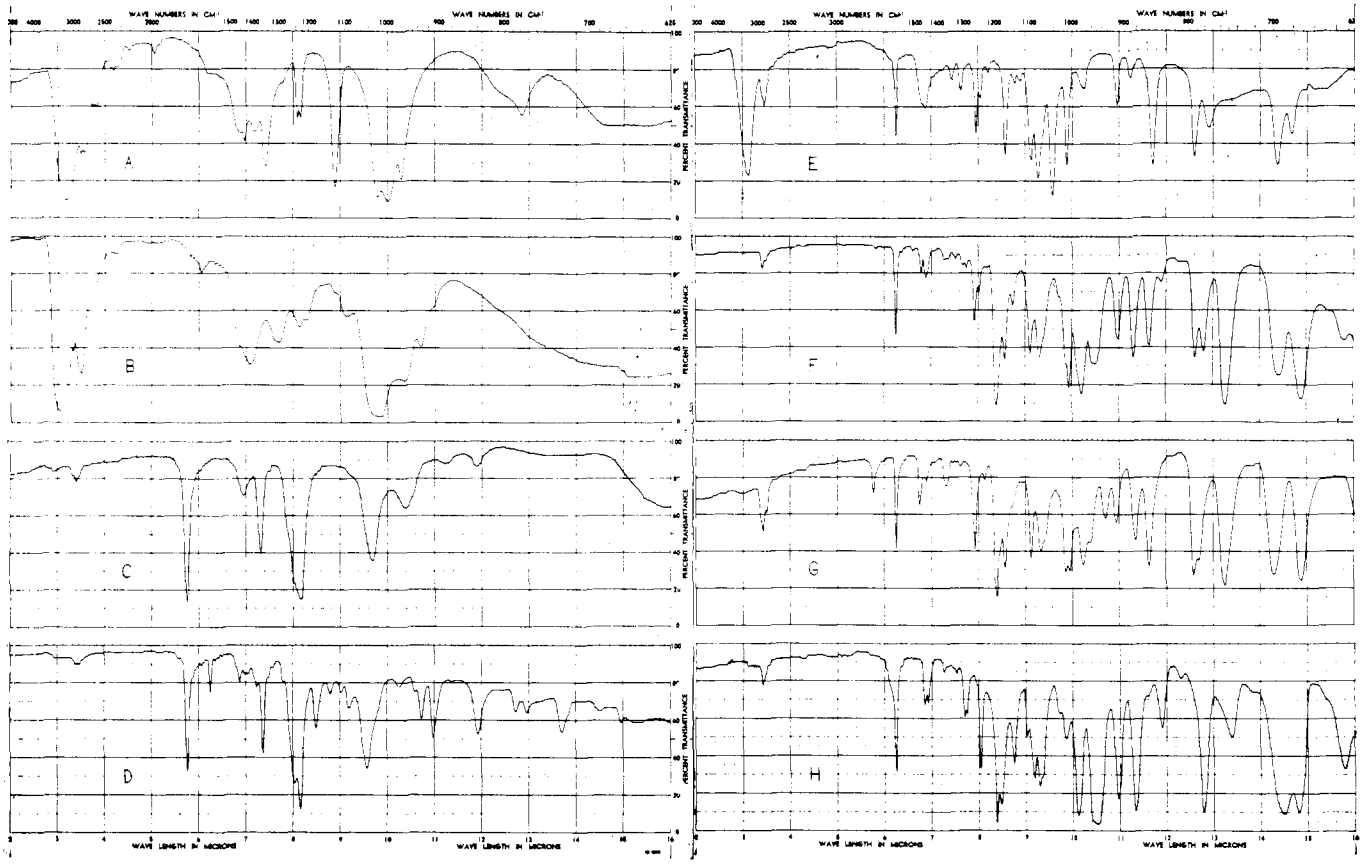


Figure 1. Infrared spectra

A. 2-Butyne-1,4-diol (KBr)
B. *cis*-2-Butene-1,4-diol (film)
C. *cis*-2-Butene-1,4-diol diacetate (film)
D. 1,4,5,6,7,7 - Hexachlorobicyclo - [2,2,1]-hept - 5 - ene - 2,3 - dimethanol diacetate (solid)
E. 1,4,5,6,7,7-Hexachlorobicyclo-[2,2,1]-hept-5-ene-2,3-dimethanol (KBr)
F. Thiodan, technicol (solid)
G. Thiodan, isomer, m.p. 108° C. (solid)
H. Thiodan, isomer, m.p. 212° C. (KBr)

top shelf of an end window counter. The radio-count was corrected for background, dead time, and self-absorption. The latter correction was made with barium carbonate prepared from an NBS solution of sodium carbonate containing 1280 decompositions per second per ml. Counts were reproducible to ±2%.

OTHER INTERMEDIATES AND THIODAN-5a,9a-C_2^{14}. The other compounds were assayed using the apparatus and procedure of Thorn and Shu (*6*), followed by plating and counting the barium carbonate.

Because this procedure is relatively time-consuming and the substances to be analyzed were of relatively high radioactivity, a modification of the procedure of Schweibel, Isbell, and Moyer (*5*) was used. One milliliter of a dilute solution of the substance in dimethylformamide was counted in a stainless steel planchet under an end window counter whose efficiency was determined against a standard sample of benzoic acid-C^{14} dissolved in dimethylformamide. The standard, obtained from the New England Nuclear Co., contained 9860 decompositions per second per mg. and was counted at an average efficiency of 764 (±) 16 decompositions per minute per count per minute. Assay by this procedure gave results within 2% of the results obtained by the combustion procedure.

A further check on this procedure was made by burning a sample by the method of Thorn and Shu. A portion of the barium carbonate which resulted was treated with acid to evolve carbon dioxide, which was then transferred to a 250-ml. ionization chamber. The activity was determined by the resistor leak method and gave results within 2% of the method of Schweibel *et al.*

J. Chemical Analysis of Thiodan. A 20- to 30-mg. sample of Thiodan was weighed in a 1-ml. beaker on a micro- or semimicrobalance and transferred to a 125-ml. iodine flask which contained 10 ml. of methanol and 2 ml. of methanolic 10% sodium hydroxide. The mixture was refluxed for 2 hours to hydrolyze the Thiodan to sodium sulfite. The mixture was allowed to cool and the condenser was washed down with 5 ml. of methanol and 10 ml. of water. The solution was made just acid to phenolphthalein by the dropwise addition of 5*N* sulfuric acid and then just alkaline with a few drops of sodium hydroxide to prevent loss of sulfur dioxide. The sample was transferred to a flask containing exactly 10.00 ml. of standard 0.05*N* iodine solution and 5 ml. of 5*N* sulfuric acid. Three 10-ml. washings with water were sufficient to complete the transfer. The excess iodine was titrated with standard 0.02*N* sodium thiosulfate solution until only a light yellow color remained; then 5 ml. of 2% starch solution were added and the titration was continued until the blue color disappeared completely. Care was taken to use adequate stirring, a white background, and good lighting. A blank determination was also carried out. The Thiodan content was computed on the basis of the iodine consumed by the sulfur dioxide which was produced during hydrolysis of Thiodan.

K. Infrared Spectrophotometric Method for Determination of Low and High Melting Isomers of Thiodan. Thiodan exists in two isomeric forms (melting points 108° and 212° C.). The infrared spectrum of each isomer has a band near 8 microns which was used to estimate the quantity present. However, accurate results could not be obtained with samples of impure Thiodan. It was established that the isomer contents of the Thiodan-5a,9a-C_2^{14} samples were roughly the same as the isomer content of commercial Thiodan.

The method involved making absorbance measurements of the samples dissolved in carbon disulfide at 7.95 and 8.05 microns for the low- and high-melting isomers, respectively.

A Baird Model 4-55 infrared spectrophotometer was employed at 2*N* slit program and 2*X* abscissa. Solutions for calibration were prepared using binary mixtures (by weight) of pure isomers made up to 5 ml. with carbon disulfide. The solutions were placed in a 0.2-mm. sodium chloride cell and the spectrum was scanned from 7.7 to 8.3 microns at the rate of 0.5 micron per minute. A base line was drawn between the transmittance maxima at 7.8 and 8.2 microns, absorbances were calculated, and graphs were plotted of absorbance at the respective wave lengths *vs.* concentration of the individual isomers. Both plots gave a straight line through all points.

Samples of Thiodan for analysis were treated in the same manner as the calibration standards.

L. Crystallization of Radio-Tagged Thiodan. Small samples of Thiodan-5a,9a-C_2^{14} were dissolved in warm hexane, filtered, and crystallized by cooling with ice water. Another crop was obtained in each case by further cooling with carbon dioxide ice, and the remaining mother liquor was evaporated to dryness. Each crop and residue were assayed chemically for Thiodan, a radio-count was determined, and the isomer ratio was estimated. The original samples assayed 85 to 93% Thiodan with a low melting isomer–high melting isomer ratio of about 2 to 1. Upon crystallization, the purer first two crops had higher radioassays than the residues. These data indicated that the non-Thiodan portion was not radioactive. The various crops had different isomer ratios from the original samples, but no generalization could be made as to the direction of enrichment. When recrystallized Thiodan-5a,9a-C_2^{14} was crystallized with recrystallized commercial Thiodan of different isomer composition, the radioactivity of the resulting crops, which differed in isomer ratios, followed as would be expected. When radio-Thiodan and commercial Thiodan, neither of which had been recrystallized, were mixed and then crystallized from hexane, the radioactivity varied with the purity of the resulting crops. These data indicated that radio-Thiodan and commercial Thiodan had the same isomer content, and that the impurities in radio-Thiodan were not radioactive.

Because crystallization of Thiodan-5a,9a-C_2^{14} would alter the isomer content, it was preferable to use the unpurified material, and because the impurities did not appear to be radioactive, it was feasible to use the radio-Thiodan in biological experiments.

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INSECTICIDE TOXICITY

Preparation and Biological Activity of a Series of Halogenated Ethyl and Vinyl Dimethyl Phosphate Esters

A. M. MATTSON, R. W. FAY, T. B. GAINES, and G. W. PEARCE

Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Savannah, Ga.

Practically all of the work reported in the synthesis (*1*, *4–7*) and biological activity (*4*, *5*, *7*) of insecticidally active halogenated ethyl or vinyl dialkyl phosphates has been done on chloro-substituted compounds with passing mention of the possibility of preparing mixed bromo-chloro compounds (*6*, *7*). However, recently a report on the insecticidal properties of dimethyl 1,2-dibromo-2,2-dichlorophosphate was given by Gojemerac and Waples (*2*). This compound is available commercially under the name "Ortho-Dibrom" (California Spray Chemical Corp., Moorestown, N. J.).

The present report deals with the preparation and comparative toxicity to flies and rats of a series of tertiary phosphate esters with two methyl groups as constant constituents. The variations in the tertiary group consisted of a series of bromo, chloro, and bromo-chloro–substituted ethyl or vinyl groups.

Compounds Synthesized

The compounds synthesized are shown in Table I, together with the method of synthesis. The tetrahalogenated compounds were prepared by bromination or chlorination of the dimethyl dichlorovinyl and dibromovinyl esters. The dichlorovinyl compound (I) was a specially purified commercial product (Montrose Chemical Co., Newark, N. J.). Dimethyl 2,2-dibromoethyl phosphate (IV), the bromine analog of DDVP (I), was prepared by Perkow's reaction (7). The reaction was run in an ether solution at a lower reaction temperature (−10° to 0° C.) than that given by Perkow because the reaction was extremely vigorous. The reaction mixture was washed with aqueous sodium bicarbonate and several times with water. After drying with anhydrous sodium sulfate, the ether was removed under reduced pressure. Compounds II and V were obtained by bromination of I and IV. Brominations were done in carbon tetrachloride solutions with a slight excess of the calculated amount of bromine for a 24-hour period. Compounds III and VI were obtained by chlorinating I and IV. Chlorination was done by bubbling dry chlorine gas through carbon tetra-

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Compounds Synthesized

The compounds synthesized are shown in Table I, together with the method of synthesis. The tetrahalogenated compounds were prepared by bromination or chlorination of the dimethyl dichlorovinyl and dibromovinyl esters. The dichlorovinyl compound (I) was a specially purified commercial product (Montrose Chemical Co., Newark, N. J.). Dimethyl 2,2-dibromoethyl phosphate (IV), the bromine analog of DDVP (I), was prepared by Perkow's reaction (7). The reaction was run in an ether solution at a lower reaction temperature (−10° to 0° C.) than that given by Perkow because the reaction was extremely vigorous. The reaction mixture was washed with aqueous sodium bicarbonate and several times with water. After drying with anhydrous sodium sulfate, the ether was removed under reduced pressure. Compounds II and V were obtained by bromination of I and IV. Brominations were done in carbon tetrachloride solutions with a slight excess of the calculated amount of bromine for a 24-hour period. Compounds III and VI were obtained by chlorinating I and IV. Chlorination was done by bubbling dry chlorine gas through carbon tetra-

A series of dimethyl phosphate esters was prepared in which the third ester substituents were a series of bromo, chloro, and mixed bromo-chloro–substituted ethyl and vinyl groups. Among these compounds were the 1,2,2,2-tetrabromoethyl, 1,2,2,2-tetrachloroethyl, 1,2-dibromo-2,2-dichloroethyl, 2,2-dibromo-1,2-dichloroethyl, and 2,2-dibromovinyl esters of dimethyl phosphate. Tests were made on their insecticidal activity toward houseflies and their toxicity to white rats. When compared with 2,2-dichlorovinyl dimethyl phosphate (DDVP), several compounds showed equal or greater effectiveness against houseflies, with a lesser toxicity to white rats.

chloride solutions for 3 to 4 hours while cooling the reaction mixture in a water bath. The carbon tetrachloride solutions of the reaction products were treated in the same manner as the ether solutions discussed in the previous paragraph.

Refractive indices and chemical analyses (Table I) showed a satisfactory degree of purity of these compounds. All but one of the compounds were liquids at room temperature. The tetrabromo compound (V) solidified after refrigeration, and stayed solid at room temperature. The melting point was very broad with complete liquefaction at approximately 45° C.

Toxicity to Rats and Chickens

Acute oral LD_{50} values were found for male rats using peanut oil solutions. The compounds are listed in Table II in order of increasing toxicity. Of the compounds containing only one kind of halogen, the bromine compounds are much less toxic to rats than their chlorine-containing analogs. Two of the compounds (II and VI) have both bromine and chlorine and differ only in the position of the halogen atoms. The compound (II) which has the bromine on the 1-ethyl carbon atom shows markedly less toxicity than does the compound (VI) which has chlorine in this position. However, on the basis of these limited data, no generalizations can be made regarding this phenomenon. Comparison of the toxicities on a molar basis does not change the order of the compounds, even though there is such a wide difference in molecular weights.

A dermal LD_{50} value of about 800 mg. per kg. was found for compound II using male rats. Severe edema was produced in rats at 400 mg. per kg. and above. The lesions healed, however, on surviving rats. Dermal toxicity studies were not made on the other compounds. The toxicity of this same compound (II) seemed to be greater to chickens when given subcutaneously in peanut oil in the abdominal region than would be expected from oral LD_{50} values found for rats. At a dermal dose of 100 mg. per kg. both of two chickens treated died, while at 50 mg. per kg. one of two chickens treated died. At dosages ranging from 200 to 600 mg. per kg., none of six chickens tested survived. No evidence of paralysis, which occurs with some organic phosphorus compounds, was found. Severe lesions occurred at sublethal dosages. Lesions of varying severity occurred in 10 chickens treated with 40 mg. per kg., although all the fowls tested survived until discontinued (29 days). Perforation of the abdominal wall at the place of injection occurred in three chickens at this level. These chickens seemed otherwise unaffected. The severity of these lesions caused by dermal application on rats and subcutaneous injection in chickens seems to be noteworthy, even though the levels of dosage were relatively high. It is possible that some impurities were responsible for this effect. However, the chemical analysis indicates that this material had a purity of at least 95%. Any impurities responsible for these lesions must be very active.

Table I. Methods of Synthesizing Halogenated Ethyl and Vinyl Esters of Dimethyl Phosphate and Values for Chemical Analyses

General formula. $(CH_3O)_2P(O)OR$

	R Groups[1]					
	I $CH{=}CCl_2$	II $CHBrCBrCl_2$	III $CHClCCl_3$	IV $CH{=}CBr_2$	V $CHBrCBr_3$	VI $CHClCBr_2Cl$
	Source					
	Commercial	I + 2 Br	I + 2 Cl	$(CH_3O)_3P$ + Bromal	IV + 2 Br	IV + 2 Cl
Mol. wt.	221.0	380.8	291.9	309.9	469.7	380.8
Ref. index, 25° C.	1.4516	1.5083	1.4714	1.5005	1.5530	1.5180
Carbon, %						
Theory	...	12.6	16.4	15.5	10.2	12.6
Found	...	12.1	15.7	14.6	10.4	12.3
Halogen, %						
Theory	...	60.6	48.6	51.6	68.1	60.6
Found	...	59.6	46.7	51.4	68.0	58.7
Phos., %						
Theory	...	8.14	10.6	10.00	6.60	8.14
Found	...	8.33	11.5	9.28	6.62	8.29

Table II. Acute Oral Toxicity[a] to Rats[b] of Halogenated Ethyl and Vinyl Tertiary Esters of Dimethyl Phosphate

General Formula. $(CH_3O)_2P(O)OR$

Compound	R Groups	LD_{50} Mg./kg.	LD_{50} Confidence (19/20) limits[c]	Relative Toxicity[d]
IV	$CH{=}CBr_2$	253	202–316	1
II	$CHBrCBrCl_2$	250	219–285	1
V	$CHBrCBr_3$	184	156–217	$1^1/_2$
I	$CH{=}CCl_2$	80	62–104	3
VI	$CHClCBr_2Cl$	49	45–54	5
III	$CHClCCl_3$	14.3	11.7–17.4	18

[a] Given in peanut oil.
[b] Sherman strain male rats.
[c] Method of Litchfield and Wilcoxon (*3*).
[d] Based on value of 1 for compounds IV and II.

Fly Toxicity Studies

The toxicity tests toward houseflies (Roberds strain) were done by topical applications of acetone solutions of the compounds. The LD_{50} and LD_{90} values shown in Table III indicate that all the compounds had insecticidal activity great enough to be of interest as insecticides if their other properties warranted such use. The compounds are listed in order of increasing toxicity. The order of toxicities for LD_{50} and LD_{90} values is the same, except for interchanging of the positions of I and V. The relative toxicities of these compounds, based on a value of 1.0 for the least effective (compound III), vary

Table III. Toxicity to Houseflies (Roberds) by Topical Application of Halogenated Ethyl and Vinyl Tertiary Esters of Dimethyl Phosphate

General formula. $(CH_3O)_2P(O)OR$

Compound	R Group	Micrograms per Fly		Relative Toxicity[a]	
		LD_{50}	LD_{90}	LD_{50}	LD_{90}
III	$CHClCCl_3$	0.147	0.325	1.0	1.0
IV	$CH{=}CBr_2$	0.123	0.243	1.2	1.3
I	$CH{=}CCl_2$	0.076	0.100	1.9	3.3
V	$CHBrCBr_3$	0.068	0.163	2.2	2.0
VI	$CHClCBr_2Cl$	0.059	0.093	2.5	3.5
II	$CHBrCBrCl_2$	0.048	0.082	3.0	4.0

[a] Based on value of 1.0 for compound III.

Table IV. Toxicities Toward Houseflies and Rats of Halogenated Ethyl and Vinyl Esters of Dimethyl Phosphate

General formula. $(CH_3O)_2P(O)OR$

Compound	R Group	LD_{50}, Mg./Kg.		LD_{50} Ratio, Rat/Fly
		Rat, oral	Fly[a], topical	
II	$CHBrCBrCl_2$	250	2.7	93
V	$CHBrCBr_3$	184	3.8	48
IV	$CH{=}CBr_2$	253	6.8	37
I	$CH{=}CCl_2$	80	4.2	19
VI	$CHClCBr_2Cl$	49	3.3	15
III	$CHClCCl_3$	14	8.2	2

[a] Average fly weight = 18 mg.

over the range 1 to 3 for LD_{50} values and 1 to 4 for LD_{90} values.

Comparison of Rat and Fly Toxicities

A comparison of the toxicities of these compounds toward rats and flies can be made by comparing the values for the rat-fly LD_{50} ratios (Table IV). The higher the figure the more specificity the compound has for flies and the more favorable should be its use as an insecticide. The compounds are listed in descending order of these ratios. The compounds containing only bromine give more favorable ratios than those having only chlorine, and the mixed bromo-chloro compound having the bromine on the 1-carbon atom of the ethyl group has a better ratio than its bromo-chloro isomer. This order is similar to that found for the relative toxicities toward rats. The variation in ratios showing a gradation of from 2 to 93 represents a 45-fold difference in relative rat-fly toxicities.

One interesting point is the relative toxicity of the mixed bromo-chloro compounds to rats. The two compounds (II and VI) have similar LD_{50} values for flies but differ by a factor of 5 in their LD_{50} values for rats. This large difference in rat toxicity could be due to differences in the rate of absorption of the compounds from the intestinal tract or in the chemical stability of the compounds in the digestive tract, or to differences in specificity of these compounds toward various enzymes found in the rat but not common to the fly. Thus these compounds may be of potential use in comparing the enzyme systems of rats and flies in relation to inhibition by organic phosphorus compounds.

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INSECTICIDE POTENTIATION

Effect of EPN on in Vivo Metabolism of Malathion by the Rat and Dog

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University of Wisconsin, Madison, Wis.

The phenomenon of "potentiation" in organophosphates, where certain combinations of compounds display an unexpectedly high toxicity, was first described in 1957 (*5*) and has been frequently confirmed (*4*). One of the best combinations for potentiation is EPN, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate, with malathion, *S*-[1,2-bis-(ethoxycarbonyl)ethyl] *O,O*-dimethyl phosphorodithioate, and one of the most susceptible animals is the dog.

The low toxicity of malathion to the mammal is due to its extensive degradation by carboxyester hydrolysis (*6*). Cook *et al.* (*3*) showed that EPN in vitro inhibited the degradation of malathion by liver in vitro, and Murphy and DuBois (*4, 7*) found that it inhibited the degradation by liver in vitro of malaoxon (the oxidized form of malathion which is the actual toxicant). One would, therefore, suspect that potentiation results from an increased level of malaoxon at some target site as a result of reduced destruction of malathion and/or malaoxon. However, Seume and O'Brien (*9*) showed for several rat tissues in vitro that EPN inhibited the oxidation as well as the degradation of malathion, so that EPN led to reduced levels of malaoxon in spite of increased levels of malathion. They suggested that EPN produced potentiation by increasing the persistence of malaoxon at some target site, rather than by raising its short-term concentration. In support of this, they showed that with the rat EPN in vivo or in vitro

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The low toxicity of malathion to the mammal is due to its extensive degradation by carboxyester hydrolysis (*6*). Cook *et al.* (*3*) showed that EPN in vitro inhibited the degradation of malathion by liver in vitro, and Murphy and DuBois (*4, 7*) found that it inhibited the degradation by liver in vitro of malaoxon (the oxidized form of malathion which is the actual toxicant). One would, therefore, suspect that potentiation results from an increased level of malaoxon at some target site as a result of reduced destruction of malathion and/or malaoxon. However, Seume and O'Brien (*9*) showed for several rat tissues in vitro that EPN inhibited the oxidation as well as the degradation of malathion, so that EPN led to reduced levels of malaoxon in spite of increased levels of malathion. They suggested that EPN produced potentiation by increasing the persistence of malaoxon at some target site, rather than by raising its short-term concentration. In support of this, they showed that with the rat EPN in vivo or in vitro

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The mechanism by which pretreatment with EPN increases the subsequent toxicity of malathion was investigated. In both the rat and dog EPN resulted in a marked shift in the initial detoxification site of the malathion molecule from the carboxyester to the thiolophosphate bond. The percentage of the administered malathion excreted as metabolites in urine was increased by EPN in the dog but unchanged in the rat. Malathion levels in rat tissues were increased by EPN, whereas malaoxon levels in rat blood were reduced. Potentiation appears to result from an increased persistence rather than an increased concentration of malaoxon in the tissues.

Table I. Compounds and Their Order of Elution from Columns

Name Used in Text	Chemical Name	Formula	Source	Pair of Solvents Which Elutes
A. Celite columns				
Malathion	*S*-(1,2-bisethoxycarbonyl) ethyl *O,O*-Dimethyl phosphorodithioate	$(MeO)_2P(S)SCH(CH_2COOEt)COOEt$	See methods	Iso-octane–methanol
Malaoxon	*O,O*-Dimethyl *S*-(1,2-biscarboethoxy) ethyl phosphorothiolate	$(MeO)_2P(O)SCH(CH_2COOEt)COOEt$	American Cyanamid Co. and G. D. Thorn	Same
B. Ion exchange columns				
Dimethyl phosphate	Dimethyl phosphate	$(MeO)_2POOH$	Victor Chemical Co.	250 ml. 0.01*N* HCl 250 ml. 0.1*N* HCl
Malathion monoacid	*O,O*-Dimethyl *S*-(1-carboethoxy 2-carboxy) ethyl phosphorodithioate	$(MeO)_2P(S)CH(CH_2COOH)COOEt$	American Cyanamid Co.	50 ml. 0.01*N* HCl + 200 ml. 99% MeOH 50 ml. 0.1*N* HCl + 200 ml. 99% MeOH
Malathion diacid	*O,O*-Dimethyl *S*-(1,2-biscarboxy) ethyl phosphorodithioate	$(MeO)_2P(S)CH(CH_2COOH)COOH$	American Cyanamid Co.	Same
Dimethyl phosphorothioate	*O,O*-Dimethyl phosphorothioate	$(MeO)_2POSH$	American Cyanamid Co.	60 ml. 0.1*N* HCl + 190 ml. 99% MeOH 60 ml. 1*N* HCl + 190 ml. 99% MeOH
Unknown A	...	...	...	Same
Unknown B	...	...	...	Same
Desmethyl malathion	*O*-Methyl *O*-hydrogen *S*-(1,2-biscarboethoxy)ethyl phosphorodithioate	$MeOP(S)(HO)SCH(CH_2COOEt)COOEt$	See methods	60 ml. 1*N* HCl + 190 ml. acetone 30 ml. concd. HCl + 30 ml. H_2O + 190 ml. acetone
Dimethyl phosphorodithioate	*O,O*-Dimethyl phosphorodithioate	$(MeO)_2PSSH$	American Cyanamid Co.	Same

produced a shift in the nature of the degradation products of malathion in vitro, from primarily products of carboxyester hydrolysis to primarily products of phosphorothiolester cleavage. This confirmed the view of Cook *et al.* (*3*) that the EPN acted by inhibition of a carboxyesterase.

In the present study the EPN-malathion metabolic relationship has been examined in vivo. From the foregoing studies one would anticipate that EPN would produce a reduction in total excretion of malathion metabolites, a shift in the nature of the metabolites from primarily products of carboxyester cleavage to primarily products of phosphorothiolester cleavage, and an increase in malathion levels and malaoxon persistence in various tissues. These effects should perhaps be more pronounced in the dog than the rat, because the dog is more susceptible to potentiation.

Methods and Materials

Synthesis. Radioactive malathion was prepared as described by Krueger and O'Brien (*6*). The yield was 50% and the specific activity was about 15 mc. per gram.

Desmethyl malathion (Table I) was synthesized by a procedure suggested by R. W. Young, American Cyanamid Co. Equimolar malathion and potassium *O,O*-dimethyl phosphorodithioate were refluxed in acetone for 4 hours. The acetone was evaporated off and the resulting mixture was partitioned between water and chloroform. The water phase was chromatographed on the ion exchange column (see below) and gave two peaks, one which cochromatographed with known dimethyl phosphorodithioate and the other which was assumed to be desmethyl malathion. This identification is only tentative, because difficulties in extraction and purification have so far prevented the isolation of the pure compound.

Treatment and Handling of Animals. Radioactive malathion and nonradioactive EPN were administered intraperitoneally in 10% ethanol and 90% propylene glycol. Usually with the rats, 10 mg. of EPN was dissolved in 4 ml. of the ethanol-glycol mixture and 50 mg. of EPN was dissolved in 6 ml. of the ethanol-glycol mixture. Doses and EPN-malathion intervals are de-

scribed in the "Results" section. Thirty-four 150-gram male rats (Holtzmann Co., Madison, Wis.) were treated and held individually in metabolism cages, allowing for the separate collection of urine and feces during a 48-hour period. Feces were discarded and urine samples were collected periodically. Sample volumes and radioactivity were determined and then the samples were quick-frozen and stored for subsequent metabolite analysis.

The two adult female mongrel dogs used had been handled and trained for use in physiological experiments. The dogs were held separately in metabolism cages for the 72-hour duration of the experiment. They were fasted 12 hours prior to and 12 hours after malathion administration. The doses were administered intraperitoneally in 5 ml. of 10% ethanol and 90% propylene glycol. Ten-milliliter blood samples were drawn periodically from the femoral vein by syringe using heparin as an anticoagulant. Urine samples were taken by catheter to ensure total samples up to 72 hours.

Cholinesterase Determinations. For rat brain studies, rats were treated with nonradioactive malathion and EPN according to the schedule given in Table II. The brains were removed, homogenized in cold 0.9% sodium chloride, and diluted with saline to a final concentration of 0.75 mg. per ml. Warburg flasks were prepared containing 1.25 ml. of the homogenate, 1 ml. of 0.033*M* phosphate buffer at pH 7.2, and 0.25 ml. of 1.25% sodium bicarbonate, while 0.2 ml. of 2% acetylcholine bromide was placed in the side arm. Manometric assay was made at 38° C. under 5% carbon dioxide for 30 minutes.

For dog blood studies, the blood was cooled in ice immediately and centrifuged to remove plasma, and the erythrocytes were washed twice with cold 0.9% sodium chloride. The original blood volume was restored by using distilled water. Warburg flasks were prepared containing 1 ml. of hemolyzed cells, 1 ml. of 0.033*M* phosphate buffer at pH 7.2, and 0.5 ml. of 1.0% sodium bicarbonate, while 0.2 ml. of 3% acetyl-β-methylcholine was placed in the side arm. Cholinesterase activity was measured manometrically at 38° C. under 5% carbon dioxide in nitrogen for 30 minutes.

Extraction of Metabolites. For studies on the whole rat, the animals were decapitated and each was homogenized separately in 3 liters of chloroform with a 4-quart Waring Blendor. After blending 5 minutes, dry ice was added to the brei to freeze the protein out of suspension. This allowed the chloroform to be filtered off readily. The chloroform extracts were concentrated by evaporation and extracted with water adjusted to pH 7 with sodium bicarbonate. The protein fraction was blended with 400 ml. of 0.001*N* hydrochloric acid and filtered, and a fraction of the filtrate was readjusted to pH 7 and extracted with chloroform. The activity of the two aqueous fractions was summed to give chloroform "inextractables," and of the two chloroform fractions to give "extractables."

Blood was collected from decapitated rats in iced vessels using sodium citrate as an anticoagulant. The dog blood used was a portion of that taken for cholinesterase assays. The whole blood was extracted three times with equal volumes of chloroform. Fifteen milliliters of water and of chloroform were added, and the mixture was shaken and centrifuged at 2000 r.p.m. The chloroform fractions were combined, as were the water fractions, and counted. In a separate experiment it was shown that none of the radioactivity in the chloroform was removed by extraction with water.

Urine Analysis. The hydrolysis products of malathion in urine were separated by ion exchange columns modified from those described by Plapp and Casida (*8*). The pairs of solvents used are given in Table I. Five to 10 ml. of urine adjusted to pH 1 and containing dissolved known compounds for cochromatography were used on a 2.5 × 20 cm. column, with a flow rate of 4 ml. per minute. Fractions of 12 ml. each were collected and analyzed for total radioactivity and for phosphorus according to the method of Allen (*1*).

Blood Analysis. Partition chromatography with 1.25 × 20 cm. Celite columns (*2*) was used to separate malathion and malaoxon derived from blood. The chloroform extracts from blood were evaporated to dryness and dissolved in the iso-octane–methanol mixture, added to the column, and eluted without pressure. Twenty 5-ml. fractions were collected. Identification was based on cochromatography with known compounds.

Table II. Per Cent Cholinesterase Inhibition of Rat Brain in Vivo

	Time of Sacrifice,[a] Hours		
	0.5	1.0	4.0
a. EPN	36.5	6.5	8.5
b. Malathion	10.0	22.0	6.0
c. EPN + malathion	68.0	17.0	15.0

[a] Zero time taken as time of injection of malathion (b and c) or 2 hours after injection of EPN (a). In (c), EPN-malathion interval was 2 hours. Ten rats as controls, 3 rats for each treatment.

Table III. Effect of EPN on Nature of Metabolites in Whole Rat and in Rat Blood

	% as Chloroform-Extractables[a]	% of Chloroform-Extractable[b] as Malaoxon
Whole rat		
No EPN	18	...
With EPN	49	...
Blood		
No EPN	0.52	16.4
With EPN	1.76	0.0

EPN-malathion interval 3 hours. Animals killed 0.5 hour after malathion. Results for blood and for whole rat are from different animals. Results means of 3 (whole rats) or 2 samples of blood each from 2 rats.

[a] Remainder as chloroform-inextractables.

[b] Remainder as malathion.

Results

The Rat. Doses of 5 mg. of EPN per kg. plus 25 mg. of malathion per kg. were lethal to rats when the EPN-malathion interval was 1 hour. Doses of 2.5 mg. of EPN per kg. followed by 25 mg. of malathion per kg. were not lethal to the dog or rat and were selected for use throughout. On varying the interval between administration of 2.5 mg. of EPN per kg. and 25 mg. of malathion per kg. to rats it was found that symptoms of organophosphate poisoning (lachrymation, fasciculation, salivation) were evident with 0.5- to 2-hour intervals but not thereafter, nor were there symptoms in control rats which received either malathion or EPN alone.

Cholinesterase Inhibition. Table II shows that with an EPN-malathion interval of 2 hours, EPN potentiated the effect of malathion against brain cholinesterase 0.5 hour after the malathion; at later times no potentiation of inhibition was observed.

Tissue Metabolites. The levels of certain groups of metabolites in the whole rat and in rat blood were studied. The chloroform-extractable fraction containing malathion plus malaoxon was further analyzed in the case of blood, but not with the whole rat because this fraction contained too much fat for successful chromatography.

Table III shows that EPN raised the proportion of chloroform-extractables in the whole rat and in blood about threefold. However, malaoxon was detected in blood in the control animals but not in those treated with EPN. The total amount of metabolites in blood was about 3.3 p.p.m. (average of all rats).

Urinary Excretion. The rate of excretion of malathion metabolites was studied for various EPN-malathion intervals. Figure 1 shows that the curve for rats with malathion only does not differ greatly from that in which the rats were pretreated 2 hours prior with EPN. Full data were also obtained for other intervals, with substantially similar results. Thus for EPN-malathion inter-

Table IV. Identity of Malathion Metabolites in Rat Urine

	Compound	Control	EPN-Malathion Interval, Hours 0.5	2	3
1.	Dimethyl phosphate	6	6	5	8
2.	Malathion monoacid	12	36	26	14
3.	Malathion diacid	48	14	31	14
4.	Dimethyl phosphorothioate	10	16	13	21
5.	Unknown A	3	2	2	4
6.	Unknown B	0	4	1	1
7.	Desmethyl malathion	11	8	9	14
8.	Dimethyl phosphorodithioate	4	8	6	8
9.	Total products of carboxyester cleavage	59	50	57	28
10.	Total products of phosphorothiolester cleavage	24	37	33	51
11.	Total unknowns	3	5	3	5

Results expressed as percentages of total radioactivity recovered from column, including that which did not form peaks (8 to 14% of total). "Products of carboxyester cleavage" = 2 + 3. "Products of phosphorothiolester cleavage" = 1 + 4 + 7 + 8. Urine collected between 0 and 48 hours.

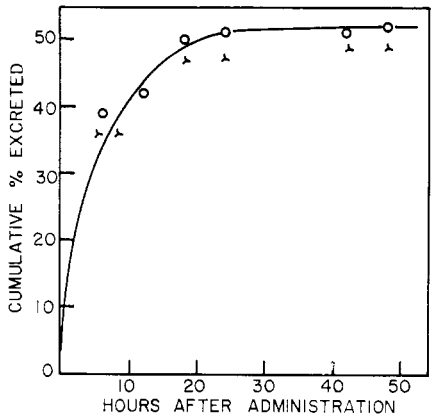

Figure 1. Urinary excretion of malathion metabolites by the rat

O Average for 6 rats administered malathion intraperitoneally at 25 mg. per kg.
Average for 4 rats administered EPN + malathion intraperitoneally at 2.5 and 25 mg. per kg., respectively. EPN administered 2 hours prior to malathion

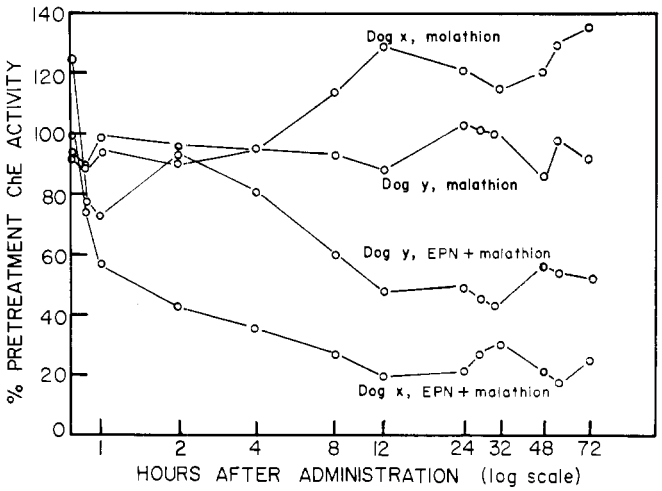

Figure 2. Effect of malathion and EPN plus malathion on dog erythrocyte cholinesterase in vivo

Time in hours after malathion administration
EPN at 2.5 mg. per kg. administered intraperitoneally 3 hours prior to malathion. In both cases malathion administered intraperitoneally at 25 mg. per kg.

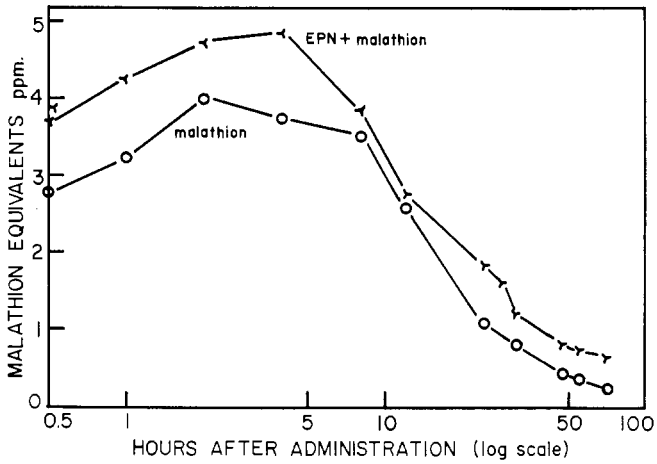

Figure 3. Total malathion equivalents in whole blood of dog X

For conditions see Figure 2

vals of 0.5, 1, 2, 3, 4, and 5 hours, the average cumulative per cent excretion 5 to 6 hours after the malathion was administered was 39, 36, 41, 41, 38, and 41 compared with the control value for malathion alone of 39%.

Ion exchange columns were used to identify and measure the proportion of urinary metabolites for various EPN-malathion intervals of from 0.5 to 7 hours. A part of these data is shown in Table IV, for urine accumulated from 0 to 48 hours. The most important observation was that the malathion diacid content was greatly reduced by EPN, while the various products from phosphorothiolate cleavage were correspondingly increased. Most of this increase was due to *O,O*-dimethyl phosphorothioate and dimethyl phosphate. This shift from products of carboxyesterase hydrolysis increased with increasing EPN-malathion interval up to 3 hours. After this time a steady return to normal was found. Thus in the urine accumulated from 0 to 12 hours (not shown in the table) the percentage as products of carboxyesterase hydrolysis increased, the figures for EPN-malathion intervals of 3, 4, 5, 6, and 7 hours being 26, 37, 40, 46, and 46%; the percentage as products of thiolophosphate hydrolysis decreased, the values being 59, 39, 42, 34, and 29.

The Dog. From the results of the rat studies a dose of 2.5 mg. of EPN per kg. and 25 mg. of malathion per kg. and EPN-malathion interval of 3 hours were selected for the dog. Two adult female mongrel dogs weighing 21 and 18 kg. were used (dogs X and Y). Dog X was treated first with malathion alone and then in a subsequent experiment with EPN plus malathion, while dog Y was treated with EPN plus malathion and later with malathion alone. This experimental design reduced the biological variation inherent in metabolism studies. The time interval between experiments was approximately 3 weeks, at which time no further radioactivity was being excreted from the first experiment. In both cases malathion alone gave no symptoms, but the combination caused central nervous system depression, and a reduction of heart rate—e.g., for dog X from 80 per minute to 62 at 15 minutes, recovering thereafter to 72 per minute at 75 minutes.

Cholinesterase Inhibition. Figure 2 shows that malathion alone had no inhibitory effect upon erythrocyte cholinesterase in vivo, while the EPN plus malathion gave pronounced inhibition.

Blood Metabolites. Figure 3 shows for one dog that EPN caused only a small increase in the total blood radioactivity; in the other dog a similar picture was seen, except that at 0.5 hour the malathion equivalents were raised more by EPN and after 2 hours the curves for the second dog were almost identical with and without EPN.

Table V. Identity of Malathion Metabolites in Dog Urine

Collection Interval	0–2 Hr.		4–8 Hr.		8–12 Hr.		12–24 Hr.		32–48 Hr.		56–72 Hr.	
	Control	EPN	Control	EPN	Control	EPN	Control	EPN	Control	EPN	Control	EPN
Dimethyl phosphate	1	8	1	8	1	7	1	9	1	5	0	2
Malathion monoacid	56	23	43	14	39	14	28	8	33	12	40	15
Malathion diacid	11	2	18	5	22	4	35	3	20	6	17	8
Dimethyl phosphorothioate	5	27	7	27	6	25	9	40	10	38	9	36
Unknown A	0	5	2	5	0	4	1	4	0	0	0	0
Unknown B	0	9	0	8	0	11	2	9	0	0	0	2
Desmethyl malathion	19	19	22	17	22	21	22	15	22	16	20	19
Dimethyl phosphorodithioate	5	4	5	4	6	5	10	4	10	5	8	6
Total products of carboxyester cleavage	67	25	61	19	61	18	63	11	53	18	57	23
Total products of phosphorothiolester cleavage	30	58	35	56	35	58	42	68	43	64	37	63
Total unknowns	0	14	2	13	0	15	3	13	0	0	0	2

Calculations as in Table IV.
Results average of two dogs.
Results expressed as percentages of total radioactivity recovered from column.

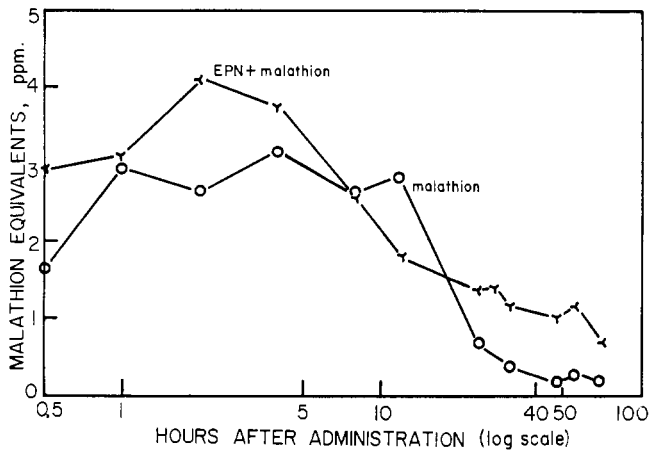

Figure 4. Total chloroform-inextractables in blood of dog X

For conditions see Figure 2

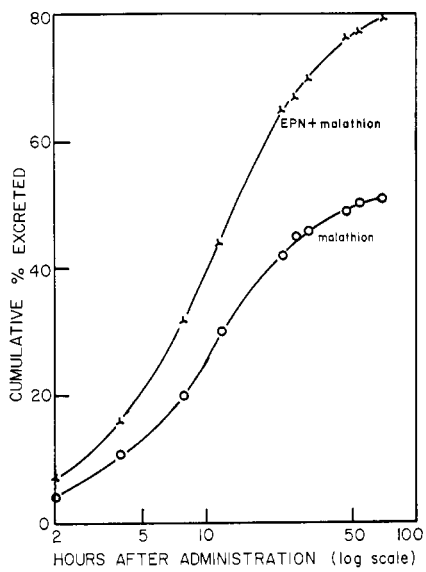

Figure 5. Urinary excretion of malathion metabolites by dog X

For conditions see Figure 2

Figure 4 shows that the chloroform-inextractables were very similar with and without EPN for dog X. In dog Y a comparable picture was found. The levels of chloroform-extractable metabolites were also studied, but were erratic and EPN had apparently opposite effects in the two dogs, raising the level in one, lowering it in the other. The levels varied between 0 and 2.2 p.p.m. expressed as malathion.

URINARY EXCRETION. Contrary to expectation, EPN increased the over-all urinary excretion of malathion metabolites. Figure 5 shows results for dog X. With dog Y the excretion of malathion metabolites was the same with or without EPN up to 10 hours after administration, at which time a difference occurred which yielded final excretion figures after 72 hours of 51% without EPN and 65% with EPN.

The nature of the urinary metabolites was studied for six collection periods between 0 and 72 hours (this procedure, which was more precise than for the rat, was made possible by catheterization and by the larger volumes of urine involved). The results for the two dogs were remarkably similar, seldom differing by more than 5%. Table V shows the averages for the two dogs. For any given treatment, the variation of metabolites with time was fairly small: In the controls, the main change was an increase in malathion diacid at the expense of malathion monoacid; with EPN treatment, the dimethyl phosphorothioate content increased with time. The difference between the control and EPN-treated animals was consistent at all times, in that products of carboxyesterase hydrolysis were severely reduced from about 60 to about 20%; malathion monoacid and malathion diacid were involved about equally in this reduction. There was a marked increase in products of phosphorothiolester cleavage caused by EPN, due almost entirely to extra dimethyl phosphorothioate.

Discussion

Of the anticipated results, the only unequivocal effect in both animals was a marked shift induced by EPN in the nature of the urinary metabolites, reflecting a change from the usual detoxification site, the carboxyester group, to the phosphorothiolate group.

A reversal of expectation was the finding that urinary excretion of malathion products was hardly affected by EPN in the rat, and was substantially increased by EPN in the dog. Because in the whole rat and in rat blood the amount of chloroform-inextractables—i.e., ionic compounds derived from carboxyester and phosphorothiolester cleavage, which should be readily excreted—was greatly reduced by EPN (Table III), it must follow that in the rat the products of phosphorothiolester cleavage are more readily excreted than the products of carboxyester cleavage. A similar conclusion is reached for the dog, in which the chloroform-inextractables of blood were little increased by EPN, yet their urinary output was considerably increased. The phenomenon may be connected with the fact that the carboxyacids are weaker acids than the phosphates, or

possibly with the larger molecular size of the carboxyacids—e.g., malathion monoacid, mol. wt. 302, compared with dimethyl phosphorothioate, mol. wt. 142.

It had been hoped to study fecal excretion in the dog, but the animals had to be fasted and were also disturbed by the restriction of the metabolism cage; thus dog X did not defecate during the whole of the control period.

In the rat, the fraction of chloroform-extractables (malathion plus malaoxon) was greatly increased in blood and in the whole animal; in the blood the malaoxon level was reduced. These observations parallel those of Seume and O'Brien for rat tissues in vitro. In the dog, the chloroform-extractable picture in blood was obscure: No conclusions could be drawn. The levels of total radioactivity of chloroform-inextractable metabolites in the blood were raised somewhat at first, but these effects had disappeared 10 hours after treatment.

These results do not permit a final verdict on the ultimate mechanism of potentiation. It appears certain that in vivo, as in vitro, carboxyesterase(s) is (are) inhibited by EPN. That this inhibition gives rise to an increase in malathion level has been shown only in the rat (for blood and whole animal). The rat results confirm the hypothesis of Seume and O'Brien (*9*) that there is an actual reduction of the concentration of malaoxon at the hypothetical target site and not an increased level, unless measurements on blood and the whole body are a very inaccurate reflection of events at the target site. If this is so, the effectiveness of EPN-potentiated malathion must be caused by an increased persistence of malaoxon at the target site, rather than an increased concentration. A final conclusion can be based only upon a more precise study of potentiation in vivo.

Acknowledgment

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INSECTICIDE DECOMPOSITION

Clay-Catalyzed Decomposition of Insecticides

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LARGE QUANTITIES OF INSECTICIDES are formulated in the form of dusts or wettable powders in which the toxicant is mixed with a finely ground, natural clay which dilutes the insecticide to concentrations suitable for agricultural use, and imparts the desirable properties of flowability and dispersibility to the dust. Various mineral clays (attapulgite, kaolinite, montmorillonite, etc.) are used as supposedly chemically inert diluents. Prior to these studies, it had been recognized that clay-diluted chlorinated insecticides occasionally decompose, sometimes becoming sufficiently hot to ignite spontaneously. Investigations (*3*) showed that the decomposition was promoted by the acid sites of the clay and that appropriate neutralization could reduce decomposition to negligible rates. Since the introduction and widespread use of clay-deactivators (*3, 5*) this problem of decomposition has virtually disappeared.

Early studies of the decomposition of dieldrin in solution showed that the reaction is catalyzed by mineral acids or even wet ferric chloride. Active clays were examined with indicator dyes and found to be surprisingly strong acids, especially when dry. Acidic diluents are shown to catalyze the decomposition of DDT, aldrin, toxaphene, chlordan, dieldrin, heptachlor, Aramite, and endrin. Although these compounds have the carbon-chlorine bond in common, the decomposition reactions do not appear to involve only this bond. With the epoxidized insecticides (dieldrin and endrin), the epoxy group disappears and the formation of carbonyl groups is indicated by infrared studies. With endrin, the main product of decomposition has been identified as a ketone of unchanged molecular weight. A wide variety of diluents has been investigated but most of the work has been done with Velvex (a kaolinite clay) or Attaclay (an attapulgite clay).

Two experimental innovations were used extensively: indicator dyes to

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Two experimental innovations were used extensively: indicator dyes to

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Decomposition of insecticides occurs in wettable powders and dusts as a result of the catalytic action of the clay diluent. The heat evolved is occasionally sufficient to cause spontaneous ignition. Rates of reaction of these insecticides in the presence of various clay diluents increase with increasing acidity of the clay surface. Treatment of the clays with volatile organic bases or basic-acting substances, such as urea or hexamethylenetetramine, reduces the acid strength and diminishes the rate of decomposition accordingly. Sodium clays are usually sufficiently neutralized with one molecule of the appropriate base for each sodium ion. Reaction rates were determined from data obtained by infrared analysis or bioassay of samples from isothermal storage, and by differential thermal analysis. With 25% concentrates the reactions were zero order, and half times of reaction decreased upon dilution with clay reaching a minimum at two molecules of insecticide per exchange ion.

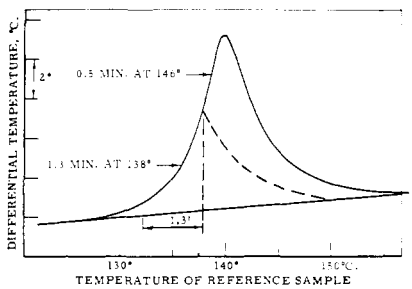

Figure 1. Exothermic peak obtained in differential thermal analysis of 25% dieldrin-Attaclay mixture

predict catalytic activity of acidic solids, and differential thermal analysis to measure reaction rates.

Experimental Methods

Acid Strength from Indicator Color Changes. The acid strength of a clay surface can be estimated with the method suggested by Walling (*10*) and extended by Benesi (*1*). This method consists of contacting separate samples of the clay under study with each member of a set of appropriate indicators (herein called Hammett indicators), and noting the resulting colors of the adsorbed indicators. If one indicator gives its acid color and a second its basic color when adsorbed on a given clay, the acid strength of that clay must lie between those of the two indicator acids.

The Hammett indicators used to estimate acid strengths of clay surfaces are listed in Table I. This class of indicators is used to estimate acid strengths of solid surfaces in terms of Hammett and Deyrup's H_0 scale (*4*), which can be considered an extension of the pH scale into media where hydrogen ion activity is no longer equivalent to its stoichiometric concentration.

Amine Titration. The method used to titrate clay surfaces is that developed by Benesi (*2*), who extended the method first employed by Johnson (*6*). It consists of measuring the amount of *n*-butylamine needed to neutralize the clay surface to each of the Hammett indicators in an inert solvent such as benzene. Clay surfaces are not uniformly acidic but, instead, give amine titers that continuously decrease with decreasing basicity of the Hammett indicators.

Table I. Properties of Hammett Indicators

Indicator	pK_a	Basic Color	Acid Color
Phenylazonaphthylamine	+4.0	Yellow	Red
Butter yellow	+3.3	Yellow	Red
Benzeneazodiphenylamine	+1.5	Yellow	Purple
Dicinnamalacetone	−3.0	Yellow	Red
Benzalacetophenone	−5.6	Colorless	Yellow
Anthraquinone	−8.2	Colorless	Yellow

Table II. Properties of Insecticide Diluents

Diluent		Surface Area, Sq. M./G.	% Loss on Drying[a]	pH in Water[b]	H_0	
Name	Type				As received[c]	Dried[a,c]
Velvex	Kaolinite	24	1.0	4.6	−3 to −5.6	−5.6 to −8
Barden	Kaolinite	...	...	...	−3 to −5.6	...
Pikes Peak	Montmorillinoid group	112	4.3	5.1	−3 to −5.6	−3 to −5.6
Rev dust	...	...	...	...	−3 to −5.6	...
Diluex	Attapulgite	125	7.0	9.5	+1.5 to −3	−5.6 (tr)
Attaclay	Attapulgite	126	5.9	7.2	+1.5 to −3	−5.6 (tr)
Celite 209	Diatomite	26	4.9	5.2	+1.5 to −3	+1.5 to −3
Celite 400	Diatomite	26	4.1	6.0	+1.5 (tr)	+1.5 (tr)
Pyrax ABB	Pyrophyllite	3	0.1	6.2	+1.5 (tr)	+1.5 (tr)
Talc	Talc	...	7.1	...	+3.3 (tr)	...
Hi-Sil 101	Silica	100	...	9.4	+3.3 (tr)	+3.3 to +1.5
CCC ($CaCO_3$)	Calcite	...	...	...	+3.3 (tr)	...

[a] Dried at 120° C. for 16 hours.
[b] pH of a 1% water suspension.
[c] tr represents transition color.

Reaction Rates by Differential Thermal Analysis. It was found that reaction rates could be measured rapidly and accurately by differential thermal analysis (DTA). The DTA apparatus used for most of the work consisted of the following principal parts: a nickel calorimeter block heated by a vertical, symmetrically wound furnace; a heating program control unit; a Photopen linear chart recorder used in conjunction with a galvanometer, connected in series with the platinum *vs.* platinum-rhodium differential thermocouple to record differential temperatures; and a Brown Electronik recorder connected to an iron *vs.* constantan thermocouple to record the block temperature. The sample (containing the insecticide) of about 1 gram was pressed firmly into a hole in the nickel block and a reference sample of the diluent under study was pressed into another hole symmetrically located in the block. Junctions of the differential thermocouple were inserted into the center of each sample and the block was sealed with a metal cap. The nickel block was inserted into the furnace and heated at a constant heating rate—about 4° C. per minute for most studies. The differential thermocouple junctions were opposed in series to give a chart reading of differential temperature. If heat were given off or taken up by decomposition of the insecticide, it was registered as a deflection of the pen.

The interpretation of the DTA chart can lead to determination of the heat,

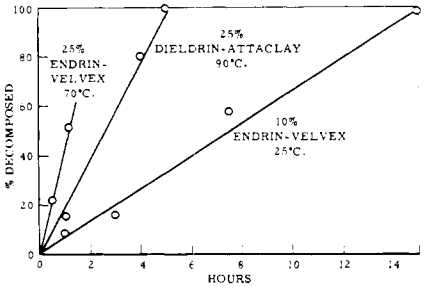

Figure 2. Time dependence of decomposition of dieldrin or endrin, showing zero-order characteristics

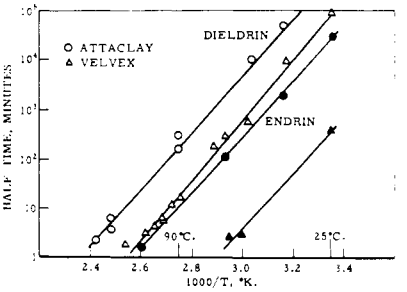

Figure 3. Temperature dependence of half time of decomposition of 25% dieldrin or endrin in mixtures

rate, and order of reaction (7). A typical DTA chart is reproduced in exact shape in Figure 1, and illustrates the determination of half times of decomposition. The heat of reaction, proportional to the area under the curve, was determined by calibration with clay-naphthalene mixture, using the known heat of fusion of naphthalene (36 cal. per gram). The inflection point of the heating curve occurs near the maximum and the cooling curve is the relaxation curve for the hot sample (as obtained with naphthalene-clay mixtures). By reference to Kissinger (7), it can be seen that these features distinguish a zero-order reaction. A slight rounding of the very top of the peak occurs in Figure 1 because in that particular sample, as is explained later, the order is zero for the first 80% of the reaction and increases during the last 20%. The half time of reaction was determined graphically as indicated in Figure 1.

Because most reactions were zero order, the top of the heating curve represents the end of the exothermic reaction. At higher temperatures the curve is purely a cooling curve and its shape is determined by the heat transfer coefficient from the sample to the block. The half time of reaction was obtained by shifting the cooling curve until it bisected the area (dashed curve). The time from this intersection to the peak (or to the start of the temperature rise) is the half time of reaction, and the temperature of reaction is the average temperature over the period (average temperature of the reference sample plus the differential temperature).

Isothermal Decomposition Rates. To obtain temperature coefficients for predicting storage stabilities, it was necessary to measure reaction rates at lower temperatures. Small sealed samples were stored at various controlled temperatures for periods of weeks or months and the rate of decomposition was determined by infrared analysis (5) of the toxicants after chromatographic extraction with solvents. In studies with Aramite, toxicant concentrations were determined by bioassay with the two-spotted spider-mite, *Tetranychus telarius* (L.), using Aramite as the standard.

Materials

Unless otherwise specified, the toxicants used in this study were commercial grade materials and are referred to by common or trade names. Some measurements were made with the recrystallized active compounds of aldrin, dieldrin, and endrin. The composition and purity of these samples were as follows: recrystallized aldrin (99.3%) containing 99.3% of 1,2,3,4,10,10-hexachloro-1,4,4*a*,5,8,8*a* - hexahydro - 1,4 - *endo*, *exo*-5,8 - dimethanonaphthalene (abbreviated HHDN); recrystallized dieldrin (99.9%) containing 99.9% of 1,2,3,4,-10,10 - hexachloro - 6,7-epoxy - 1,4,4*a*,-5,6,7,8,8*a* - octahydro - 1,4 - *endo*,*exo* - 5,8-dimethanonaphthalene (abbreviated HEOD); recrystallized endrin (98.3%) containing 98.3% of 1,2,3,4,10,10-hexachloro-6,7,-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydro-1,4-*endo*,*endo*-5,8-dimethanonaphthalene.

The mineral diluents (mostly clays) are also referred to by common or trade name. Their descriptions, together with a list of properties which include surface areas and surface acidities, are given in Table II.

The H_0 values listed (Table II) give upper and lower limits of the H_0 of the strongest acid sites on the clay surface. As examples, a clay having an H_0 between +1.5 and −3.0 gives a purple color with benzeneazodiphenylamine and a yellow color with dicinnamalacetone; an H_0 of +1.5 (tr) not only means that benzeneazodiphenylamine gives a transition color (brown or orange), but that butter yellow gives a red color and dicinnamalacetone a yellow color.

It is interesting to note that there is poor correlation between H_0 values and the pH values listed in Table II. For example, Diluex gives an alkaline reaction in water even though it is strongly acid to Hammett indicators and, as will be shown later, is highly active for insecticide decomposition. There is an obvious explanation for the above discrepancy. The H_0 values are a measure of the strongest acid sites on the clay surface. However, the pH value of a clay-water suspension is the result of hydrolyses of all portions of the clay surface. In the case of a clay like Diluex (which contains 7% magnesium oxide) one can visualize a process whereby alkaline portions of the surface produce more than enough OH^- to neutralize the H^+ originating from the acidic portions of the surface.

Kinetics of Dieldrin and Endrin Decomposition

Clay-toxicant mixtures containing 25% of dieldrin or endrin were used for the detailed kinetic studies, and most of these measurements were made with Attaclay (an attapulgite clay) or with Velvex (a kaolinite clay).

Heats of Decomposition. With DTA measurements, the heat of decomposition of the 25% concentrates was about 33 cal. per gram for either technical dieldrin or endrin on Attaclay or Velvex. The heats of decomposition of 25% concentrates of recrystallized dieldrin (HEOD) and recrystallized endrin were about 36 cal. per gram which corresponds to 144 cal. per gram of toxicant or 55 kcal. per mole.

Order of Reaction. The order of reaction is best illustrated with measurements of decomposition during isothermal storage (Figure 2). Here it is shown that dieldrin or endrin, when mixed with various diluents, decomposes at a constant rate independent of the concentration of undecomposed material. This is a zero-order reaction which indicates that the reaction occurs on sites that are completely covered by reacting molecules.

The reaction mechanism probably involves vaporization of the solid toxicant, diffusion through air spaces to the clay particle, diffusion through pores within the clay particle, adsorption, reaction to produce less strongly adsorbed products, desorption, and diffusion of the products away from the clay surface. Because the vapor pressure of toxicants at the temperature of the reaction is very low (10^{-3} mm. of mercury for endrin at 25° C.), it seemed possible that diffusion might limit the above process. However, a calculation of diffusion rates (11) indicated that even for the most active system (endrin on Velvex) the time of diffusion is negligible compared to the time of reaction.

The number, n, of molecules of toxicant per acid site is given by

$$n = \frac{10^6 W}{MS(100 - W)}$$

where W is the weight % of toxicant; S, the micromoles of acid sites per gram of clay; and M, the molecular weight of the toxicant. For a 10% endrin-Velvex mixture, $n = 11$. Thus, for a reaction time of 840 minutes, the average residence time on the acid sites was about 75 minutes.

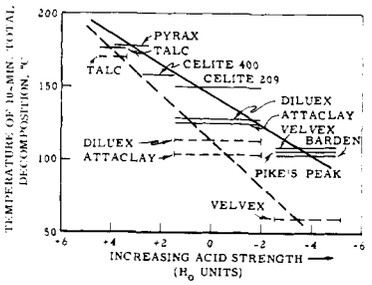

Figure 4. Effect of acid strength of carriers on stability of 25% endrin or dieldrin dust concentrates

– – – and carrier names on left for endrin
—— and carrier names on right for dieldrin

Table III. Reaction Rates of Dieldrin with Pure Kaolinites

Cation	Cation Concn., μMoles/G.	Total Acidity,[a] μMoles/G.	H_0	$t_{1/2}$ by DTA Min.	$t_{1/2}$ by DTA ° C.	$t_{1/2}$ at 65° C., Min.
Natural	...	25	−3 to −5.6	3	110	400
H^+	...	24–31	−5.6 to −8	2	90	30
NH_4^+	24	...	−3 to −5.6	3	102	150
Na^+	26	19–22	−3 to −5.6	2	114	400
Ca^{+2}	15	10–20	+1.5 to −3	1.5	115	330
Al^{+3}	8	10–12	−5.6 to −8	3	87	30

[a] By butylamine titration.

Effect of Temperature. Rates of decomposition of 25% dieldrin-Velvex, dieldrin-Attaclay, endrin-Velvex, and endrin-Attaclay mixtures were determined over a wide range of temperatures by isothermal storage, and also at more elevated temperatures by DTA measurements. These have been correlated by the Arrhenius relation:

$$k = \frac{1}{2t_{1/2}} = Ae^{-E/RT}$$

where $t_{1/2}$ is the half time of the reaction; A, the frequency factor; and E, the activation energy. This relation is illustrated in Figure 3 in which log $t_{1/2}$ is plotted as a function of $1/T$. The slope is $E/2.303R$; A can be calculated from the temperature of the intercept ($\log t_{1/2} = 0$).

The results shown in Figure 3 cover an unusually wide range of reaction rates, from half times of 1 to 100,000 minutes, yet the straight lines of the Arrhenius relation fit the data very well. The frequency factor, A, appears to depend mainly on the clay. With endrin and dieldrin on Attaclay A is $10^{11.1}$ and $10^{11.4}$, respectively, while on Velvex A is $10^{13.1}$ and $10^{13.4}$, respectively.

The diagrams allow prediction of intermediate rates and temperatures, and illustrate the use of DTA measurements to predict storage stabilities for any system where A is known. The values of A for Attaclay and Velvex are nearly the same for endrin and dieldrin; if they are the same for these clays with other toxicants, then the rate of decomposition at any temperature can be determined by a single DTA measurement.

Effect of Acidity of Diluent. Dieldrin and endrin were mixed with a number of different diluents covering a wide range of acidity and each mixture was subjected to DTA measurements. The results are shown in Figure 4 where the temperature causing a half-time decomposition of 5 minutes is shown as a function of the H_0 of the diluent. A range of H_0 values is shown for each diluent; this is the best estimate (based on amine titration data) of the actual H_0 values of the stronger acid sites of the diluents as used in these experiments. This figure illustrates the marked effect of the acidity of the diluent on the rate of decomposition. Higher temperature for a 10-minute decomposition denotes greater storage stability. The effect of the H_0 of the diluent on half time may be expressed by $d \log t_{1/2}/dH_0 = 0.3$ to 0.4 for dieldrin and $d \log t_{1/2}/dH_0 = 0.4$ to 0.5 for endrin.

Effect of Exchangeable Cations. Many natural clays have about 1 μmole of cation exchange site for every square meter of surface. Kaolinites with 25 square meters per gram of surface have 25 μmoles of exchangeable cations per gram of clay, and attapulgites with 125 square meters per gram of surface have 125 μmoles per gram. Most natural clays used for agricultural diluents have sodium ions in the exchange sites. Although hydrogen ions can be put into the exchange sites, hydrogen clays are not stable and are not found to any extent in nature.

The effect of different cations on the H_0 value and catalytic activity of kaolinites was determined with samples of Velvex prepared in several pure ionic forms (*8*): H^+, NH_4^+, Na^+, Ca^{+2}, and Al^{+3} (Table III). Natural clay and sodium kaolinite appear identical in H_0 values and catalytic activity. The hydrogen kaolinite and the aluminum kaolinite are more strongly acidic and are much more active catalysts, promoting tenfold faster rates of reaction.

Catalytic Activity of Neutralized Clays

Because dieldrin and endrin formulated with neutral diluents, such as talc or calcium carbonate ($H_0 \geqq +3.3$), decompose much more slowly than when formulated with acidic diluents, such as kaolinite ($H_0 \leqslant -5.60$) or attapulgite ($H_0 \leqslant -3$), it was of interest to compare the catalytic activity of talc with kaolinite neutralized to $H_0 = +3.3$. The catalytic activities were found to be approximately the same. A wide variety of bases can be used to neutralize the acidity of clays, but the maximum H_0 attainable and the amount of base needed to attain any given H_0 value differ with each base and with each diluent.

Population of Acid Sites in Various Diluents. By means of the previously described titration of acid sites with butylamine in benzene (amine titer), the number of acid sites per gram of clay has been determined for Velvex, Diluex, Attaclay, and Wyoming Bentonite (Figure 5). The amine titer at any point on these curves represents the number of acid sites, expressed in millimoles per gram of clay, having a strength equal to or greater than the H_0 value at that point. The length of the vertical lines indicates the experimental uncertainty of the titration measurement. The total number of acid sites per unit surface of each of these clays is about 1 μmole per square meter, the exchange capacity.

Kinetics of Decomposition on Neutralized Clays. The neutralized Velvex or Attaclay formulations of dieldrin and endrin were so stable that rates could not be measured at ordinary storage conditions. The heats of reaction with neutralized clays at elevated temperatures are generally greater than with unneutralized clays. This increase might be interpreted as a reaction of the decomposition products with the base; such a secondary reaction would tend to reduce the neutralizing effect of the base and cause the rate of reaction to increase during course of decomposition. However, no such increase has been observed. The further decomposition of the primary decomposition products may be the reason for the greater heats at the elevated temperatures.

The decomposition reactions remain zero order, as is illustrated in Figure 6 where the solid line represents zero order and a dashed line first order for comparison. The temperature dependence of reaction rate was found to be governed again by the Arrhenius equation, in which the frequency factor, A, was the same as found with the unneutralized clays, but in which the neutralization increased the activation energies, E. Figure 7 shows a fan of Arrhenius relations for 25% endrin on Attaclay in which the solid lines represent changes in E for various neutralizing treatments, all drawn at constant A ($10^{11.1}$). Maximum and minimum activation energies are labeled. As is shown, the effectiveness of a neu-

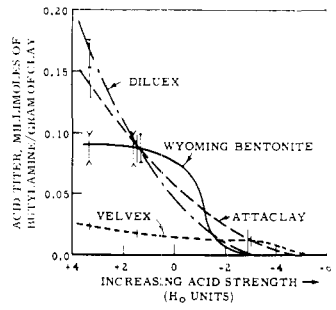

Figure 5. Acidity of clays dried at 120° C.

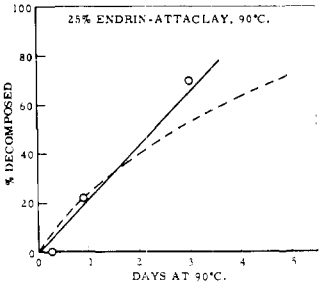

Figure 6. Time dependence of decomposition of 25% endrin-Attaclay mixture

Attaclay neutralized with 2.5% urea and 2.5% NH_4HCO_3
—— Zero order
– – – First order

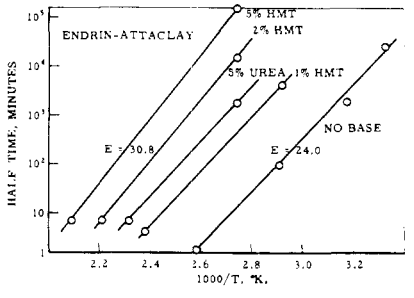

Figure 7. Temperature dependence of 25% endrin-Attaclay mixtures with added bases

tralizing treatment may be expressed by the increase of the activation energy, E.

Comparison of Various Neutralizers. A wide variety of bases have been tested, including inorganic bases, nitrogen bases, and oxygen bases (including water). Some require application in water slurry and some may be sprayed in liquid form on the diluent, while volatile solids can be mixed with the clay and the neutralization accomplished through diffusion of the vapor.

Inorganic bases such as sodium hydroxide are moderately effective, slowing the reaction of dieldrin on Velvex at 100° C. from a half time of 8 minutes to 25 weeks. However, this treatment is effective only if the clay is neutralized

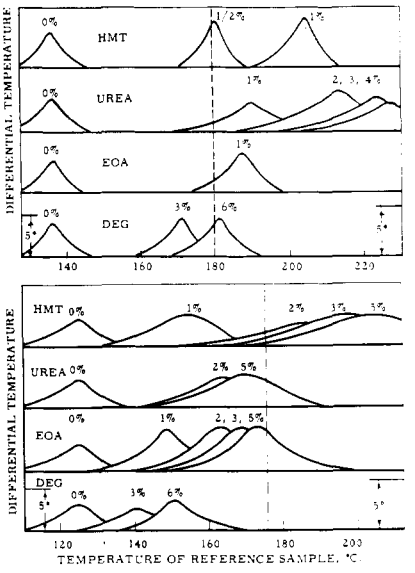

Figure 8. DTA results for neutralization

Top. Of 25% dieldrin-Attaclay mixtures
Bottom. Of 25% endrin-Attaclay mixtures

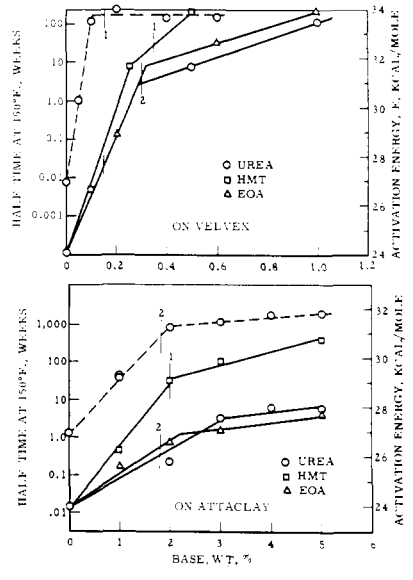

Figure 9. Dependence of storage stability on concentration of added base

– – – Dieldrin
—— Endrin

when suspended in an aqueous slurry. Merely adding the same amount of sodium hydroxide in water (10 wt. % of the clay) does not slow the reaction but does increase the heat of reaction. Another disadvantage of the sodium hydroxide treatment is that, though all exposed surfaces may be neutralized, subsequent grinding exposes new surfaces as acidic as the fresh clay; this makes a sodium hydroxide treatment impractical commercially.

Figure 8, top, shows the original DTA charts for decomposition of 25% dieldrin-Attaclay mixtures with various neutralizers, illustrating the effect of

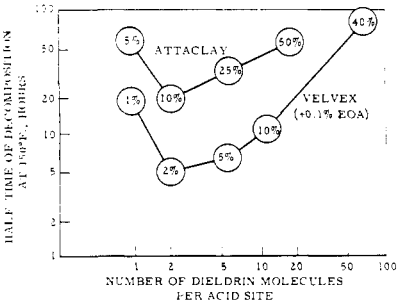

Figure 10. Effect of dieldrin concentration (inside circles) on storage stability at 150° F. calculated from DTA measurements

change in concentration of bases. The stabilizing effect of neutralizers is shown by the shift of the peaks to higher temperatures as the concentration of neutralizer is increased. The three nitrogen bases [hexamethylenetetramine (HMT), urea, and ethanolamine (EOA)] are much more active than an oxygen base [diethylene glycol (DEG)] (*9*), and HMT is definitely more active than the other nitrogen bases. The vertical dashed line shows the DTA peak temperature which indicates a 20-week half time of decomposition at 150° F. (65.5° C.). All peaks to the right of this line indicate a greater storage stability than this arbitrary standard.

Figure 8, bottom, shows DTA data for 25% endrin-Attaclay mixtures. The decomposition temperatures are much lower because of the greater reactivity of endrin. In this case HMT stands out clearly as being much more effective than urea or ethanolamine, and is the only base giving peaks to the right of the critical stability line. Other strong nitrogen bases, such as cyclohexylamine and dicyclohexylamine, were as effective as ethanolamine, but it took 3% of the weakly basic aniline to give the same results as 1% of these stronger bases.

Figure 9 shows the reaction times at 150° F. of 25% endrin or dieldrin mixtures with Velvex or Attaclay to illustrate the effect of concentration of various bases. The corresponding activation energies, E, are also shown, so that rates of decomposition at any temperature may be calculated for these compositions. A regular increase in stability is obtained on adding more base until a critical level is reached, after which further addition of base increases the stability only slightly. The changes in slope generally correspond to some stoichiometric ratio of molecules of base to acid sites. For urea on Velvex with dieldrin, the change of slope is at 16 μmoles of urea per gram of clay, which corresponds to one urea molecule for each strongly acid site. With HMT and endrin on either Attaclay or Velvex, the change of slope also occurs at one molecule of HMT per acid site (vertical lines in Figure 9). On the

other hand, for urea with dieldrin on Attaclay, or urea and ethanolamine with endrin on either clay, the change of slope comes at two molecules of base per acid site. This is evidence that the decomposition reaction takes place only on the acid sites in the clay.

Estimation of Neutralization by H_0 Measurements. Earlier, in Figure 4, the relation between the H_0 of unneutralized diluents and their catalytic activity was shown to be fairly reliable. However, the same relation is not as reliable with neutralized clays, for, although the same slope fits the data, the scatter of points is much broader. Perhaps the organic bases have some influence on the performance of the indicator dyes. It appears that an H_0 value in excess of +3.3 for neutralized clays is not necessarily an indication of a 20-week half time of decomposition at 150° F.

Commercial Storage Problems

Calculation of Isothermal Decomposition Rates. In the previous section it was shown that the rates of decomposition of 25% dust concentrates at various temperatures could be calculated with the Arrhenius equation, using the half time of reaction as a measure of the rate of decomposition. However, with compositions containing less toxicant, the half time of reaction is shorter because n, the number of toxicant molecules per acid (or exchange) site, is less. For example, a 25% dieldrin-Velvex concentrate (n = 33) may be stabilized with urea so that half the dieldrin decomposes in 140 days at 150° F. (total decomposition in 280 days); this amounts to an average reaction time (t_r) of a dieldrin molecule on an acid site of 8.5 days, where $t_r = 2t_{1/2}/n$. The same reaction time occurs in a 4% dieldrin dust containing the same clay. As here there are only four molecules of dieldrin per acid site, however, the time for half-decomposition (2 molecules per site) would equal 17 days as compared with 140 days for the 25% concentrate.

The decrease of half times of decomposition with decrease in n is shown in Figure 10 to occur only in compositions where n is large and average reaction times (t_r) are independent of dieldrin concentration—i.e., true zero-order kinetics result. However, when n approaches unity, the values of t_r and $t_{1/2}$ increase. At low concentrations (n<1), all of the toxicant is adsorbed onto acid sites and no more solid dieldrin remains as a reservoir to keep the dieldrin vapor at saturation. Consequently, the thermodynamic activity and chemical reactivity of the adsorbed dieldrin decline rapidly as n decreases below unity. Under these conditions, t_r increases rapidly during the reaction and the order of reaction increases. (Inhomogeneity of composition can also result in an apparent increase in both half times and order of reaction.)

The steep increase in half times ($t_{1/2}$) as n decreases below 2 is illustrated in Figure 10 with data from DTA measurements. Thus, half times of reaction decrease on dilution to a minimum and then increase on further dilution. The toxicant concentration of the minimum (where n = 2) depends largely on the concentration of acid sites of the clay diluent. This is further illustrated by isothermal storage data at 195° F.; a tenfold decrease in $t_{1/2}$ occurs upon diluting a 25% dieldrin-Velvex concentrate (n = 33) to a 2% dust (n = 2), whereas a sevenfold increase in $t_{1/2}$ occurs upon diluting a 25% dieldrin-Attaclay concentrate (n = 6) to a 2% dust (n = 0.4).

Table IV. Storage Stability of 25% Dieldrin Dust Concentrates in Fifty-Pound Bags at 150° F.

Carrier	Base	% of Dieldrin Decomposed at 150° F. After 1 Week Calcd.	After 1 Week Exptl.	After 8 Weeks Calcd.	After 8 Weeks Exptl.
Attaclay	None	100	100	100	100
Attaclay	5% urea	0.05	0	0.4	0
Attaclay	5% NH_4HCO_3	0.6	0	5	0
Attaclay	1% NH_3, 4% H_2O	...	0	...	0
CCC, 95% / Hi-Sil 101, 5%	None	...	0	...	0
Phosphodust	None	...	0	...	0
Pyrax ABB	None	0.2	0	2	0

Table V. Stability of Field-Strength Dieldrin or Endrin Dusts on Unneutralized and Neutralized Diluents

Type of Diluent	Deactivator, Type and Amt.[a]	Toxicant[b]	Decomposition of Toxicant, Wt. %, When Stored for 14 Days at 130° F. Exptl.	14 Days at 130° F. Calcd.	10 Months at 75° F. Exptl.	10 Months at 75° F. Calcd.
Calcium carbonate	0	Dieldrin	0	...	0	...
	0	Endrin	0	...	...	...
Talc[c]	0	Dieldrin	10–90	13	0–20	<1
	Urea, 1%	Dieldrin	0–3	...	...	...
	HMT, 0.5%	Endrin	0	...	...	...
Pyrophyllite	0	Dieldrin	20	13	0	<1
	Urea, 1%	Dieldrin	2	...	...	...
	0	Endrin	>95	70	...	...
	HMT, 0.25%	Endrin	0	...	...	...
Kaolinite	0	Dieldrin	>95	100	60–100	100
	Urea, 1%	Dieldrin	4	<1	...	...
	HMT, 1%	Endrin	0	<1	5	<1
Attapulgite	0	Dieldrin	90	75	...	...
	Urea, 1%	Dieldrin	0–4	2	...	...
	HMT, 4%	Endrin	4–17	<1	0–3	<1
	HMT, 5%	Endrin	0–4	<1	...	...
Diatomite	0	Dieldrin	>95	70	45	60
	Urea, 1%	Dieldrin	2	...	...	...
	HMT, 3%	Endrin	12	...	...	...
	HMT, 4%	Endrin	2	...	...	...
Montmorillonite	0	Dieldrin	>95	...	10–50	...
	Urea, 1%	Dieldrin	>95	...	3–50	...
	Urea, 3%	Dieldrin	0	...	...	...
	HMT, 3%	Endrin	3–60	...	...	...
	HMT, 5%	Endrin	0–6	...	...	...

[a] Amount of deactivator is expressed as weight % of diluent replaced.
[b] Toxicant concentration was 1 to 5% in all cases.
[c] "Soapstone" from Valley Chemical Co., Greenville, Miss.

"Spontaneous" Decomposition in Commercial Storage. The above calculations of rates are made for isothermal decomposition of the mixture. However, if the mixtures are stored in large bags, drums, or bins, the evolution of heat resulting from a very slow rate of decomposition can warm up the center of the mixture to temperatures well above that of the outside air and the rate of decomposition increases. In separated 50-pound bags, the rate of cooling of the interior by conduction of heat to the outside is about 0.1 cal. per gram of mixture per day per degree centigrade difference in temperature between the center and surface of the bag. Under such conditions, heat evolution in the center of 0.2 cal. per gram per day can be controlled by conduction, but if the heat evolved from the reaction reaches 0.3 cal. per gram per day the center of

Table VI. Quantities of Urea or HMT Necessary to Stabilize Dieldrin and Endrin Field-Strength Dusts

Carrier, Trade Name	Urea Required for Dieldrin Dusts, Wt. %	HMT Required for Endrin Dusts, Wt. %
CCC	0	0
Flo-Float	0	0
Kaluent	0	0
Tobacco dust diluent	0	0
Slaked lime	0	0
Hi-Sil 233	0	5
Silene EF	0	...
Celite 209	1	3
Clear Lake talc	0	0.25
Sierra White talc	1	0.25
Southwest talc	1	0.5
Mil-Slip talc A	1	0.5
Mil-Slip talc B	1	0.5
Frianite M-3X	1	0.5
Diacron	1	2
Diluex	1	5
Attaclay	1	4
Carriclay	1	...
Pyrax ABB	1	0.25
Pyro clay	1	0.25
Phosphodust	1	1
Dusting sulfur	1	0.25
Natka clay	1	1
Tako clay	1	1
Barden AG clay	1	1
Extender clay	1	1
Velvex	1	1
FF absorptive clay	2	3
Rev dust	3	5
Pikes Peak clay	3	5
Dilu-Dust	3	...

Table VII. Rates of Decomposition of Various Insecticides as Measured by DTA

Toxicant	Carrier	Base	Measured $t_{1/2}$ Min.	Measured $t_{1/2}$ °C.	$t_{1/2}$[a] at 150° F.	E[a]	h[b]
25% aldrin[c]	Velvex	None	3	150	3 weeks	31.5	120
	Velvex	0.1% urea	>20	240	>10 years	>38	...
	Attaclay	None	4	199	2 years	30.5	145
	Attaclay	0.2% urea	6	228	>10 years	32.5	155
25% toxaphene	Velvex	None	8	120	13 hours	29.3	28
	Velvex	1% urea	10	153	15 weeks	32.0	80
	Attaclay	None	6	158	5 weeks	28.2	84
25% chlordan	Velvex	None	6	109	11 hours	28.2	37
	Velvex	1% urea	9	161	35 weeks	32.6	68
25% heptachlor	Attaclay	None	5	140	5 days	27.0	43
25% DDT	Velvex	None	2.5	143	8 days	30.3	100
	Velvex	1% urea	5	193	10 years	34.5	56

[a] Estimated by assuming A to be $10^{11.4}$ and $10^{13.4}$ for Attaclay and Velvex, respectively.
[b] Calories evolved per gram of toxicant (by DTA).
[c] 99.3% pure HHDN.

the bag will heat up slowly for a while and then in a day or two it will heat very rapidly until all of the toxicant is decomposed. This results from the fact that the rate of cooling by conduction rises linearly with temperature, while the rate of heating due to the reaction rises exponentially with temperature.

Effect of Moisture. In the preceding section on rates of decomposition, all clays were dried for 1 day at 90° to 110° C. before testing. Rates of decomposition are slower on clays containing more adsorbed water, so the rates predicted by the previously described calculations are rates expected only for dry, active clays. Indicator dyes show that clays with H_0 values of -5.6 can be neutralized to about $+1.5$ by water vapor alone. The neutralization with water vapor is neither sufficiently complete nor permanent for safe storage conditions.

Because of the considerable effect of water on rates at temperatures below 100° C., isothermal storage behavior cannot be correlated except for systems with dry clay. However, in most DTA measurements the water is driven off and the results correlate with those for dry clay.

Experiments with 50-Pound Samples. Fifty-pound batches of 25% dieldrin dust concentrates were formulated with various diluents and deactivators as shown in Table IV and these were stored in multiwall bags placed inside open steel drums in a constant-temperature room at 150° F. The dieldrin content was determined periodically by infrared analysis. For purposes of comparison, Table IV has both experimental and calculated values for the amount decomposed; the agreement is satisfactory.

Stabilization of Field-Strength Dusts. Dusts of the type used in crop-dusting (containing 1 to 5% of toxicant) were prepared with a variety of carriers, deactivators, and with both dieldrin and endrin (Table V). Samples were stored at 130° F. in pint Mason jars and analyzed for toxicant concentration by infrared analysis. Calculated amounts of decomposition are shown for comparison with the experimentally determined values. For the purpose of calculation, it was assumed that, except with attapulgite, zero-order kinetics apply in this range, and the frequency factor, A, of the Arrhenius relation is the same for talc, pyrophyllite, kaolinite, and diatomite.

Table V shows that some of the diluents which are only slightly acidic (talc, pyrophyllite, diatomite) nevertheless require neutralization with bases for formulation with dieldrin or endrin as field-strength dusts. An arbitrary level of safe storage stability of 5% decomposition in 2 weeks at 130° F. has been adopted for field-strength dusts instead of the 150° F. for concentrates. The quantities of urea or hexamethylenetetramine necessary to stabilize dieldrin and endrin field-strength dusts made with various carriers are shown in Table VI.

Decomposition and Stabilization of Other Toxicants

The generality of the clay catalysis of decomposition reactions in pesticidal dusts or dust concentrates has been demonstrated with a number of other toxicants. Table VII shows the rates of decomposition at the elevated temperatures obtained in DTA measurements. It is useful to compare the estimated half times of reaction at 150° F. Without bases, the rates of decomposition are seriously high for chlordan, toxaphene, heptachlor, and DDT. The use of urea to inhibit decomposition is highly effective in all cases. The decomposition of Aramite, determined by bioassay of 25% dusts containing Velvex or Attaclay stored at 65° C., was quite parallel. Rates of decomposition were faster than with the above pesticides, faster on Velvex than on Attaclay, and subject to the deactivating influence of urea. Urea-neutralization of Velvex, Barden, or Attaclay carriers in 25% Aramite concentrates increased decomposition times 10- to 100-fold. Yaffe (*12*) has recently discussed the need of stabilizing Aramite formulations.

Effect of Bases on Other Properties. Investigation of formulations with and without added bases showed that urea and hexamethylenetetramine have no deleterious effect from the standpoint of insecticidal activity (by bioassay), phytotoxicity, grindability, and compatibility with common wetting and dispersing agents.

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PHYTOTOXICITY OF HERBICIDES

Reduction of 3-Amino-1,2,4-triazole Phytotoxicity in Tomato Plants

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The phytotoxicity of 3-amino-1,2,4-triazole was studied using tomato plants to determine the efficacy of selected compounds in reducing growth and chlorophyll toxicities. When the concentration of 3-AT was $1 \times 10^{-4}M$, equimolar solutions of certain purine precursors simultaneously applied caused a reduction of 3-AT growth inhibition; but this did not occur when all concentrations were five times this amount. Adenine, hypoxanthine, and guanine and their ribosides acted like the purine precursors. Riboflavin, FMN, and FAD reduced not only 3-AT growth inhibition at $5 \times 10^{-4}M$, but also the inhibition of chlorophyll formation. A hypothesis of the mechanism of 3-AT phytotoxicity is presented.

THE compound 3-amino-1,2,4-triazole (3-AT), also known as aminotriazole and Amitrol, is an effective defoliant and herbicide. Green plants, treated with sublethal dosages of 3-AT, manifest toxic effects in two ways—growth of embryonic tissues is inhibited, and leaves developed after treatment with 3-AT are devoid of chlorophyll. In addition, albinistic leaves in several species lack chloroplasts (*16*, *18*). Thus, it may be concluded that 3-AT mainly affects developing rather than mature tissues.

Evidence related to the biochemical mechanism is limited. Possibly, the interaction of 3-AT with several metals to form stable complexes is responsible for the toxicity symptoms; however, Hall, Johnson, and Leinweber (*6*) found that plants inhibited by 3-AT were not benefited by treatment with excess metal ions.

The structural similarity between 3-AT and pyrrole makes it attractive to speculate that the phytotoxicity of 3-AT is in some way related to porphyrin or porphyrinlike substances. In support of this hypothesis, Heim, Appleman, and Pyfrom (*7*) and Pyfrom, Appleman, and Heim (*16*) have shown that crystalline catalase activity and catalase activity of plants treated with 3-AT are depressed. Likewise, Margoliash and Novogrodsky (*12*) have shown that 3-AT causes a complete inhibition of crystalline preparations of liver and erythrocyte catalase in the presence of hydrogen peroxide. On the other hand, Bogorad (*1*), in his studies on the enzymatic synthesis of porphyrins from porphobilinogen (PBG), found that 3-AT up to $5 \times 10^{-3}M$ had no adverse effects on a system containing PBG and porphobilinogen deaminase.

The present report provides further evidence concerning the biochemical effect of 3-AT inasmuch as the 3-AT phytotoxicity was reduced by appropriate physiological compounds.

Procedure

Chemicals. Technical grade 3-amino-1,2-4-triazole was supplied by the American Cyanamid Co. and was recrystallized twice before use. 4-Aminoimidazole (4-AI) was prepared in aqueous solution according to the method of Rabinowitz (*17*).

Biological Assay. The effect of 3-AT on plants was studied by the observation of growth and chlorophyll development of the apparent first leaf of tomato plants subsequent to treatment of the growing tip of the plant with 3-AT and other compounds. Tomato plants, *Lycopersicum esculentum* Mill. var. Bonny Best, were grown in the greenhouse at 60° to 85° F. in vermiculite watered periodically with a complete nutrient solution (*8*) until the plants had reached the 8- to 12-leaf stage.

The growing tips of plants at this stage were treated with various chemical solutions. To accomplish this, the apparent third leaf was excised and a powder funnel was fitted around the terminal 3 cm. of the plant by means of a split rubber stopper sealed to the stem with anhydrous lanolin. The terminal leaflets of the apparent first and second leaf were removed to afford a higher proportion of juvenile tissue for treatment and to provide well defined points from which growth measurements could be made (Figure 1). With such an arrangement, the growing tip and the first two leaves could be totally submerged in the desired solution for a given period, at the termination of which the solution could be siphoned away. After treatment, the plants were maintained under normal greenhouse conditions.

The period of time during which the plant tip was exposed to the 3-AT solution is designated in this paper as treatment time. The inhibition of growth caused by treatment with $10^{-4}M$ 3-AT rapidly approached a value of about 50% for a treatment time of 12 hours. When the effect of two compounds on the same plant was tested, they were applied simultaneously in the same solution, as experience indicated that the inhibition caused by treatment with 3-AT could be partially reduced merely by an im-

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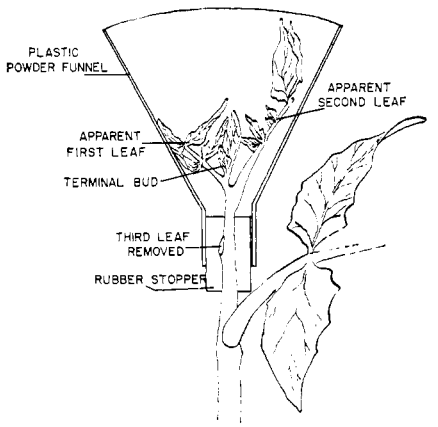

Figure 1. Diagram of setup used in the biological assay

mediate second treatment with an aqueous solution.

For any one set of plants the growth response was proportional to the concentration of 3-AT. For example, in one experiment with $10^{-5}M$, $10^{-4}M$, and $10^{-3}M$ 3-AT the growth was 90, 70, and 35% of the controls, respectively. However, as is evident from the data reported, the per cent inhibition of growth caused by $10^{-4}M$ 3-AT in different experiments was variable. The plants were less inhibited at high greenhouse temperatures—i.e., greater than 90° F.—and light quantity might have contributed to the noted variation.

The standard assay involved treatment of tomato plants with water for controls, a $10^{-4}M$ 3-AT solution, and a $10^{-4}M$ 3-AT solution containing equimolar amounts of one or more compounds to be assayed for efficacy in reduction of toxicity. None of the chemicals used showed any visible reaction with 3-AT at the concentrations employed. Treatment time was usually 3 hours; variations from this procedure are noted where applicable.

The effect on growth of 3-AT and of 3-AT plus the compound to be tested was quantitatively evaluated as follows: The length of the apparent first leaf was measured at the start of the experiment. For most of the experiments, this value was 10 to 15 mm. Six days after treatment, a second measurement was made. From these data, growth increments were calculated as follows:

$$\text{Growth increment} = \frac{\text{length of first leaf at 6 days} - \text{length of first leaf initially}}{\text{length of first leaf initially}}$$

These values were then related to that of the control plants as follows:

$$\%\ \text{of control} = \frac{\text{growth increment of treated plant}}{\text{growth increment of control plant}} \times 100$$

To evaluate the statistical significance of the difference between the growth of the plants treated with 3-AT only and that of plants treated with 3-AT plus a second compound, the t test was applied to the means of the respective growth increments. The significant differences found are indicated in the tables. The means of the growth increments of plants treated solely with guanine, hypoxanthine, inosine, 4-aminoimidazole (4-AI), 4-amino-5-imidazolecarboxamide (4-A-5-IC), glutamine, histidine, and aspartic acid did not differ significantly at the 5% level from those of the control plants.

The first leaf and terminal bud 6 days after treatment were assayed for chlorophyll content relative to the water-treated control plants. After extraction of the pigment according to Petering, Wolman, and Hibbard (*15*), the procedure of Milner *et al.* (*14*) was used. From these data, expressed as Klett units per gram of fresh weight, the relative chlorophyll content was calculated as per cent of the control plants.

Table I. Efficacy of Selected Compounds in Reducing 3-AT Growth Inhibition

Expt. No.	No. of Plants	Growth Increment of Controls × 100	Growth Increment as % of Control: 3-AT Plus Compound Indicated: 3-AT $10^{-4}M$	Adenine $10^{-4}M$	Guanine $10^{-4}M$	Hypoxanthine $10^{-4}M$
19[a]	8	305	82.5		94.8[b]	
10	5	471	80.0			87.6
18[a]	7	302	73.2		91.1[c]	98.5[c]
20	8	479	70.3	101[c]		93.6[c]
11	6	424	70.2		85.5[b]	
21	7	567	67.8	86.4[c]		
27[d]	10	415	66.6	55.5		54.6
12	5	730	66.5			91.2[c]
5	4	371	59.9			62.4
23[d]	8	635	59.8	53.7	60.9	61.6
22	8	427	50.3		44.2	
				Adenosine $10^{-4}M$	Guanosine $10^{-4}M$	Inosine $10^{-4}M$
24	8	535	83.1	95.1[b]	94.0[b]	104[c]
10	6	471	80.0			92.5[b]
4[e]	4	256	78.4			93.3[c]
25[f]	8	537	78.0	97.6[c]	90.9[b]	99.2[c]
3	6	363	70.0			79.9[c]
12	5	730	66.5			94.1[c]
				4-AI[g] $10^{-4}M$	4-A-5-IC[g] $10^{-4}M$	
24	8	535	83.1	101[c]		
19[a]	8	305	82.5	96.1[b]		
25[f]	8	537	78.0	95.3[c]		
26[h]	10	525	75.8	86.6[b]		
11	6	424	70.2		80.7	
27[d]	10	415	66.6	54.4	54.4	
16	10	349	64.6	96.9[c]		
26[i]	10	525	54.6	70.6[c]		
15[i]	12	512	45.6		54.2[c]	

[a] 6-hr. treatment.
[b] Mean of growth increment significantly different at 5% level from that of 3-AT alone.
[c] Mean of growth increment significantly different at 1% level from that of 3-AT alone.
[d] All concns. $5 \times 10^{-4}M$.
[e] Experiment of 4 days.
[f] All concns. $2.5 \times 10^{-4}M$.
[g] Purine precursors.
[h] 6-hr. treatment; all concns. $2 \times 10^{-4}M$.
[i] 6-hr. treatment; all concns. $4 \times 10^{-4}M$.
[j] 12-hr. treatment.

Results and Discussion

Reduction of 3-AT Growth Inhibition by Purines. Table I indicates that the growth inhibition of the tomato leaf caused by 3-AT was significantly reduced when adenine, guanine, or hypoxanthine was supplied simultaneously with the inhibitor, although reduction did not occur in all experiments. Nevertheless, except for experiment 10, the data are consistent with the interpretation that the purines tested resulted in significant partial reduction of growth inhibition caused by 3-AT, provided the inhibition effected by 3-AT alone was not too severe. When the concentrations were all $5 \times 10^{-4}M$, as in experiment 27, the purines were ineffective in reversing 3-AT growth inhibition.

In accordance with these observations the effect of various ribosides was of interest. Table I further indicates that under the assay conditions employed adenosine, guanosine, and inosine also significantly reduced growth inhibition caused by 3-AT.

As purines and their ribosides were effective for minimizing growth inhibition, a number of precursors known to be involved in purine biosynthesis in other systems were assayed. The data of Table I indicate that 4-aminoimidazole and possibly 4-amino-5-imidazolecar-

boxamide were effective agents. On the other hand, comparable experiments with glycine, histidine, aspartic acid, and glutamine failed to demonstrate that these amino acids reduce growth inhibition caused by 3-AT.

The partial reduction of growth inhibition by purines and metabolically related compounds indicates that 3-AT may in some manner interfere with normal purine metabolism. Disruption of purine metabolism could logically account for decreased growth, as the relationship of nucleic acid and protein synthesis is well established.

Reduction of 3-AT Growth Inhibition by Riboflavin. For some organisms, purines not only stimulate riboflavin production (*11*) but also become incorporated into the riboflavin molecule (*9, 13, 19*). This metabolic relationship suggests that an effect comparable to that of purines on 3-AT inhibition might also be produced by riboflavin. The data of Table II indicate that riboflavin as well as FMN and FAD is effective in reducing growth inhibition caused by 3-AT. In these experiments, even in series showing strong inhibition by 3-AT, there is conclusive evidence for significant reduction of toxicity.

Reduction of 3-AT Chlorophyll Inhibition by Riboflavin. Although the purines and related compounds indicated in Table I were effective in partially reducing the inhibitory effect of 3-AT on growth, none of these chemicals caused any visible reduction of chlorophyll inhibition. However, Table II indicates, for tomato tips treated simultaneously with flavin mononucleotide (FMN) and 3-AT, a very marked reduction of the chlorophyll inhibition. FMN alone did not influence the chlorophyll content of plants not treated with 3-AT. Riboflavin and flavin adenine dinucleotide (FAD) also reduced in a similar manner the degree of chlorophyll inhibition caused by 3-AT. Even plants under conditions which had as low as 25% or less of the chlorophyll found in control plants when treated with 3-AT alone formed as much as 75% or more of the normal chlorophyll content when treated with 3-AT solutions including riboflavin derivatives.

Rogers' work indicates that the toxicity of 3-AT is not related to porphyrin or chlorophyll synthesis itself (*18*). Also, Hall, Johnson, and Leinweber (*6*) have reported that chlorophyll synthesis occurred in etiolated cotton cotyledons which had been sprayed with 3-AT. Furthermore, studies in the authors' laboratory have failed to reveal any inhibition by 3-AT of porphyrin and bacteriochlorophyll production by *Rhodopseudomonas spheroides* cultured under the conditions of Lascelles (*10*). The obvious inhibition of growth and of normal plastid formation in newly formed 3-AT treated tissues points rather to the interpretation that the essential action of 3-AT is to disrupt the metabolism of purines and riboflavin and their derivatives.

The inhibition of plastid formation and consequently of chlorophyll synthesis by 3-AT would seem from the present study to result from the effectiveness of 3-AT in blocking the synthesis of certain metabolites, chiefly riboflavin, necessary for normal chloroplast development. A comparable effect on chloroplast development in albinistic *Euglena* has been reported by De Deken-Grenson (*2*), who noted that amino acids and yeast RNA selectively promote chloroplastic growth under conditions where growth was unaffected.

Additional data which will be reported shortly indicate that 3-AT inhibits riboflavin synthesis in the yeast *Eremothecium ashbyii*. Also, albinistic tissues of corn and pea plants treated with 3-AT show much less riboflavin than do tissues taken from comparable parts of untreated plants.

The inhibition of vitamin B_2 production is of interest with other studies which have shown that riboflavin content in tomato plants increases with high light intensity (*3*), long photoperiods (*4*), and high temperatures (*5*), factors which were not kept constant in these studies and which might have contributed to the variability of data observed.

Table II. Efficacy of Riboflavin and Its Derivatives in Reducing 3-AT Growth and Chlorophyll Inhibition

Expt. No.	No. of Plants	Growth Increment Controls × 100	Growth Increment as % of Control: 3-AT 5 × 10^{-4}M	3-AT Plus Compound Indicated: Riboflavin 5 × 10^{-4}M	FMN 5 × 10^{-4}M	FAD 5 × 10^{-4}M	Riboflavin + ATP 5 × 10^{-4}M
36	8	467	70.4		80.2[a,b]		
32	9	529	66.9	87.9[c]			98.7[c]
35	10	649	69.4	93.1[c]	87.6[c]	87.2[c]	
37	10	381	68.6		91.9[a,c]		
34	5	492	60.1	83.3[c]			
39	8	389	53.6	68.1[c]	86.5[c]		77.3[c]
40	10	444	52.6	90.8[c]			101[c]
			Relative Chlorophyll Content as % of Control				
35			82.0	97.5	96.0	95.1	
34			54.0	85.7			
39			25.2	59.0	93.7		84.7
36			22.9		82.2[a]		
32			20.8	74.8			78.0
37			17.5		85.1[a]		
40			11.6	76.5			91.2

[a] FMN was 5 × $10^{-5}M$.
[b] See [b], Table I.
[c] See [c], Table I.

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Colorimetric Microdetermination of 1-Chloro-2-nitrobenzene in Pineapple

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The herbicide, 1-chloro-2-nitrobenzene, is extracted from pineapple tissue and determined colorimetrically by measuring the intensity of the azo dye formed after reduction, diazotization, and coupling with *N*-(1-naphthyl)-ethylenediamine. This sensitive micromethod can determine 0.01 p.p.m. of the herbicide in pineapple.

A METHOD for the determination of 1-chloro-2-nitrobenzene (Monsanto Chemical Co.'s herbicide *o*-nitrochlorobenzene, ONCB) in pineapple tissue in connection with residue studies is needed. The method described below is similar to that used by Averell and Norris (*1*) for the determination of parathion and by Young and Gortner (*3*) for the estimation of monuron after hydrolysis to *p*-chloroaniline. Sherwood (*2*) suggested the application of the parathion method to the analysis of ONCB.

Experimental

Reagents. Attapulgus clay–Celite adsorbent. Mix two parts of Attapulgus clay with 1 part of Celite.

Sodium nitrite, 1% solution (w./v.). Keep in refrigerator and discard after a week.

Ammonium sulfamate, 10% solution (w./v.). Keep in refrigerator and discard after a week.

N-(1-Naphthyl)-ethylenediamine dihydrochloride, 1% solution (w./v.). Filter before using and keep in refrigerator. Discard after a week.

o-Nitrochlorobenzene standard, melting point 32.3° C. (Monsanto Chemical Co. No. A61).

Procedure. Assemble opposite longitudinal wedges of at least four fruits from a replicate treatment to make a sample. Cut into small pieces, mix, and weigh 150 grams into a Waring Blendor. Add 100 ml. of water and 150 ml. of benzene, blend for 1 minute or until the sample is homogenized, and pour the mixture into two 250-ml. centrifuge bottles. Stopper tightly with cork stoppers, centrifuge at 1500 r.p.m. for 10 minutes, and siphon off 100 ml. of the supernatant liquid.

Remove pigments, which appear to be completely extracted by the benzene, by passing the extract through a column of Attapulgus clay–Celite adsorbent prepared as follows: Place a wad of glass wool at the bottom of a 125-ml. open-top cylindrical separatory funnel or a chromatographic column of similar size and add a mixture of 5 grams of adsorbent and about 20 ml. of benzene. Apply suction until the benzene is just removed from the top of the column. Do not suck dry, as this will allow the extract to channel subsequently.

Collect the decolorized extract in a 200-ml. tall-form beaker and wash the column with 60 ml. of benzene. Add an acid-washed Hengar boiling stone, cover, and evaporate the mixture by boiling on a hot plate at low heat until about 10 ml. remain. Add 10 ml. of 95% ethyl alcohol, 10 ml. of 1*N* hydrochloric acid, and 0.2 ± 0.01 gram of zinc powder. Cover, digest, and evaporate the mixture at low heat to a volume of 5 ml. This takes about 20 minutes. Filter through a 9-cm. Whatman No. 42 filter paper directly into a 25-ml. volumetric flask and wash to a volume of about 20 ml.

Introduce 1 ml. of a 1% sodium nitrite solution, mix, and allow to stand for 10 minutes. Add 1 ml. of a 10% ammonium sulfamate solution, stopper, and invert the flask to remove all the nitrite from the neck of the flask. Allow to stand for 10 minutes with occasional shaking to remove the nitrogen evolved. Add 1 ml. of 1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution, dilute to volume, mix, and allow to stand for 20 minutes.

Read the absorbance on a colorimeter using a 540-mμ filter and obtain micrograms of ONCB by dividing by the absorbance factor determined below. Divide the result by 100 to get parts per million of ONCB in the sample.

Inasmuch as the color follows Beer's law, the absorbance factor giving absorbance per microgram of ONCB may be more conveniently used for calculating instead of employing a standard curve. Run 0- to 30-γ standards in benzene by proceeding at the reduction step using ethyl alcohol, hydrochloric acid, and zinc. Calculate the absorbance per microgram of ONCB for each standard and take the average value. Standard readings obtained on the Evelyn photoelectric colorimeter are given in Table III.

Results and Discussion

Removal of Solvent in Extracts. A serious problem in the determination of volatile organic chemicals is the removal of solvent after extraction without loss of the volatile chemical, particularly when special laboratory equipment is not employed. Table I shows that a complete loss of ONCB occurs when extracts are taken to dryness even by directing a fine stream of air over the surface of the extract in an open beaker at room temperature. Evidently no loss of ONCB occurs if the extract is evaporated to 10 ml. in a covered beaker and the reduction step with zinc and hydrochloric acid, to form *o*-chloroaniline, is carried out in the presence of benzene before diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine. Controls on non-ONCB-treated pineapple fruits have consistently shown negligible amounts of color, indicating that in the presence of benzene and fruit extract, the formation of any aromatic amine compound is unlikely during the reduction step. The absence of appreciable amounts of any naturally occurring aromatic nitro or amine compound is also indicated.

Effect of Acidity During Color Development. In the determination of parathion, Averell and Norris (*1*) specified the use of an amount of hydrochloric acid in excess of that required to react with the quantity of zinc present. The excess must be sufficient to give a

Table I. Effect of Method of Benzene Removal on Loss of ONCB in Extract

Method	ONCB Present, γ	ONCB Found, γ	ONCB Lost, %
Boiled in open beaker to 10 ml., then aired just to dryness	57	0	100
Aired just to dryness	57	3.0	95
Boiled in covered beaker to 10 ml., followed by reduction in presence of benzene	57	56.3	1
Same	34.2	35.4	0

Table II. Effect of Acidity on Development of the Test Color with 34.2 γ of ONCB

Zinc, G.	HCl, Meq.	HCl Excess, Meq., Calcd.[a]	pH	Absorbance	Absorbance/γ/ 25 Ml.[b]
0.2	6	0	1.1	0.441	0.0129
0.1	2.5	0	1.1	0.455	0.0133
0.2	10	3.9	0.7	0.553	0.0162
0.2	10	3.9	0.9	0.561	0.0164

[a] Not directly related to pH, owing to incomplete reaction with zinc.
[b] Obtained on the Evelyn photoelectric colorimeter with filter 540.

Table III. Standard Absorbance Readings Obtained with Filter 540 on the Evelyn Photoelectric Colorimeter

ONCB, γ/25 Ml.	Transmittance, %	Absorbance		A^a/γ
10.7	67	0.174		0.0163
21.4	43¾	0.359		0.0168
32.1	29¾	0.527		0.0164
			Av.	0.0165

[a] A = absorbance.

pH of 0.6 to 1.0 during the coupling reaction in order to achieve maximal color development. Table II gives data on ONCB which confirm this effect. Similar results were obtained on smaller amounts of ONCB. An excess of hydrochloric acid is essential for proper color development.

Adherence to Beer's Law. Absorbance readings obtained on the Evelyn photoelectric colorimeter with filter 540 using matched colorimeter tubes bear a direct linear relationship with varying amounts of ONCB as shown in Table III. The mean absorbance factor, $\frac{\text{Absorbance}}{\gamma}$, is therefore used for the calculations.

Of the various green Evelyn filters available, filter No. 540 is best inasmuch as it transmits light maximally at 540 mμ where maximal absorption of the test color occurs. This is shown by the absorption spectrum in Figure 1 obtained on the Beckman DK-2 spectrophotometer using a 1-cm. cuvette.

Recovery and Sensitivity. Recovery of micro quantities of ONCB added to fresh pineapple fruit tissue and analyzed by the method described above is presented in Table IV. Average recovery of 93% is considered excellent particularly in view of the small amounts of ONCB present.

The method is extremely sensitive—0.01 p.p.m. may be determined. On the Evelyn instrument using the ⅞-inch colorimeter tube, a reading of 96% transmittance is obtained at this concentration.

Literature Cited

(1) Averell, P. R., Norris, M. V., *Anal. Chem.* **20,** 753–6 (1948).
(2) Sherwood, L. V., Monsanto Chemical Co., St. Louis, Mo., private communication, November 8, 1957.
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Table IV. Recovery of ONCB Added to Pineapple Fruit Tissue[a]

Added, P.P.M.	Recovered, P.P.M.[b]	Recovery, %
0.07	0.06	86
0.07	0.07	100
0.21	0.20	95
0.21	0.19	90
0.36	0.33	92
0.36	0.35	97
	Av.	93

[a] 100-gram sample.
[b] Corrected for a blank value of 0.01 p.p.m. apparent ONCB.

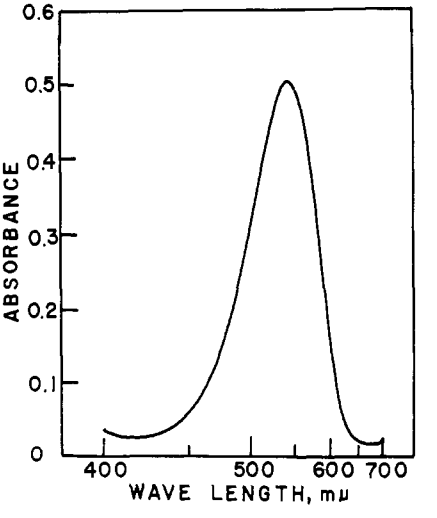

Figure 1. Absorption spectrum of ONCB test color, 55 γ ONCB per 25 ml.

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HERBICIDE RESIDUES

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G. H. BATCHELDER and G. G. PATCHETT

Stauffer Chemical Co., Richmond, Calif.

ETHYL - *N*,*N* - DI - *n* - PROPYLTHIOLCARBAMATE (EPTC), also known as Eptam, is a promising selective herbicide. A sensitive method which determines the di-*n*-propylamine formed upon hydrolysis of EPTC in concentrated sulfuric acid has been developed for the determination of crop residues. The amine is reacted with carbon disulfide in the presence of ammonia and cupric ion in a two-phase benzene-water system to form, in benzene, the cupric di-*n*-propyldithiocarbamate complex which has an intense absorption peak at 440 mμ. Detection of quantities of EPTC down to 4 γ enables determination of 0.02 p.p.m. of residue by the described procedure.

The colorimetry is based upon the method of Dowden (*1*) for determination of secondary alkylamines, which previously had been adapted to the determination of octamethylpyrophosphoramide (OMPA) by Hall, Stohlman, and Schechter (*3*). OMPA does not interfere, however, when 100 γ are processed through the steam distillation procedure. Possible interference by free amines is eliminated by acidifying the steam distillate prior to extraction of EPTC in preparation for hydrolysis. A wide

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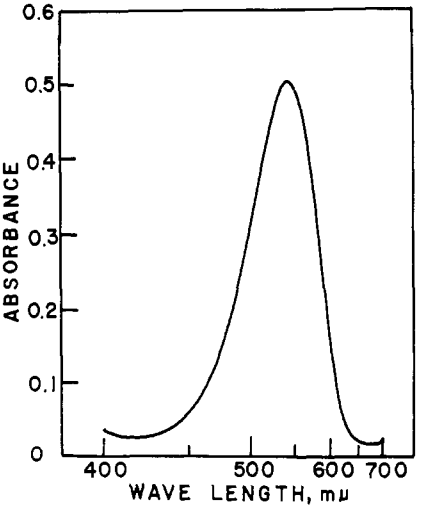

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The colorimetry is based upon the method of Dowden (*1*) for determination of secondary alkylamines, which previously had been adapted to the determination of octamethylpyrophosphoramide (OMPA) by Hall, Stohlman, and Schechter (*3*). OMPA does not interfere, however, when 100 γ are processed through the steam distillation procedure. Possible interference by free amines is eliminated by acidifying the steam distillate prior to extraction of EPTC in preparation for hydrolysis. A wide

EPTC, ethyl-*N,N*-di-*n*-propylthiolcarbamate, is hydrolyzed in sulfuric acid to di-*n*-propylamine from which is formed the cupric dithiocarbamate complex having an absorption peak at 440 mμ. Crop and soil extraction procedures are presented.

variety of crops have exhibited background values of less than 0.02 p.p.m., excluding turnips which seriously interfere with the colorimetry.

EPTC is extracted from samples with hexane or by direct steam distillation. The latter method was employed originally by Fang (*2*) in radiotracer studies of EPTC residues. Interference with the colorimetry is eliminated effectively by a combination of procedures utilizing steam distillation, benzene extraction of acidified amine solution, and determination of background absorbance prior to color formation.

Special Reagents

Copper-Ammonia Reagent. Dissolve 1.0 gram of cupric sulfate pentahydrate in 5.0 ml. of water and dilute to 250 ml. with concentrated ammonium hydroxide.

EPTC Standard Solution. One hundred micrograms of EPTC per ml. of iso-octane.

Sample Extraction and Cleanup

Hexane Extraction Method. Macerate 800 grams of sample with 1600 ml. of hexane in a 1-gallon Waring Blendor until thoroughly blended. A stable emulsion usually forms but can be broken by mixing in approximately 1000 grams of anhydrous sodium sulfate and allowing to stand overnight in a sealed container. Filter 800 to 900 ml. of hexane and reserve for further processing.

Concentrate a 400-ml. aliquot of hexane extract, representing 200 grams of sample, to about 15 ml. by rapid distillation through a three-ball Snyder column. Wash the column with 5 ml. of hexane, cool, and add 100 ml. of water. Replace the Snyder column with a distillation head and condenser, and steam-distill the EPTC and the residual hexane plus about 80 ml. of water into a 125-ml. separatory funnel. Add 5 drops of concentrated hydrochloric acid to the distillate and extract the aqueous phase with the codistilled hexane. Draw off the aqueous phase and carefully transfer the hexane to a dry 40-ml., glass-stoppered, graduated centrifuge tube, avoiding the introduction of any water droplets into the tube. Return the aqueous phase to the separatory funnel, re-extract with an additional 10 ml. of hexane, and transfer the hexane as before to the centrifuge tube. Care must be taken to prevent introducing water droplets into the centrifuge tube because dilution of the sulfuric acid will seriously retard the rate of hydrolysis of EPTC. The combined hexane, representing 200 grams of sample, is now ready for hydrolysis and colorimetry.

Direct Steam Distillation Method. Add 400 grams of crop sample macerated with 1000 ml. of water, or 400 grams of soil sample and 1000 ml. of water, to a 4-liter wide-mouthed Erlenmeyer flask. Add 25 ml. of glacial acetic acid and distill about 400 ml. of distillate into a 500-ml. separatory funnel. Add 5 drops of concentrated hydrochloric acid to the distillate and extract the EPTC from the aqueous phase with two 30-ml. portions of iso-octane. Combine the iso-octane extracts, which represent 400 grams of sample, and store them for subsequent hydrolysis and colorimetry.

Hydrolysis and Colorimetry. Add 1 ml. of 96% sulfuric acid to a 40-ml. centrifuge tube containing either the hexane extract or an aliquot of iso-octane extract representing 200 grams of sample. Residues up to 3.5 p.p.m. can be determined without adjusting the indicated sample size. Shake vigorously for 1 minute to extract the EPTC into the acid phase. Allow the phases to separate and discard the solvent phase by withdrawing it with a suction tube. (Small amounts of residual solvent will not interfere.) Heat the tube in an 85° ± 1° C. glycerol bath for 20 minutes to hydrolyze the EPTC. Twice during the heating period gently roll the tube to rinse the sides with hot acid.

Cool the tube in an ice bath and cautiously dilute the cold acid to 20 ml. with water. Add 1 drop of phenolphthalein solution and neutralize by the dropwise addition of 50% sodium hydroxide while swirling and cooling. Promptly acidify by the dropwise addition of concentrated hydrochloric acid, add 4 extra drops of acid, and dilute to 25 ml. with water. Extract the slightly acidic aqueous solution with two 10-ml. portions of benzene to remove benzene-soluble background color from the amine hydrochloride in the aqueous phase. Withdraw and discard each benzene portion with a suction tube, taking care to remove as much of the benzene as possible after the last extraction.

Add 1 drop of phenolphthalein solution and add 50% sodium hydroxide dropwise until the solution is basic. Add 2 extra drops of sodium hydroxide. Introduce 10.0 ml. of benzene and extract the free amine from the basic solution. Withdraw the aqueous phase and replace it with 15 ml. of water plus 2 drops of 50% sodium hydroxide. Shake vigorously to wash the benzene layer. Allow the phases to separate, transfer about 4 ml. of the benzene layer into a test tube, and dry over 1 gram of granular anhydrous sodium sulfate to remove turbidity. Transfer the clarified benzene into a 1-cm. cell and determine the background absorbance at 440 mμ, against a benzene blank, with a Beckman DU spectrophotometer (or equivalent). Return the benzene to the centrifuge tube containing the major portion of benzene and the dilute sodium hydroxide. Add 0.5 ml. each of copper-ammonia reagent and carbon disulfide. Without delay or interruption shake moderately for 4 minutes. Allow the solution to clear and then dry a portion of the benzene, as before. Read the

Table I. Preparation of Standard Curve for EPTC[a]

EPTC, γ	Net Absorbance, 440 Mμ
0	0.003
4	0.012
10	0.027
20	0.050
50	0.121
100	0.239
200	0.475
400	0.947

[a] EPTC was added in 30 ml. of iso-octane to a 40-ml. centrifuge tube and processed through the colorimetric procedure.

Table II. Recovery of EPTC Added to Hexane Extracts[a]

Crop	EPTC Added, p.p.m.	EPTC Recovered, %
Alfalfa		
Fresh	0.25	88
Dried	1.00	76
Beans, dried	0.10	100
	0.05	80
Beets	0.20	80
Clover	0.50	84
Corn	0.50	78
	0.10	100
Onions	0.50	72
Peppers	0.50	87
Potatoes	0.20	75
Snap bean plants	0.10	100
Spinach	0.50	88
Strawberries	0.20	85
Sugar beets	0.05	80
Tomatoes	0.50	79

[a] Aliquots of raw hexane extract representing 200 grams of crop were fortified with EPTC and processed through the hexane concentration, steam distillation, and colorimetric determination procedures.

Table III. Recovery of EPTC Added to Macerated Crops

Crop	EPTC Added, p.p.m.	EPTC Recovered, %
Hexane Extraction Method		
Beans, dried	0.20	90
Carrots	0.50	78
Snap bean plants	0.10	100
Sugar beets	0.20	105
Tops	0.20	90
Direct Steam Distillation Extraction Method		
Blackberries	1.0	101
	0.1	100
Cantaloupes	1.0	90
	0.1	80
Onions, dried	1.0	86
Peas	1.0	79
Soil		
Sandy loam	6.0	92
Clay	2.0	99

absorbance at 440 mμ within 10 minutes after the color is formed.

During color formation, solution temperature should be 20° to 25° C. for optimum results. The reaction may not be completed at lower temperatures and side reactions can occur at higher temperatures. After addition of reagents, a delay in starting the 4-minute shaking period also can result in side reactions. The color formed in absence of sample extractives is normally stable after drying, but slow fading has been observed in the presence of crop extractives.

Determine the amount of EPTC in the sample by referring the net absorbance (the difference between absorbance before and after color formation) to a standard curve prepared by processing aliquots of the EPTC standard solution in a similar manner. Typical absorbance values are listed in Table I.

The colorimetry is not significantly affected by the small quantities of sample extractives introduced into the concentrated sulfuric acid. Typical recovery values are presented in Table II for EPTC added to a variety of crop extracts.

Discussion

Thirty consecutive colorimetric determinations of 400 γ of EPTC over a 7-week period of routine analyses by a single operator have resulted in a relative average deviation of ±3.4%.

EPTC is miscible in all proportions with most organic solvents but is only slightly soluble in water (375 ± 15 p.p.m. at 20° C.). It is readily extracted with hexane from a variety of crops (Table III). The hexane extraction procedure has been employed for most crop residue analyses to date. However, the recently adapted direct steam distillation method of extraction is being used currently for all soil and most crop samples. The latter method has the significant advantages of combining the extraction and cleanup steps, and of eliminating the handling of large quantities of hexane. Crops with a high starch content, such as potatoes and dried beans, cannot be extracted conveniently by this method because of excessive foaming and scorching. Some typical recoveries of EPTC added to macerated crops, employing both extraction methods, are given in Table III.

Acknowledgment

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(1) Dowden, H. C., *Biochem. J.* **32,** 455–9 (1938).
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HERBICIDE RESIDUES

Improved Extraction Procedure for the Determination of EPTC Residues in Potatoes

W. H. GUTENMANN and D. J. LISK

Pesticide Residue Laboratory, Department of Entomology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

An improved method of extracting the herbicide, EPTC, from potatoes is described. Potatoes are blended with isopropyl alcohol and Skellysolve B (*n*-hexane). After filtering, hexane separates and is collected. The aqueous isopropyl alcohol is extracted with hexane. The combined hexane extracts are evaporated and EPTC is determined. Stable emulsions do not form when isopropyl alcohol is used. Recovery of 0.1 p.p.m. of EPTC from potatoes ranged from 93.5 to 99.0%. Analysis of potatoes treated with EPTC for weed control showed no residue.

Ethyl *N*,*N*-di-*n*-propylthiolcarbamate (EPTC, Eptam) is a selective pre-emergence herbicide which will control grassy and certain broadleaf weeds. A method by Batchelder and Patchett (*1*) for determining EPTC residues has been developed. It involves extraction of the herbicide from crop material with Skellysolve B (*n*-hexane) and separation from plant waxes and pigments by steam distillation. EPTC is then hydrolyzed to di-*n*-propylamine. The amine is determined colorimetrically after reaction with carbon disulfide in the presence of cupric ion and ammonia to form the yellow cupric dithiocarbamate.

Extraction of EPTC from potatoes by blending with hexane produces emulsions which are difficult to break. Recovery of EPTC using the above method after hexane extraction is erratic. Extraction with hexane–isopropyl alcohol eliminates the formation of stable emulsions and good recovery of EPTC is obtained.

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An improved method of extracting the herbicide, EPTC, from potatoes is described. Potatoes are blended with isopropyl alcohol and Skellysolve B (*n*-hexane). After filtering, hexane separates and is collected. The aqueous isopropyl alcohol is extracted with hexane. The combined hexane extracts are evaporated and EPTC is determined. Stable emulsions do not form when isopropyl alcohol is used. Recovery of 0.1 p.p.m. of EPTC from potatoes ranged from 93.5 to 99.0%. Analysis of potatoes treated with EPTC for weed control showed no residue.

Ethyl *N*,*N*-di-*n*-propylthiolcarbamate (EPTC, Eptam) is a selective pre-emergence herbicide which will control grassy and certain broadleaf weeds. A method by Batchelder and Patchett (*1*) for determining EPTC residues has been developed. It involves extraction of the herbicide from crop material with Skellysolve B (*n*-hexane) and separation from plant waxes and pigments by steam distillation. EPTC is then hydrolyzed to di-*n*-propylamine. The amine is determined colorimetrically after reaction with carbon disulfide in the presence of cupric ion and ammonia to form the yellow cupric dithiocarbamate.

Extraction of EPTC from potatoes by blending with hexane produces emulsions which are difficult to break. Recovery of EPTC using the above method after hexane extraction is erratic. Extraction with hexane–isopropyl alcohol eliminates the formation of stable emulsions and good recovery of EPTC is obtained.

Table I. Recovery of 0.1 P.P.M. EPTC from Potatoes

Code	Check Value, γ	Recovery, % (Corrected for Check)
A	0.3	100.0
B	0.7	96.0
C	0.8	93.5
D	0.3	94.0
E	0.2	99.0
F	0.7	99.0

Extraction Procedure

Thoroughly macerate about a dozen potatoes in their own liquid in a 1-gallon blender. Weigh a 200-gram portion of the blended tissue, transfer to a 1-quart blender, and blend with 200 ml. of isopropyl alcohol (reagent grade) for 30 seconds. Add 300 ml. of Skellysolve B (technical) to the mixture and blend for 2 minutes. Pour the contents into a large, coarse porosity, sintered-glass funnel, and filter by suction. Use 25 ml. of hexane (used hereafter to designate technical Skellysolve B) to rinse the blender and funnel. Then transfer the contents of the suction flask to a 500-ml. separatory funnel and allow the layers to separate. Drain the aqueous isopropyl alcohol (lower) layer into a beaker and the upper hexane layer into a 500-ml. Erlenmeyer flask with a 24/40 standard-taper joint. Re-extract the isopropyl alcohol–water mixture twice with 50-ml. portions of hexane, using the latter to rinse the suction flask and beaker each time. Combine the hexane extracts, and distill off the solvent as in step 2 of the hexane extraction method (*1*). Then use the subsequent steps of the method (*1*) for cleanup and determination of the herbicide.

Results and Discussion

Recovery studies with potatoes were made using the isopropyl alcohol–hexane extraction procedure. Twenty micrograms (0.1 p.p.m.) of EPTC in hexane was added to each of six 200-gram portions of blended potato tissue. The herbicide was then blended in, 200 ml. of isopropyl alcohol was added, and the procedure was completed as described. Check samples were also carried through the procedure. Table I shows the recoveries and check values obtained.

The average of the six recoveries was 96.9%. The average of the six check values was 0.5 γ of EPTC.

Potato plants, 8 to 10 inches tall, were treated with EPTC in 1958 for weed control. Using a randomized block design, a comparison was made between two granular formulations, clay and vermiculite, and an emulsifiable concentrate. One application of each formulation was made on July 23 using a hand sprayer. The granular clay formulation was applied at a rate of 3 pounds per acre. The granular vermiculite and emulsion formulations were applied at both 3 and 4.5 pounds per acre. The potato plants were also treated with DDT, parathion, Maneb, and sodium arsenite. The tubers were harvested on October 11. Analyses of the tubers for EPTC from each of the treatments using the method described showed no residue.

Acknowledgment

This experiment was done in cooperation with R. D. Sweet, Vegetable Crops Department, New York State College of Agriculture, Ithaca, N. Y.

Literature Cited

(1) Batchelder, G. H., Patchett, G. G., J. Agr. Food Chem. **8**, 214 (1960).

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SEED DISINFECTION

Fungicide and Dye Distribution in Liquid Seed Treatment

OLLE LINDSTRÖM

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The mercurial distribution produced in liquid seed treatment (Panogen process) was determined by a new chemical method which permits mercury assay of individual kernels of seed. Distribution statistics based on whole kernel assay differs but slightly from the statistics with the radioactive method, where only part of the kernel surface was examined. Because of vapor action, here illustrated by simultaneous treatment with nonvolatile tracer and labeled mercurial, a reasonably uniform mercurial distribution is obtained in laboratory and commercial treatment, irrespective of treatment conditions and liquid dosage. More or less uniformly colored treated seed is of no value in judging the mercurial distribution.

The mechanism of liquid seed treatment (Panogen process) was recently studied by radioactive tracer methods with particular attention to distribution (*3*). This method accounts only for the amount of fungicide present on that part of the kernel facing the window of the Geiger tube, roughly one sixth of the total kernel surface. Since then methods have been developed for the assay of the total mercury content of single kernels of treated seed. Gamma counting is performed at near 4π geometry by scintillation techniques (*2*). Irradiation of treated seed in a nuclear reactor produces by neutron activation the radioactive isotope mercury-197, which is determined by radioactive techniques (*5*). Finally, a new chemical method with spectrophotometric mercury determination has been developed for the mercury assay of single kernels of treated seed (*2*). This method, called flame analysis, is used in the present investigation.

The effects of important variables in seed treatment were studied, particularly the liquid dosage and treatment conditions. Commercially treated seeds were included. The relationships between distribution statistics based on beta counting and whole kernel assay are illuminated. Regression analysis is used to get the relation between fungicide uptake and kernel size. The importance of vapor action is demonstrated in an experiment involving simultaneous treatment with a nonvolatile tracer and the labeled mercurial fungi-

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cide. Estimation of the dye distribution on the treated seed is frequently (and erroneously) used in the field to judge the effectiveness of the seed treatment procedure. This was illustrated by means of an objective method for evaluation of the distribution of the dye.

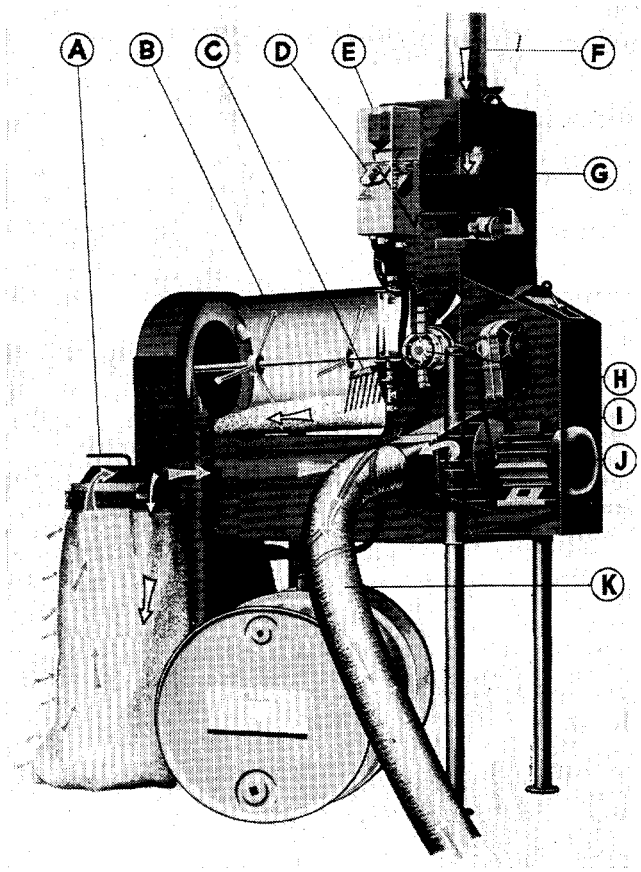
Materials and Methods

Seed Treatment Materials. Radioactive distribution studies were performed with radioactive Swedish Panogen 4925, containing 0.8% weight by volume of mercury as cyano(methylmercuri)guanidine labeled with mercury-203 (*3*). In a demonstration of vapor action 1.5% thallium nitrate tagged with thallium-204 was added to this Panogen formulation then containing two radioisotopes. Experimental formulations with 0.8, 1.5, 3.0, and 4.5% weight by volume of mercury as methylmercurihydroxide and 1.6 and 4.7% as cyano(methylmercuri)guanidine were used in studies of the influence of the liquid dosage on fungicide and dye distribution. Dye concentrations and solvents were varied in these experimental formulations. The following solvent-water ratios were used: methanol 1 to 4 (T_1); ethylene glycol 1 to 4 (T_2), 1 to 2 (T_3), and 2 to 1 (T_4). Distribution measurements were also performed on seeds commercially treated with Swedish Panogen 4925 containing 0.8% weight by volume of mercury as cyano(methylmercuri)guanidine.

Seed Treatment Procedures. Laboratory treatments were performed with 100-gram batches of seed in a rotating bottle (*3*). Two techniques were used for the addition of the disinfectant: liquid poured directly on seeds (poor initial distribution) and liquid poured on walls of the bottle.

The reproducibility of the mechanical mixing process of the laboratory procedures is poor because of the minute amount of liquid added to the small batch of seed. Larger batches are therefore required for tests in which small differences in dye distribution are to be detected. Tests were performed with 20-kg. batches in a drum (diameter 23 inches and length 20 inches), revolving at 42 r.p.m. (This batch size was considered sufficient for reproducibility but is less than the optimum batch to get a good initial coverage in this drum.) The required amount of the disinfectant was added to the seed in the revolving drum in 1 minute. The drum was revolved 10 minutes, after which samples were taken. This mixing time was considered necessary to get representative samples.

Commercially treated seed samples were also examined. The Panogen seed treaters used in these tests were: Models J and JS (capacity 3 tons of small grain per hour); Model K (capacity 10 tons of small grain per hour); and Model A (capacity 4.5 tons of small grain per hour). [Conversion factors: 1 ton (metric) = 1000 kg. = 2205 pounds ~ 36.4 U.S. bushels of wheat; 1 fluid ounce per bushel (wheat) ~ 108 ml. per kg.]



A. Discharge of treated seed
B. Mixing drum
C. Distributing fingers
D. Dipper
E. Constant-level reservoir
F. Inlet spout
G. Scale hopper
H. Feed pump
I. Exhaust fan
J. Electric motor
K. Pipe for exhaust gas

Figure 1. Section through Panogen seed treater, Model A illustrating principle of operation

Figure 1 shows the design and function of the Panogen seed treater, Model A.

Nucleonic Techniques. Beta counting was performed as described (*3*). In case of seeds treated with doubly labeled Panogen containing mercury-203 and thallium-204 the two beta activities were separated by means of a 30 mg. per sq. cm. absorber. Figure 2 shows transmission curves for seed samples treated with mercury-203 or thallium-204, and a mixed sample with 43% mercury-203 and 57% thallium-204 activity. The absorber reduces the mercury-203 activity by 50% and the thallium-204 activity by 10%. If C_{no} denotes the activity observed with no absorber and C_{ab} the activity observed with the 30 mg. per sq. cm. absorber, the appropriate activities, C, are

$$C_{Hg^{203}} = \{C_{no} - 2.0 \times C_{ab}\}/0.8 \quad (1)$$

$$C_{Tl^{204}} = C_{no} - \{C_{no} - 2.0 \times C_{ab}\}/0.8 \quad (2)$$

Chemical Analytical Methods. Mercury in single kernels of treated wheat was determined by flame analysis (*2*). The mercurial is located in the surface layer of the seed and is easily brought into solution by digestion of the surface layer with a drop of nitric acid. The technique previously described in detail for analysis of wheat may be used also with oats, rye, barley, and cotton.

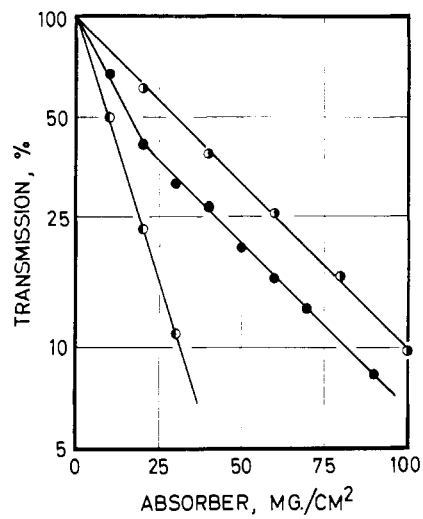


Figure 2. Transmission curves for beta activity of seeds treated with mercury-203 and thallium-204

◐ Only Hg^{203}
◑ Only Tl^{204}
● Mixture of Hg^{203} (43%) and Tl^{204} (57%)

Table I. Evaluation of Dye Distribution on Treated Wheat Seed by Pairwise Comparison

(10 experienced and 10 inexperienced examiners)

Code[a]	Treatment Conditions: Dosage, ml./100 g.	Solvent	Dye concn., % w./v.	Dye concn. on seed	Score (Total Number of Preferred Choices): Experienced	Inexperienced
$F_1T_1H_1$[b]	0.2	T_1	0.35	7	65	65
$F_1T_1H_2$[b]	0.2	T_1	0.40	8	52	51
$F_1T_1H_1$[c]	0.2	T_1	0.35	7	51	50
$F_1T_2H_1$	0.2	T_2	0.35	7	41	43
$F_1T_1H_2$[c]	0.2	T_1	0.40	8	41	41
$F_2T_1H_2$	0.04	T_1	2.00	8	16	17
$F_2T_2H_1$	0.04	T_2	1.75	7	12	12
$F_2T_1H_1$	0.04	T_1	1.75	7	2	1

[a] Dosage: F_1 0.2 ml./100 grams; F_2 0.04 ml./100 grams. Solvent: T_1 methanol-water 1:4; T_2 glycol-water 1:4. Dye applied to seed: H_1 7 p.p.m.; H_2 8 p.p.m.
[b,c] Duplicates of indicated factor combination.

Table II. Seed Treatment with Radioactive Panogen, Active Mercurial Labeled with Mercury-203, and Thallium-204 Nitrate Used as Nonvolatile Tracer

Kernel No.	Observed Activity, C.P.M.: C_{no} (no absorber)	C_{ab} (30 mg./sq. cm. absorber)	Calculated Tracer Activities: Hg^{203} C.p.m.	Hg^{203} Rel. activity	Tl^{204} C.p.m.	Tl^{204} Rel. activity
Seeds Counted 2 to 5 Hours after Treatment						
1	206	66	92	1.0	114	0.8
2	234	76	103	1.1	131	0.9
3	265	101	79	0.8	186	1.3
4	215	69	96	1.0	119	0.9
5	210	66	98	1.0	112	0.8
6	232	87	73	0.8	159	1.1
7	310	108	118	1.2	192	1.4
8	207	64	99	1.0	108	0.8
9	242	82	98	1.0	144	1.0
10	1206	475	320	3.4	900	6.4
Seeds Counted 4 Days after Treatment						
11	161	55	64	1.0	97	0.6
12	292	118	70	1.1	222	1.4
13	287	112	79	1.2	208	1.3
14	202	83	45	0.7	157	1.0
15	189	70	61	0.9	128	0.8
16	181	72	46	0.7	135	0.9
17	184	65	68	1.0	116	0.7
18	1194	497	125	1.9	1069	6.9
19	306	117	90	1.4	216	1.4
20	195	69	71	1.1	124	0.8

(Cottonseeds are cut into smaller pieces prior to nitric acid extraction.) After rinsing with a small portion of distilled water, the aqueous solution is atomized and burned in an oxyhydrogen flame. The combustion gases are purified and assayed for mercury by means of a mercury vapor spectrophotometer. The coefficient of variation for the total analysis is about 7%. This variation is connected with the sample preparation, as only 1% variation is associated with the assay of the mercury content of the aqueous solution (*2*). The detection limit is about 0.02 γ of mercury in this particular application.

Control analyses of larger seed samples were performed by conventional wet digestion and colorimetric dithizone assay (*3*).

Distribution Parameters. The mercurial distribution is characterized by the same kind of distribution parameters that were introduced for the beta counting populations (*2, 4*). The main group of normal kernels, N-kernels, is obtained by exclusion of the few rich kernels, R-kernels, carrying more than twice the average N-kernel mercurial content. The group of N-kernels is treated as a normal distribution and is characterized by its coefficient of variation, also called the spreading error. The R-kernel population is described by three parameters: (1) R = number of R-kernels divided by total number of kernels; (2) S = total quantity of fungicide carried in excess by the R-kernels present, above the N-kernel average, divided by the amount of fungicide added in the process; (3) $T = S/R + 1$, which is the relative dose for the R-kernels referred to the N-kernel average.

Evaluation of Dye Distribution. Commercial seed dressings contain dye for coloring the treated seed to distinguish it from nontreated seed. Frequently the outcome of the seed treatment procedure is considered equivalent to the more or less uniform dye distribution achieved in the mixing process. Some experiments were therefore made to compare dye distribution with fungicide distribution.

Dye distribution should be evaluated as it is in the field—by visual examination. To get reliable and objective results, a statistical procedure is required with a sufficient number of independent examiners and a randomized testing sequence. No distribution parameters are obtained as for the analytical methods, but a ranking list is used.

Samples are compared by twos (all possible different combinations) and the examiner has to decide which is most uniformly colored—a decision has to be made even if the two samples are actually identical. If there are n samples $\left\{n^2 - \sum_1^t v\right\}$ different comparisons have to be made. The tester has to examine the pairs in a randomized order without knowing the identity of the samples. The test is conducted by an interviewer, who records the results. Scores are obtained by summing the total number of comparisons in favor of the actual sample.

Table I reports the result of a test involving two groups of test persons, one group with a long experience in this particular field and a second group with none. Although detectable differences between the seed samples were slight, scores reported by the two groups of examiners were essentially the same. This indicates that the method should give fairly objective results if a sufficiently large number of examiners are consulted. [A closer inspection of the data in Table I demonstrates that the variation between the replicates (laboratory-treated seeds) apparently is larger than the differences to be expected between the two levels of dye concentration in the disinfectant, 0.40 and 0.35%. A poorer dye distribution is obtained for the low volume dosages.]

Experimental Results and Discussion

Demonstration of Vapor Action. The role played by vapor action (*3*) in the Panogen process was demonstrated in a straightforward way by means of a formulation containing nonvolatile thallium nitrate tracer and radioactive mercurial. Table II contains data for wheat seeds treated with this formulation; 3200 counts were taken for each reading, the background being 25 c.p.m. Mercury-203 and thallium-204 activities were calculated by Equations 1 and 2.

The mercurial distribution is more uniform than the distribution of the thallium tracer. The relative activity figures—actual activity divided by the

Table III. Distribution Study Involving Beta Counting and Whole Kernel Assay of Wheat Treated with Radioactive Panogen

Kernel No.	Kernel Weight, W, Mg.	Beta Activity B, C.P.M.	Kernel Assay A, γ Hg	B/W, C.P.M./Mg.	$B/W^{2/3}$, C.P.M./$(Mg.)^{2/3}$	$B/W^{1/3}$, C.P.M./$(Mg.)^{1/3}$	$A/W \times 10^3$, P.P.M.	$A/W^{2/3}$ γ Hg/$(Mg.)^{2/3}$	$A/W^{1/3}$ γ Hg/$(Mg.)^{1/3}$
1	33.5	117	0.51	3.5	11	36	15	0.049	0.16
2	45.8	202	0.94	4.4	16	57	21	0.073	0.26
3	35.2	150	0.65	4.3	14	46	18	0.061	0.20
4	26.0	126	0.64	4.8	15	43	26	0.074	0.22
5	38.2	109	0.59	2.9	10	32	15	0.053	0.18
6	48.2	155	0.73	3.2	12	43	15	0.055	0.20
7	41.8	95	0.78	2.3	8	27	19	0.065	0.23
8	41.7	129	0.66	3.1	11	37	16	0.055	0.19
9	40.1	121	0.63	3.0	10	35	16	0.054	0.18
10	56.8	123	0.80	2.2	8	32	14	0.054	0.21
11	49.9	121	0.85	2.4	9	33	17	0.063	0.23
12	44.9	100	0.53	2.2	8	28	12	0.042	0.15
13	50.8	127	0.67	2.5	9	34	13	0.049	0.18
14	40.2	124	0.63	3.1	11	36	16	0.054	0.18
15	46.5	93	0.66	2.0	7	26	14	0.051	0.18
16	43.4	98	0.56	2.3	8	28	13	0.045	0.16
17	37.7	92	0.38	2.4	8	27	10	0.034	0.11
18	48.5	131	0.81	2.7	10	36	17	0.061	0.22
19	51.7	108	0.47	2.1	8	29	9	0.034	0.13
20	36.0	100	0.49	2.8	9	30	14	0.045	0.15
21	37.0	80	0.46	2.2	7	24	12	0.041	0.14
22	47.1	87	0.38	1.8	7	24	8	0.029	0.11
23	36.0	92	0.38	2.6	8	28	11	0.035	0.12
24	43.1	89	0.40	2.1	7	25	9	0.032	0.11
25	51.5	110	0.53	2.1	8	30	10	0.038	0.14
26	46.2	77	0.42	1.7	6	21	9	0.033	0.12
27	44.0	119	0.71	2.7	10	34	16	0.057	0.20
28	36.9	116	0.64	3.1	10	35	18	0.057	0.19
29	41.8	93	0.58	2.2	8	27	14	0.048	0.18
30	55.3	95	0.24	1.7	7	25	4	0.017	0.06
Av.	43.2	113	0.59	2.7	9.3	32	14	0.049	0.17
Coeff. of variation	0.16	0.23	0.27	0.29	0.26	0.24	0.31	0.27	0.26

average for the N-kernels—furnish striking evidence. Kernels 10 and 18 have very high relative thallium-204 activities of the order of 6 to 7, indicating large initial doses of disinfectant. Because of vapor action, the relative mercury-203 activities are lower, approaching unity with increasing storage time—the relative mercury-203 activity for No. 18 is 1.9 compared to 6.9 for thallium-204, indicating that in this particular case more than 70% of the initial mercury-203 loading has volatilized.

Whole Kernel Assay *vs.* Beta Counting. Different distribution parameters are to be expected for statistics based on whole kernel assay compared to beta counting, which accounts only for the mercurial present on parts of the kernel facing the window of the Geiger tube. Table III reports analytical data for a sample of wheat seed treated with radioactive Panogen. These seeds were first subjected to beta counting and then analyzed by the flame method (2560 counts were taken with a counting error of 2 to 3%). Variation coefficients are reported in the table for primary analytical data and for primary data divided by the kernel weight, W, and by $W^{2/3}$ and $W^{1/3}$. These statistics are discussed below. Whole kernel assay gives larger coefficients of variation than beta counting in this particular example.

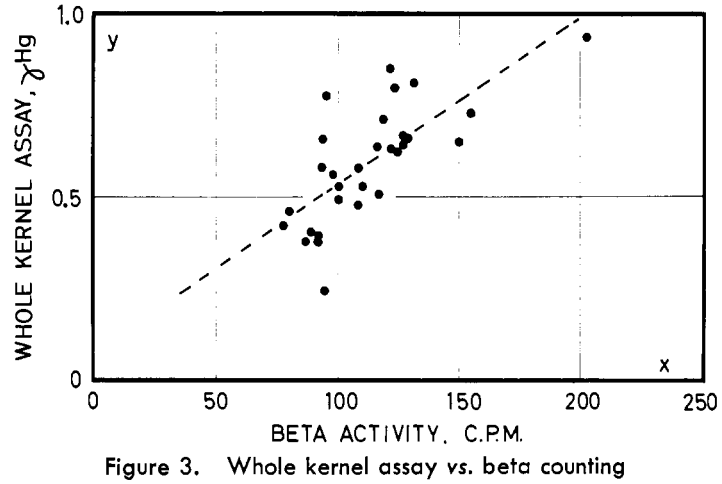


Figure 3. Whole kernel assay *vs.* beta counting

In Figure 3 total mercury contents are plotted against the observed beta activity. The equation of the regression line is $y = (0.0046 \pm 0.0017)\ x + 0.078$ (95% confidence limits), which indicates a significant though weak correlation between these two analytical quantities. The correlation coefficient is $r = 0.72$ with the 95% confidence limits 0.49 to 0.86.

It is assumed that no R-kernels are present, that all kernels are the same size, and that beta counting accounts for the mercury content of $1/f$ of the total kernel surface, f being an integer. Two distribution models are used.

In one model the f surface fractions of a kernel are assumed to be independent and randomized with respect to mercury content. Each kernel is the sum of f surface fractions picked up at random from a large universe. The coefficient of variation based on whole kernel assay is then $\sqrt{1/f}$ of the corresponding statistics based on beta counting (normal distribution). As f is estimated to be about $f = 6$, the ratio between the corresponding coefficients of variation would be ~0.4.

The other extreme is that the f sur-

face fractions of a particular kernel are not independent but are carrying equal amounts of mercury; variations may, however, occur between different kernels. This gives identical statistics for the two populations. A perfect correlation between beta counting and whole kernel assay is then to be expected, which is not the case as shown in Figure 3.

There is, however, always a small proportion of R-kernels found in the beta counting population. This R-kernel population has a major influence on the relations between the two populations studied.

If surface fractions carrying R-kernel activities ("R-surface fractions") are randomly distributed, there is but a small chance that a kernel will carry more than one R-surface fraction. The R-kernel population obtained in the beta counting will then be almost completely eliminated in the statistics based on whole kernel assay of the same sample of seed. The spreading error based on whole kernel assay then becomes smaller or larger than the corresponding beta counting statistics, depending on the properties of the beta R-kernel population.

In practice certain kernels are carrying more than the statistical share of R-surface fractions because of mechanical damage and/or improper mixing. This bias again would tend to give similar R-kernel statistics in the two populations but with the R-kernel dosage (defined by the T factor) smaller for whole kernel assay than for beta counting.

No exact relationship can be derived between the statistics based on whole kernel assay and on beta counting. The distribution studies performed, however, indicate somewhat larger spreading errors, similar R- factors, and smaller S- and T- factors for statistics based on whole kernel assay. This is probably due to a partial transformation of the beta R-kernel population into the N-kernel population observed in the whole kernel assay as discussed above.

Distribution statistics may serve as a guide for evaluating the effectiveness of the seed treatment. Beta counting would be preferred for this purpose because distribution parameters should be evaluated by means of the smallest possible samples of the object. For comparison between different treatments, whole kernel assay is also more convenient.

Comparison between Dye and Fungicide Distribution. The dye distribution obtained in laboratory seed treatment is much influenced by how the few drops of disinfectant are added to the seed batch. This variability, apparent in Table I, may also have an influence on tests, where small differences in fungicide distribution are to be detected. Drum treatment with 20-kg. batches was therefore used in the following experiments, designed to establish: factors influencing the dye distribution; the relationship between dye distribution and mercurial distribution, if any; and the effect of volume dosage on the mercurial distribution. (This treatment did not give a good initial distribution because of the small charge of seed compared to the capacity of the drum and the mode of addition of the disinfectant. It was, however, desired to enlarge any differences between the formulations studied.)

Experimental formulations containing various solvents and varying amounts of dye and fungicide were used as reported in Table IV. The largest and the smallest rate of application differed by a fac-

Table IV. Dye Distribution on Wheat for Various Experimental Formulations

Formulation	Solvent	Hg Concn., % W./V.	Dye Concn., % W./V.	Viscosity (20° C.), Cp.	Surface Tension, (20° C.), Dyn./Cm.	Rate of Application Ml./100 kg.	Rate of Application Fl. oz./bu.	Dye Distribution (13 Examiners) Score	Dye Distribution (13 Examiners) Ranking No.
I	T_1	0.8	0.35	1.0	43.0	335	3	130	3
II	T_1	1.5	0.50	1.0	43.0	98	9/10	56	10
IIIa[a]	T_1	1.5	0.60	2.6	51.2	98	9/10	30	12
IIIb	T_1	1.5	0.60	2.6	51.2	98	9/10	15	13
IV	T_1	1.5	0.90	2.6	50.3	98	9/10	84	6
Va	T_1	3.0	1.4	3.2	51.0	70	2/3	104	5
Vb	T_1	3.0	1.4	3.2	51.0	70	2/3	61	9
VI	T_1	3.0	2.1	3.2	50.7	70	2/3	131	2
VII	T_1	4.5	2.4	3.2	50.3	40	3/8	51	11
VIII	T_1	4.5	3.3	3.6	50.2	40	3/8	110	4
IXa	T_3	1.6	0.53	6.0	56.9	98	9/10	82	7
IXb	T_3	1.6	0.53	6.0	56.9	98	9/10	72	8
X	T_4	4.7	3.4	17.8	53.4	33	3/10	176	1

[a] a and b denote duplicate tests.
T_1. Methanol-water 1:4.
T_3. Glycol-water 1:2.
T_4. Glycol-water 2:1.

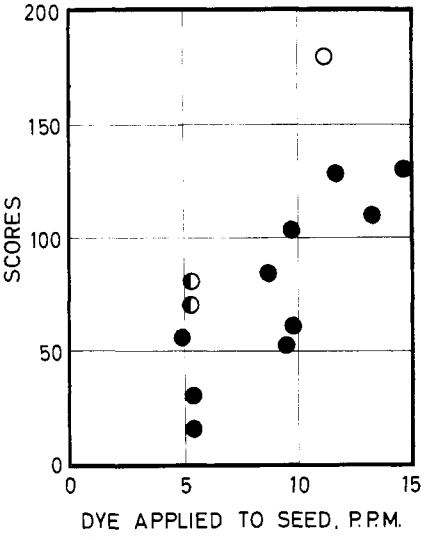


Figure 4. Score for dye distribution plotted as function of amount of dye applied to seed

Formulation types (Table IV)
● T_1 ◐ T_3 ○ T_4

Table V. Distribution Parameters for Wheat Seeds as a Function of Liquid Dosage

(Statistics based on whole kernel assay of 30 kernels. 95% confidence limits reported)

Formulation (Cf. Table IV)	Rate, Fl. Oz./Bu.	Check Analysis of Treated Seed, γ Hg/10 G.	Flame Analysis of Single Kernels, Av. Results γ Hg/kernel	Flame Analysis of Single Kernels, Av. Results P.p.m. Hg	R-Kernel Statistics R	R-Kernel Statistics S	R-Kernel Statistics T	N-Kernel Statistics, Coefficients of Variation γ Hg/kernel, A	N-Kernel Statistics, Coefficients of Variation (Kernel weight)$^{2/3}$ $W^{2/3}$	N-Kernel Statistics, Coefficients of Variation $A/W^{2/3}$	N-Kernel Statistics, Coefficients of Variation $\sqrt{A^2-(W^{2/3})^2}$ W, act. sample	N-Kernel Statistics, Coefficients of Variation $\sqrt{A^2-(W^{2/3})^2}$ W, large sample
I	3	285, 190	1.11 ± 0.19	28.0	1/28	0.05	2.4	0.41 ± 0.11	0.16 ± 0.04	0.36 ± 0.10	0.36	0.36
IIIa	9/10	152, 168	0.69 ± 0.12	16.2	3/30	0.13	2.3	0.32 ± 0.09	0.14 ± 0.04	0.32 ± 0.09	0.29	0.28
Va	2/3	205, 220	0.83 ± 0.14	21.9	2/30	0.10	2.6	0.21 ± 0.06	0.21 ± 0.05	0.24 ± 0.06	0.00	0.14
VII	3/8	197, 175	0.62 ± 0.06	18.0	0/30	0	...	0.26 ± 0.07	0.18 ± 0.05	0.24 ± 0.06	0.19	0.20
IXb	9/10	145, 162	0.71 ± 0.08	16.5	1/30	0.04	2.3	0.23 ± 0.06	0.13 ± 0.03	0.22 ± 0.06	0.19	0.17
X	3/10	236, 180	0.61 ± 0.07	15.4	0/30	0	...	0.32 ± 0.08	0.12 ± 0.03	0.33 ± 0.08	0.30	0.28
Av.		193		19.3	0.04	0.05	2.4	0.30	0.16	0.29	0.25	0.25

Table VI. Distribution Parameters for Wheat Seeds, 17.5% Moisture Content in Laboratory Treatment

(Statistics based on whole kernel assay of 30 kernels. 95% confidence limits reported)

Mode of Addition	Check Analysis of Treated Seed, γ Hg/10 G.	Flame Analysis of Single Kernels, Av. Results: γ Hg/kernel	Flame Analysis of Single Kernels, Av. Results: P.p.m. Hg	R-Kernel Statistics: R	R-Kernel Statistics: S	R-Kernel Statistics: T	N-Kernel Statistics, Coefficients of Variation: γ Hg/kernel, A	N-Kernel Statistics, Coefficients of Variation: (Kernel weight)$^{2/3}$ $W^{2/3}$	N-Kernel Statistics, Coefficients of Variation: $A/W^{2/3}$	N-Kernel Statistics, Coefficients of Variation: $\sqrt{A^2 - (W^{2/3})^2}$
On seed[a]										
a	145, 135	0.58 ± 0.08	13.1	1/30	0.05	2.5	0.28 ± 0.07	0.13 ± 0.03	0.23 ± 0.06	0.23
b		0.54 ± 0.04	11.3	0/30	0	...	0.21 ± 0.05	0.13 ± 0.03	0.17 ± 0.04	0.16
Via wall										
a	130, 125	0.63 ± 0.06	13.8	1/30	0.03	2.1	0.18 ± 0.05	0.13 ± 0.03	0.20 ± 0.05	0.14
b		0.58 ± 0.04	12.6	0/30	0	...	0.20 ± 0.05	0.12 ± 0.03	0.20 ± 0.05	0.16
Av.	134	0.58	12.7	0.02	0.02	2.3	0.22	0.13	0.20	0.17

[a] a and b are duplicates.

tor of 10. The wheat seeds, 12% moisture content, were not cleaned prior to treatment, in a further attempt to get larger differences between the tests. All treatments were made on the same occasion in a randomized order. Dye distribution was evaluated 1 week after treatment and mercurial distribution 3 to 4 weeks after treatment.

Results of the visual evaluation of dye distribution are reported in Table IV. Four groups are recognized, with maximum score for X followed by VI and I; a large group with mediocre score; and finally the poor IIIa and IIIb. Actual differences between the samples were, however, small (except between X and the poorest batches) as indicated, for example, by the differing scores for the duplicates, Va and Vb. No volume effect is recognized in Table IV in contrast to Table I. A most significant feature is, however, revealed in Figure 4, where the score is plotted as a function of the amount of dye applied to the seed in the treatment. Increase of the amount of dye apparently produces an impression of more uniformly colored seed. Some influence of the solvents used is also indicated.

Table V reports fungicide distribution parameters for some of the batches in Table IV. No agreement exists between dye and mercurial distribution: The best and the poorest formulations with respect to dye coverage exhibit about the same N-kernel distribution parameters. No conclusions can be made with respect to the R-kernel statistics, however, as larger samples are required for comparison in this case.

Reduction of the liquid dosage has apparently no adverse effects on the fungicide distribution, as shown by the data in Table V. There seems actually to be some improvement at lower dosages, though this trend is not statistically significant.

The use of a concentrated liquid disinfectant in a small dosage is the basic principle of the Panogen process. The present distribution studies, based on whole kernel assay as well as the earlier radioactive studies (*3*), show that satisfactory distribution patterns are obtained not only in the region of practical interest but also with much smaller volume dosages.

Table VII. Distribution Parameters for Commercially Disinfected Seeds Treated with Swedish Panogen 4925

(Statistics based on whole kernel assay of 30 kernels. 95% confidence limits)

Variety of Seed, Location of Treating Establishment	Moisture Content, %	Dosage, Ml./100 Kg.	Seed Treater	Distribution Parameters: Coeff. of variation (based on γ Hg/kernel)	Distribution Parameters: R	Distribution Parameters: S	Distribution Parameters: T
Spring wheat (Svenno), Hälsingborg, Sweden	17	200	Model JS	0.25 ± 0.06	0/30	0	...
Spring wheat, Skurup, Sweden	17	200	Model K	0.20 ± 0.05	2/30	0.11	2.6
Oats (Solhavre), Skara, Sweden	15	300	Model A	0.30 ± 0.08	2/30	0.08	2.2
Oats (Blenda), Håle-Täng, Sweden	18	300	Model J	0.29 ± 0.07	0/30	0	...

Comparison between Laboratory Treatments. Table VI reports a comparison between the two laboratory treatment procedures used in these studies: addition of disinfectant directly on the seed giving a less uniform initial coverage of the disinfectant than addition via the walls of the treatment bottle. Wheat seeds with a high moisture content (17.5%), which accelerates the resorption processes, were used to accentuate the differences between the two methods. In Sweden the moisture content of seed to be treated is frequently as high (sometimes even higher) as 17.5%, which is the present limit for seed of small grain to be sealed by the Swedish government. Because of the variability of the laboratory methods, representative samples made up of seven replicate treatments were prepared for the flame analysis. The analysis was run in duplicate with 30 kernels in each series. The seeds were stored 14 days prior to the chemical analysis. The checks run with the conventional analytical method compare very well with the averages from the flame analysis, as demonstrated in Table VI. In spite of the high moisture content, no significant differences are obtained between the two treatment procedures. This is in harmony with results of earlier radioactive studies (*3*).

Studies with Commercially Treated Seed. One important advantage of flame analysis is that it can be run on commercially treated seed received from the field. Distribution parameters in Table VII are of the same magnitude as obtained with seeds treated in the laboratory. A similar conclusion was reached by Arny in a biological study (*1*).

Actually the seed itself is likely to be an important source of variation in mercurial distribution. In a radioactive distribution study with a mixture of intact and damaged seed [Table XV, (*3*)] the final distribution was poorer than the initial one because of a very heterogeneous seed sample. The individual kernels in a lot of seed exhibit a large variation with respect to a number of properties, easily subjected to measurement, such as kernel weight, germination, and growth rate, and there is no reason why a similar variation should not be found with the factors governing the mercurial resorption.

Statistical Remarks

The large variation in kernel size in the seed samples studied is likely to contribute to the observed variability for whole kernel assays. To get an estimate of the importance of this source of variation, Tables III and VIII also contain the primary analytical results divided by the kernel weight, (kernel weight)$^{1/3}$, and (kernel weight)$^{2/3}$. Maximum coefficients of variation are obtained when the analytical results are divided by the kernel weight and minimum values when divided by the cube root of the kernel weight. A similar result was obtained by regression analysis on the analytical results underlying Table V. Rates varied in this experiment and therefore the primary figures for each test were converted to percentages, $p\%$, based on the averages (R-kernels excluded). A plot of $\log_{10} p$ *vs.* $\log_{10}$ kernel weight gives a scattered picture, but the larger kernels in general carry more mercury. The equation of the regression line is $y = (0.38 \pm 0.17)\,x + 1.38$. The 95% confidence limits for the slope indicate a significant correlation. For simplicity $y = {}^1/_3\,x +$ constant may be used with the mercury content proportional to the cube root of the kernel weight (a square root relation is, however, not excluded, as shown by the confidence limits). The correlation coefficient for p and (kernel weight)$^{1/3}$ is $r = 0.33$ with the 95% confidence limits 0.19 to 0.46.

In the earlier radioactive studies (*3*) it was assumed that under ideal conditions the mercury content should be proportional to the surface area of the kernel—i.e., proportional to (kernel weight)$^{2/3}$. The spreading error was then obtained by subtracting this surface variancy from the experimentally observed variancy. This procedure, exemplified in the column for $\sqrt{A^2 - (W^{2/3})^2}$ in Tables V and VI, is not justified in the light of the present findings, which indicate only a weak correlation between mercury content and kernel size. The best procedure is then to estimate the spreading error directly from the population of γ Hg/(kernel weight)$^{1/3}$, for example. This gives slightly higher coefficients of variation, as shown by Tables V and VI.

N-kernel statistics are derived assuming normal distributions. Distribu-

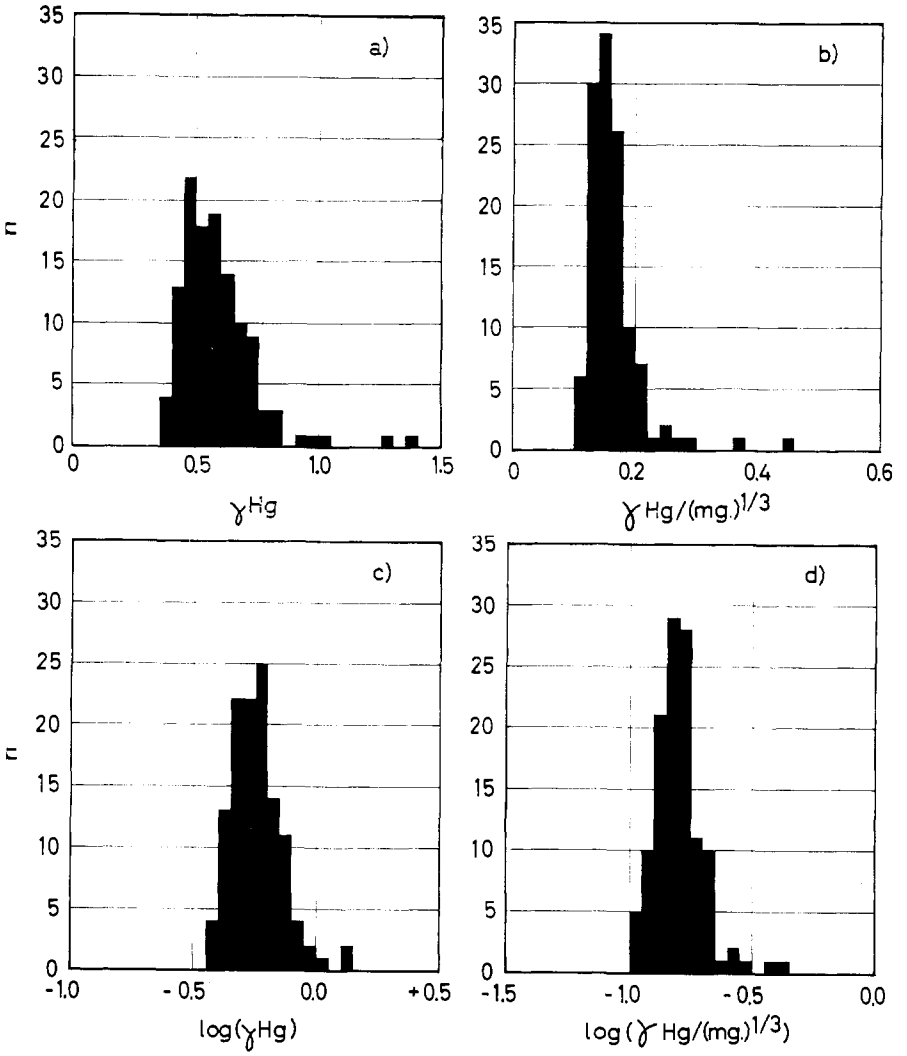


Figure 5. Histograms based on whole kernel assay

a. Primary data, A, γ Hg/kernel
b. $A/W^{1/3}$, γ Hg /(kernel weight)$^{1/3}$
c. $\log_{10} A$
d. $\log_{10} A/W^{1/3}$

Table VIII. Comparison between Coefficients of Variation Estimated in Different Ways

Reference Table V	N-Kernels Only, Primary Data				All Kernels (R + N), Primary Data		All Kernels (R + N), Logarithms		N-Kernels Only, Logarithms	
	A	$A/W^{1/3}$	$A/W^{2/3}$	A/W	A	$A/W^{1/3}$	Log A	Log $A/W^{1/3}$	Log A	Log $A/W^{1/3}$
I	0.409	0.374	0.356	0.361	0.464	0.433	0.613	0.543	0.565	0.497
IIIa	0.323	0.309	0.320	0.350	0.488	0.484	0.528	0.502	0.373	0.352
Va	0.210	0.199	0.237	0.326	0.449	0.420	0.397	0.366	0.236	0.224
VII	0.261	0.241	0.241	0.300	(0.261)	(0.241)	(0.311)	(0.272)	0.311	0.272
IXb	0.233	0.219	0.224	0.263	0.322	0.323	0.314	0.313	0.252	0.246
X	0.315	0.311	0.329	0.383	(0.315)	(0.311)	(0.385)	(0.377)	0.385	0.377
Table VI										
On seed										
a	0.282	0.248	0.228	0.234	0.374	0.395	0.348	0.345	0.279	0.254
b	0.206	0.174	0.166	0.183	(0.206)	(0.174)	(0.219)	(0.187)	0.219	0.187
Via wall										
a	0.179	0.183	0.199	0.243	0.254	0.266	0.249	0.248	0.197	0.187
b	0.201	0.194	0.201	0.247	(0.201)	(0.194)	(0.221)	(0.207)	0.221	0.207
Totals	2.62	2.44	2.51	2.88	3.37	3.23	3.58	3.36	3.23	2.77

Some samples contained no R-kernels, indicated by parenthesis in R + N columns.

tions are, however, slightly skewed with a deficit of low values, which also is apparent from the summarized data in Table XIV of (*3*).

The slightly nonsymmetrical frequency distributions observed in histograms *a* and *b* in Figure 5 are based on data underlying Table VI. An attempt is made to normalize the data by using logarithms, histograms *c* and *d*. The latter histograms are more symmetrical, which raises the question of whether normalization of primary data in this way would be a preferred route. Use of logarithms may, furthermore, diminish the differences between the R- and N-kernel populations to the extent that separation into two populations may even no longer be necessary in case of whole kernel assay, where differences between R- and N-kernels are moderate.

Table VIII illustrates these questions. The coefficients of variation reported here are based on: primary N-kernel data; primary (R+N)-kernel data; logarithms of N-kernel data; and logarithms of (R+N)-kernel data taken from the experiments in Tables V and VI. (The totals quoted in the bottom row of the table have no physical meaning but are introduced as a guide for comparison between the studied statistical procedures.) The primary N-kernel statistics show again minimum variation for $A/W^{1/3}$. Differences between statistics based on A, $A/W^{1/3}$, and $A/W^{2/3}$ are small, whereas A/W gives a higher variancy.

The practical conclusion of this is that although $A/W^{1/3}$ is the most correct choice, primary analytical data, A γ Hg/kernel, may be used as well in practice. Statistics based on A/W—e.g., p.p.m. figures—should, however, not be used. Logarithms give more symmetric distributions but do not reduce the coefficients of variation. The coefficient of variation reported in Table VIII for logarithims is the antilogarithm minus 1. (In this example an increase is actually observed due to the more sharply peaked distribution with a broad base indicated by the histograms *c* and *d* in Figure 5.) Statistics based on the combined (R + N)-kernel populations give much larger coefficients of variation, and consequently the separation into two populations seems justified also for populations based on whole kernel assay.

Conclusions

Distribution statistics based on whole kernel assay differs slightly from statistics based on the previous beta counting technique, but the same approach involving two populations, R- and N-kernels, should be used. Coefficients of variation for the N-kernel population, "spreading error," are about the same with the two methods, whereas R-kernel statistics exhibit larger differences with higher readings for beta counting R-kernels. Only a weak correlation exists between fungicide content and kernel size. The fungicide content is roughly proportional to the cube root of the kernel weight and not to the two thirds power as might be expected. In practice primary analytical results—e.g., γ Hg/kernel—may be used as well as data corrected for kernel size, in view of the weak correlation between size and mercurial uptake.

The results of the earlier radioactive distribution studies were confirmed by the present studies. Further proof is presented that the mercurial fungicide and the dye are distributed in different ways. The impression of dye uniformity is governed very much by the amount of dye used, which has no connection with the mercurial distribution. Whole kernel assay studies could also demonstrate that the small liquid dosages characteristic of the Panogen process can be reduced further, well below present volume rates, with no adverse effects on the mercurial distribution. This indicates a considerable margin of safety with the method. Laboratory treatment and large scale commercial treatment give similar distribution patterns because of the vapor action of these mercurial fungicides.

The spreading errors for the commercially treated seeds are of the order of 0.2 to 0.3, which indicates a sufficiently uniform distribution to produce but a negligible drop in disinfection efficiency (*4*). Estimates of the spreading error are furthermore conservative, as this statistics is actually mainly governed by kernels with larger amounts of mercurial. The distribution curves have no "tail" with deficient kernels.

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FEED ADDITIVES

Determination of 3,5 Dinitro-*o*-toluamide (Zoalene) in Feed Concentrates

GRANT N. SMITH

The Dow Chemical Co., Midland, Mich.

Premix concentrates containing 25% 3,5-dinitro-*o*-toluamide (zoalene) with soybean meal are being used in the feed to aid in the prevention of cecal and intestinal coccidiosis in chickens. In the manufacture of such a premix concentrate it is essential to have a rapid easy-to-handle analytical method, with a high degree of accuracy, reproducibility, and specificity, for quality control.

The method which has been developed, using the colorimetric procedure previously described (*1*), is based on the extraction of the 3,5-dinitro-*o*-toluamide from the feed concentrate with dimethylformamide. This solution is then mixed with a solution of methylamine. The reaction results in the formation of a purple-colored complex, the absorbance of which can be read at wave length 550 mμ in a Beckman spectrophotometer. The intensity of the color is proportional to the concentra-

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tion of 3,5-dinitro-*o*-toluamide in the solution.

Analytical Procedure

A 1-gram sample of premix concentrate is suspended in 200 ml. of dimethylformamide in a 600-ml. beaker. The suspension is warmed slightly on a hot plate and stirred continuously for 5 minutes to ensure complete solution of the 3,5-dinitro-*o*-toluamide. The warm solution is transferred to a 1-liter volumetric flask using dimethylformamide to wash the beaker and transfer the feed residue. The solution is cooled to 20° C. and diluted to volume with dimethylformamide. The solution is thoroughly mixed, and the insoluble feed particles are allowed to settle. A 10-ml. aliquot of the extract is then transferred to a 100-ml. volumetric flask, and the sample is diluted to 100 ml. with dimethylformamide.

After thorough mixing, a 10-ml. aliquot of the diluted extract is transferred to a 25-ml. volumetric flask and the sample is diluted to volume with a 40% aqueous solution of methylamine. The methylamine solution is previously cooled and maintained at 3° C. After thorough mixing, the absorbance of the sample is determined in a Beckman spectrophotometer at a wave length of 550 mμ. The sample should be read exactly 3 minutes after the methylamine solution is added to develop the color.

A reference standard and feed blank are run with each set of determinations. A reference standard solution is prepared by dissolving 250 mg. of a reference standard of 3,5-dinitro-*o*-toluamide in 1 liter of dimethylformamide previously adjusted to 20° C. A 10-ml. aliquot of the reference solution is transferred to a 100-ml. volumetric flask and diluted to volume with dimethylformamide (20° C.). After thorough mixing, a 10-ml. aliquot of the diluted reference standard solution is transferred to a 25-ml. volumetric flask and diluted to volume with the methylamine solution (3° C.). The absorbance of the solution is then determined in a spectrophotometer at a wave length of 550 mμ. The solution should be read exactly 3 minutes after the methylamine solution is added to develop the color.

A feed blank should be prepared by extracting a 750-mg. sample of the soybean meal present in the premix concentrate. The feed blank sample is handled in the same manner as described above for the determination of 3,5-dinitro-*o*-toluamide in the sample of premix concentrate.

The absorbances of the feed blank solution and the standard reference solution should be determined at the same time the absorbances of the various solutions of the premix concentrate are being determined.

Calculation of Percentage of 3,5-Dinitro-o-toluamide in Premix Concentrate

In the procedure described above, the percentage of 3,5-dinitro-*o*-toluamide in the premix concentrate is determined by comparison with a standard reference sample.

Manufacturing specifications for the premix concentrate prescribe that it contain 25% of 3,5-dinitro-*o*-toluamide. Therefore, a 1-gram sample of the concentrate should contain 250 mg. of the drug. In the procedure described above, the 3,5-dinitro-*o*-toluamide in a 1-gram sample of the concentrate is extracted from the soybean meal and carried through three dilution steps to give a concentration of approximately 10 γ per ml. in the final solution, the absorbance of which is read in the spectrophotometer. The reference standard and the feed blank are handled in a similar manner.

The percentage of 3,5-dinitro-*o*-toluamide in the premix concentrate samples can therefore be calculated from the following equation:

$$\% \text{ 3,5-dinitro-}o\text{-toluamide} = \frac{A - B}{C} \times 25$$

A = absorbance of the premix concentrate sample
B = absorbance of the feed blank
C = absorbance of the reference sample

Discussion and Experimental Results

The three general types of reactions proposed for the determination of aryl dinitro compounds described previously (*1*) were tested with 3,5-dinitro-*o*-toluamide in premix concentrates. The reduction reaction gave erratic results because of the difficulties in controlling the reduction of the compound. Apparently the amide group was destroyed at the same time that the nitro groups were being reduced. The free carboxyl group could then react with the amino groups as they were formed to produce a compound which could no longer be diazotized and coupled. Furthermore, most of the reduction reactions produced a mixture of mono- and diamino toluamides and toluic acids.

Treatment of 3,5-dinitro-*o*-toluamide with acetone and alkali resulted in the formation of a green complex which exhibited an absorption peak at 640 mμ. The color was very intense but extremely transient. Because of the evanescent properties of this complex it was impossible to measure the intensity of the solution. Similar reactions could be obtained with ethyl alcohol, methyl ethyl ketone, acetonitrile, pyridine, and dimethylformamide. The dimethylformamide gave the most stable complex, but here again the color was not stable enough to be used as a colorimetric procedure.

The potassium cyanide reaction gave a yellow complex which exhibited an absorption peak at 360 mμ. The color produced was very stable and not greatly influenced by environmental conditions. Two disadvantages were found with this reaction. Many of the premix concentrates contained plant pigments which had absorption peaks in the same regions of the spectrum as did the cyanide complex (300 to 400 mμ). In addition, the test was not specific for the dinitro-*o*-toluamide but would give a positive test with all the closely related compounds of the dinitro-*o*-toluamide series.

The reaction of 3,5-dinitro-*o*-toluamide with methylamine in the presence of dimethylformamide to form an intense purple complex previously described (*1*) had three distinct advantages as the basis for a colorimetric procedure for the determination of 3,5-dinitro-*o*-toluamide in premixes. The reaction was specific for the 3,5-dinitro-*o*-toluamide. None of the possible decomposition products of the drug would give a positive reaction. The complex had a very intense color which permitted the detection of microgram quantities of the drug. The absorption peak exhibited by the complex was in a portion of the visible spectrum in which plant pigments did not exhibit absorption bands.

With a large concentration of 3,5-dinitro-*o*-toluamide in the premix (25%) and a very sensitive colorimetric method, it was possible to dilute the premix extract to such an extent (the equivalent

of 1 gram of premix in 25 liters of solution) that the pigments and other components of the premix would not contribute any significant amount of color to the final solution.

It was possible to analyze the premix for 3,5-dinitro-*o*-toluamide by simply extracting the drug from the feed with dimethylformamide and reacting a diluted aliquot of the extract with methylamine or 1,3-propanediamine.

To determine if the proposed method would be reproducible, three premixes were prepared and analyzed for the drug. Each sample was analyzed on three successive days using different reagents each time to be sure that the same results would be obtained under various laboratory conditions. The data in Table I indicated that reproducible results can be obtained with this method. Preliminary experience with the method indicated that any variations that might be observed could generally be ascribed to dilution error or lack of precision in the development of the colored complex and measurement of its absorbance. The factors influencing color formation and the stability of the complex have been described in great detail in a previous paper of this series (*1*) and will not be elaborated on here. To obtain reproducible results, it is absolutely essential that the color development steps be carried out precisely as described.

A standard curve was prepared using the procedure described and, when plotted, gave a straight line having a slope of 0.056 ± 0.002 absorbance unit per microgram of 3,5-dinitro-*o*-toluamide per milliliter of solution. Because of the various factors, such as temperature, etc., which might influence this determination, it is advisable to run a reference standard with each set of determinations and to calculate the concentration of the drug in the unknown samples of premix in terms of the reference standard rather than using a standard curve.

The recovery of 3,5-dinitro-*o*-toluamide from various premix concentrates was determined by analyzing a series of feed concentrates of known composition. This was done by mixing from 200 to 350 mg. of 3,5-dinitro-*o*-toluamide with sufficient soybean meal to give 1-gram samples. The samples were handled in such a manner that there could not be any loss of the drug in mixing or transferring the samples before they were analyzed. The absorbances of the samples were compared with those of the premix feed blank and the reference standard (Table II). The feed blank generally was in the range of 0.005 to 0.009 absorbance units when compared with water. When compared with the reagent blank, the absorbance of the feed blank was insignificant and could be ignored.

Table I. Reproducibility of Analytical Procedure for Assay of 3,5-Dinitro-o-toluamide in Premix Concentrate

	3,5-Dinitro-o-Toluamide, %			
		Found		
Sample	Added to sample	1st analysis	2nd analysis	3rd analysis
1	25.2	25.2	25.0	25.0
2	24.0	24.0	24.1	24.0
3	26.2	26.2	26.0	26.1

Table II. Recovery of 3,5-Dinitro-o-toluamide from Premix Concentrate

Sample	Concn. of 3,5-Dinitro-o-toluamide in Premix, %	3,5-Dinitro-o-toluamide per Gram Sample of Premix, Mg.		Recovery, %
		Added	Found	
1	21.5	215.0	213.0	99.1
2	22.4	224.5	217.5	96.9
3	23.7	237.5	219.8	92.5
4	25.3	253.2	243.2	96.0
5	25.6	256.0	256.8	100.3
6	26.1	261.0	260.8	99.9
7	26.5	264.8	250.5	94.6
8	26.7	267.5	252.8	94.5
9	27.7	277.0	266.8	96.3
10	28.3	282.8	279.0	98.6
11	28.5	285.0	282.8	99.2
12	29.1	290.8	294.0	101.1
13	30.4	304.0	295.0	97.0
14	31.5	315.0	304.0	96.5
15	32.9	329.0	316.0	96.0
				Av. 97.2 ± 2.6

The results in Table II indicate an average recovery of 97.2 ± 2.6%, which is satisfactory for the determination of 3,5-dinitro-*o*-toluamide in premix concentrates.

Table III shows the results obtained by analyzing several lots of commercially prepared premix concentrates.

During this investigation, it was observed that 1,3-propanediamine could be substituted for the methylamine used in this reaction and the same results would be obtained. The diamine gave a more stable complex and more intense color. The use of the diamine, however, has been limited because of its availability and cost. Recently it has been indicated that the 1,3-propanediamine will be produced commercially in the near future by the Union Carbide Chemicals Co. at a price which will permit its use in routine analysis (*2*). When this material becomes available it would be desirable to use the 1,3-propanediamine in place of the methylamine and to measure the absorbance of the colored complex at 560 mμ.

Table III. Recovery of 3,5-Dinitro-o-toluamide from Commercially Prepared Samples of Premix Concentrate

	3,5-Dinitro-o-toluamide %	
Lot	Theoretical	Found
1	25.0	25.2
2	25.0	25.0
3	25.0	24.6
4	25.0	25.3
5	25.0	24.8

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Microanalysis of Piperazine

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A method of analysis for piperazine (diethylenediamine) which requires a minimum of time and sample handling is described. The material is easily extracted from animal feeds, and when the extract is suitably treated—a portion made alkaline and then reacted with 1,4-benzoquinone—a red-orange color is produced. The color reaction is specific for piperazine in feeds, as there are no related materials added to feeds that will interfere.

MEDICINALLY, piperazine has been used as an anthelmintic in humans and animals. Many uses have been developed and suggested for piperazine derivatives (*4*, *8*). Leng (*9*) describes an analytical method for determining piperazine in feeds, which, however, requires rather large samples and is too long for routine use. Cavett (*2*) and Perlmutter (*10*) investigated a method of analysis which uses steam distillation to isolate the piperazine and an acid medium for color development between the drug and *p*-quinone.

Several semimicromethods for the analysis of piperazine have been published, but all lack the sensitivity required for parts per million analysis. The use of chloroanil (*3*) (tetrachloro-1,4-benzoquinone) and *p*-quinone (*6*) has been reported for the identification of the presence of certain amines.

Reactions between carbonyl compounds and certain amines are discussed by Fuson (*7*). The necessary ingredients for such reactions appear to be an amine, an alkali, and a carbonyl compound. Some of the products are colored. In the case of *p*-quinone and piperazine, the quinoidation is enhanced by the alkaline medium and a colored complex is formed. The reaction of a quinone with an amine is also analogous to that described by Feinstein (*5*) for pyrethrins and allethrin.

Experimental

The color reaction of *p*-quinone with piperazine was found to be quantitative if certain precautions and techniques were followed.

Piperazine and its salts are highly water-soluble; hence this approach was tried first. However, a dilute acid would remove piperazine from feeds much better than water alone. Settling or centrifugation failed to give a truly clear solution for further use. Filtering the aqueous extract through a mat of Super-Cel gave a clear solution. A suitable aliquot could then be taken for color development. The color reagent is prepared largely as described by Foucry (*6*). The *p*-benzoquinone is purified by steam distillation under a hood. Then, 0.10 gram of the compound is dissolved in 2.5 ml. of glacial acetic acid in a 100-ml. volumetric flask and diluted to the mark with 95% ethyl alcohol. This solution should be freshly prepared each time.

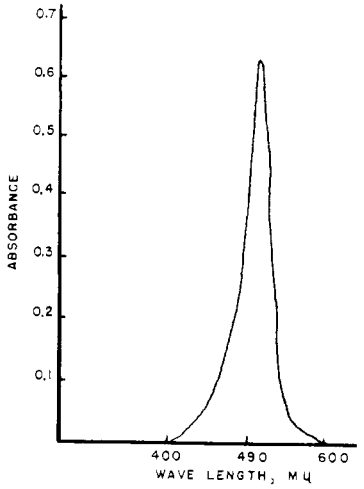

Figure 1. Absorption curve for piperazine

The concentration of potassium hydroxide solution chosen for use in the procedure provides a sufficient excess of alkali to catalyze the reaction between the piperazine and the quinone and maintain an alkaline pH.

Method

Standard Curves. Prepare a standard solution of piperazine by weighing 200 mg. of pure piperazine, or the equivalent of its salt, into a 100-ml. volumetric flask. Dilute to volume with distilled water and mix well. Transfer 0-, 5-, 10-, 15-, and 20-ml. aliquots to 100-ml. volumetric flasks; to each add 0.3 ml. of 12*N* sulfuric acid. Dilute each to volume with distilled water and mix well. Transfer a 20-ml. aliquot from each to a 200-ml. volumetric flask and dilute to volume with distilled water and mix well. Take a 5.0-ml. aliquot from each of these and place it in a test tube. Add 5.0 ml. of the quinone reagent to each tube and mix. Add 1.0 ml. of 1*N* potassium hydroxide in 50% ethyl alcohol to each tube and again mix. Heat the tubes in an 80° C. water bath for 10 minutes. Remove the tubes from the water bath and place them in a larger beaker of cold water. As soon as the tubes have cooled to room temperature, determine the absorbance of each sample. Use the zero concentration tube for a blank. The spectrophotometer is set at 490 mμ for maximum absorbance.

To prepare a standard curve in the

Table I. Recovery of Piperazine Added to Blank Feed

Piperazine, γ			
Added		Recovered	Recovery, %
50		54	108
		56	112
		48	96
		49	98
		52	104
	Av.	51.8	103.6
100		97	97
		97	97
		99	99
		99	99
	Av.	98	98.0
150		154	103
		154	103
		146	97
		147	98
	Av.	150.2	100.1
200		203	102
		200	100
		196	98
		193	96
	Av.	198	99.0
250		250	100
		250	100
		243	97
		253	101
	Av.	249	99.6

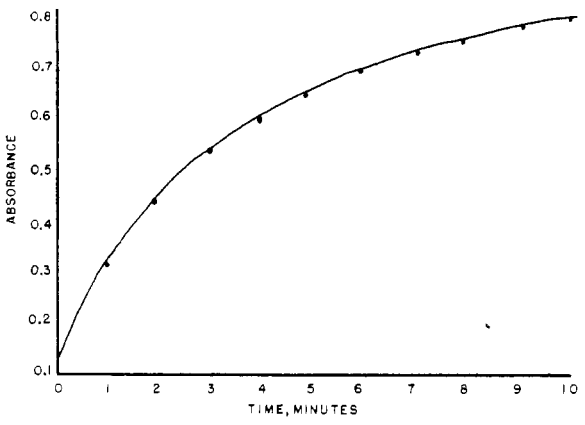

Figure 2. Effect of time on color concentration, 80° C.

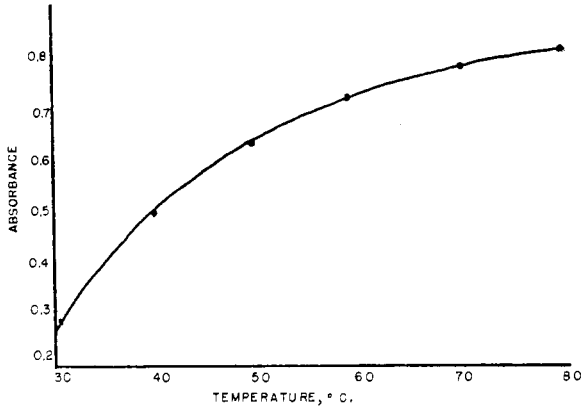

Figure 3. Effect of temperature on color concentration, 10 minutes

presence of feeds, weigh 10.0-gram samples of a blank feed into six 250-ml. Erlenmeyer flasks. A suitable blank feed should be prepared as has been previously described (*1*). Add 0-, 5-, 10-, 15-, and 20-ml. aliquots of the standard piperazine solution to the flasks. Add 0.3 ml. of 12*N* sulfuric acid to each and add sufficient distilled water to each flask to total 100 ml. of liquid. Shake the flasks intermittently for 30 minutes.

Allow the heavy particles to settle. During the settling period (10 to 15 minutes), fit a Büchner funnel into each of six 250-ml. suction flasks. Add a filter paper (S & S No. 597) to each of the funnels and cover the paper with a $^1/_4$-inch layer of Hyflo Super-Cel. Spread the Super-Cel evenly and apply suction. Pour a few milliliters of the extract from each flask over the Super-Cel mats taking care to wet them all over. Release the suction and discard the filtrate. Reconnect the flasks and suction, and pour the balance of the extract onto the mat. The filtrate must be clear; refilter if it is not. Take a 20.0-ml. aliquot from each filter flask and transfer to 200-ml. volumetric flasks, diluting to the mark with distilled water. After mixing, transfer a 5.0-ml. aliquot to a test tube and follow directions under Standard Curves beginning with ". . . add 5.0 ml. of the quinone reagent . . ."

Analysis of Samples. Weigh feed samples containing piperazine in such a way that the final solution in the determination will contain approximately 100 γ of piperazine. For feeds containing 0.05% piperazine, weigh 10.0-gram samples. To ensure reliability of results, run a reagent blank with each set of samples and use it as the blank for setting the spectrophotometer. The analysis of samples follows the preparation of a standard curve in the presence of feed, omitting the addition of the standard piperazine solution.

Results and Discussion

To test the method for applicability, several effects were studied. Feeds with varying amounts of protein and fat, as well as specific ingredients such as corn, soybean meal, cottonseed meal, meat scraps, fishmeal, alfalfa, feathers, and milk by-products, were analyzed against the laboratory prepared blank. No interference was found from any of the above listed ingredients. Recoveries of piperazine added to the above feeding ingredients ranged between 98.0 and 101.0%. The presence of medicaments such as nicarbazine, arsanilic acid, 3-nitro-4-hydroxyphenylarsonic acid, sulfaquinoxaline, and furazolidone in feeds does not interfere with the analysis. The data recorded in Table I show that piperazine added in solution in graded amounts to a blank feed may be recovered nearly quantitatively. Figure 1 shows the shape of the absorption curve using a solution containing 100 γ of piperazine in the final volume of 11.0 ml. Figures 2 and 3 show the effects of time and temperature on color development of solutions containing 150 γ of piperazine in the final aliquot and indicate that a maximum is approached at 10 minutes at 80° C. This time and temperature must be carefully controlled in the analysis of samples.

The procedure described requires a minimum of time for analysis and a minimum of sample handling. One point in the procedure requires careful attention: The temperature must be carefully controlled and the time of heating must be exact. Experiments have shown that the color increases with both heat (up to the boiling point of the alcohol) and time. The choice of the 10-minute heating period and 80° C. temperature is explained by examination of Figures 2 and 3, which show that a plateau is reached at this specific time and temperature. Heating beyond this time does not materially alter the color concentration, and a higher temperature would cause a loss of alcohol from the solution.

Experimental results were obtained using a Beckman DK-2 ratio-recording spectrophotometer. Other instruments whose wave length may be set at 490 mμ should be suitable for use.

The conditions described in the procedures and methods were maintained throughout the experimental phases of the work to develop the curves and to obtain the data presented. The alkali reagent, the experimental feed, and the quinone reagent used were equivalent for all the work reported here.

An $E^{1\%}_{1cm.}$ value of 590 has been obtained using the procedure described and Beer's law is followed over the concentration range used.

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Comparison of Enzyme and Waring Blendor Methods for Determination of Collagen in Beef

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Waring Blendor–centrifuge procedures often yield higher collagen nitrogen values in cooked than in raw beef. This may be due to inadequate blender dispersion of coagulated protein masses in cooked beef. The dispersive action of a protease inactive toward collagen was compared with that of the blender. Collagen values were lower in cooked than in raw meat by both methods, but differences between raw and cooked samples were generally greater by the enzyme method. The enzyme method yielded significantly lower collagen values for both raw and cooked beef, and these values showed better relationship to shear values and tenderness scores than those obtained by the blender method. The enzyme method offers promise for a procedure that is reproducible, rapid, and feasible for multiple sample operation.

PRESENT methods for chemical analysis of collagen in muscle tissue give inconsistent results and are time-consuming. The greatest difficulty lies in separating the soluble nitrogen-bearing components of muscle (protein and nonprotein) from connective tissue proteins. There have been many approaches to the problem, but the objectives of all methods were to facilitate the separation of muscle protein fractions and to minimize sampling variation. Miller and Kastelic (*7*) have reviewed chemical methods for determining collagen.

The Waring Blendor method of Hartley and Hall (*4*) used in this laboratory gave reproducible results for raw, but not for cooked beef. Moreover, it often indicated an apparent increase in collagen nitrogen during the cooking of meat. Hence, further work on this method seemed advisable.

The Waring Blendor method (for brevity, the blender method) consists of processing tissue and water in a Waring Blendor, adjusting to the apparent isoelectric point (*3*), pH 5.0, and washing with water by centrifugation. The high collagen values obtained by this method were attributed to inadequate dispersion of the coagulated protein masses.

Without extensive review of the literature, it is assumed that most meat researchers are aware of the controversial question of the importance of collagen to tenderness of meat. Such controversy may be expected because of the difficulty in defining the identity of collagen in tissue and estimating the amount therein. This investigation was aimed at developing some simple techniques that may help to overcome some of the difficulty mentioned above, and to permit a procedure for determining collagen that is reproducible, rapid, and feasible for multiple sample operation.

The procedures should be within the grasp of laboratory assistants without extensive and critical training and experience. Many meat research programs might be able to promote a project of this sort as one of several concurrent efforts involving the same carcasses or cuts of known history. This type of technology is not to be confused with the critically needed fundamental exploration typified by the paper of Loyd and Hiner (*6*). Their procedure would be outside the scope of many laboratories, both in the time involved and in qualified personnel. For these reasons, it was hoped to avoid the difficult-to-separate alkaline colloidal dispersions and critical hydroxyproline techniques involved in the method of Loyd and Hiner, and in the similar alkaline techniques of Lowry, Gilligan, and Katersky (*5*) and Miller and Kastelic (*7*).

In this investigation proteolytic enzymes, inactive toward collagen, were used to open up the complex connective tissue structure by hydrolysis of the simpler proteins, so that soluble nitrogenous material could be washed out by centrifugation. The objectives were to compare this procedure with the blender method, and to study the relationships among collagen nitrogen values, shear values, and tenderness scores.

Materials and Methods

Meat Used. Ten top rounds of beef, graded U. S. Good, and ripened for similar periods were purchased from a local wholesale meat company. Beginning at the proximal end, four 1.5-inch slices were cut from each top round, wrapped individually in aluminum foil, and stored at −20° F. until used.

Cooking Procedure and Shearing and Tenderness Tests. The experimental design provided for the slices from each round to be divided into two blocks. The first two slices from the proximal end of the round were in one block and the third and fourth slices in the other. One slice from each block was selected at random to be cooked under 10 pounds' pressure for 30 minutes and the other slice for 45 minutes.

A grid, prepared to conform to the shape of the semimembranosus muscle in the slices of top round, was divided into 1-inch squares. The squares were numbered, and a table of random numbers was used to select the numbers on the grid that represented the position in each slice to be used for shearing tests and chemical analyses and for organoleptic tenderness evaluation.

Prior to cooking, the slices were thawed 48 hours in a refrigerator (40° F.). Six 1-inch cores for determining raw shear values were removed from each slice. The slice was placed on a rack in an electric pressure cooker with $^1/_4$ cup of water and cooked under 10 pounds' pressure for either 30 or 45 minutes.

Six 1-inch cores were removed from the cooked slice for shear value determinations and six samples $^1/_8$ inch thick were scored for tenderness by a palatability panel. Tenderness scores were given within a range of 10 to 1; 10 represented extremely tender meat and 1, extremely tough meat. Both raw and cooked cores were sheared on a Warner-Bratzler (*9*) shearing apparatus. An average of the values obtained from 18 shears was the raw or cooked shear value for each slice. After shearing, the cores were ground

twice in a Universal No. 3 hand grinder with a fine blade, packed in 4-ounce jars with screw caps, and stored at −20° F. until analyzed for collagen nitrogen.

Chemical Analyses. The frozen, ground meat was thawed in a refrigerator. After being mixed thoroughly, duplicate 10-gram samples were analyzed for blender and enzyme collagen nitrogen.

Blender Method. In the Hartley and Hall (*4*) method, when the blender homogenate was washed with distilled water, turbidity appeared in the second or third washing. This condition was not suppressed by use of dilute calcium chloride, magnesium chloride, or sodium chloride wash solutions. The late appearance of the turbidity suggested that it might be caused by loss of some soluble muscle tissue constituent. Phosphate was suspected, and 0.06*M* phosphoric acid(approximately the concentration in muscle tissue) adjusted to pH 5.0 with sodium hydroxide was used as a wash solution. Turbidity was reduced greatly, and the solids packed firmly in centrifuging. Möhler and Kiermeier (*8*) observed that sedimentation of aqueous suspensions of finely minced meat was increased by addition of phosphate salts. Their interpretation was that an increase in cohesion occurred between protein particles.

Extraction with the phosphate wash removed about 30% of the total nitrogen from raw meat and about 10% from cooked meat. This is considerably less than the 80 to 90% removed from raw beef with 0.1*N* sodium hydroxide as reported by Miller and Kastelic (*7*). The phosphate, being essentially indifferent to muscle tissue in kind and concentration of solute, seems less likely to alter protein structure, but still capable of removing nitrogenous components soluble at the apparent isoelectric point of muscle tissue. The insoluble residue then may be autoclaved, and the soluble gelatin extracted from the residue without contamination by native soluble nitrogen-bearing components. After gelatin removal, the residue apparently contained the greater bulk of the muscle protein.

About one third of the nitrogenous material extracted from raw meat was heat-coagulable. When the coagulate from one sample was washed and autoclaved, the soluble hydrolyzate contained one twentieth as much nitrogen as the blender collagen found in the same sample. In cooking, this material is coagulated in varying degree before extraction, and in autoclaving it may be hydrolyzed partially and go into the gelatin fraction. Thus, it may help to cause apparently high values for blender collagen in cooked beef.

The turbidity that developed in washing the autoclaved residue with boiling water in the Hartley and Hall (*4*) method was avoided by use of boiling 0.2% sodium chloride solution. Rapid filtration and clear filtrates resulted.

Enzyme Method. Three enzymes (Protease 15, Rhozyme P-11, and Rhozyme A-4) were obtained from the Rohm & Haas Co. and were tried in the preliminary work to separate the soluble proteins from connective tissue in the muscle. The P-11 and A-4 gave inconsistent results and produced a colloidal dispersion during the washing with distilled water. However, the Protease-15 did not act in this manner and was used in the study. This enzyme is inactive toward native collagen (*10*).

Samples were weighed into 4-ounce screw-cap bottles and 40 ml. of water, 0.1 gram of enzyme, and enough 0.75*N* sodium hydroxide to bring the mixture to pH 7.35 were added. Each sample was mixed 1 minute with a motor stirrer and 1 ml. of toluene was added as an antiseptic.

The samples were placed in a shaker in a water bath maintained at a constant temperature of 40° C. for 16 hours, then cooled to 25° C. by placing the bottles in cold water. The samples were adjusted to pH 5.0 by the addition of 1*N* sulfuric acid. The procedure continued with the Hartley and Hall (*4*) method for washing and autoclaving.

After the first centrifugation, white floats of fat were observed on the filtrates from the samples treated with enzymes. These floats may have been clumped by the use of toluene, a surface-type antiseptic. As it was believed that some connective tissue might be occluded and lost in the washings, chloroform instead of toluene was used as a preservative. The resulting filtrates were clear with no floats; the fat apparently clumped with the chloroform in the bottom of the tube. However, after being autoclaved, the solutions were colloidal and difficult to filter. It was postulated that the fat was partially hydrolyzed and colloidally dispersed after removal of the chloroform in the autoclave, and that this condition caused the difficulty in filtering. An analysis of the filtrate containing the float indicated that only minimal quantities of collagen nitrogen were present, and not enough collagen was lost to affect the results. Therefore, the use of chloroform was discontinued, and toluene was used again. This observation is in contrast to Loyd and Hiner's (*6*) finding of an astonishing 71% of the longissimus dorsi hydroxyproline in the fat floats. The long period of alkaline digestion employed by them appears to be the principal difference in treatment of the tissue and may induce a difference in the adsorptive action of the fat.

Collagen nitrogen values were calculated as percentage of total nitrogen. Total nitrogen was determined by the AOAC method (*1*).

Table I. Average of Mean Collagen Nitrogen Values[a] Determined by Enzyme and Blender Methods

Round No.	Enzyme Method			Blender Method		
		Cooked, Min.			Cooked, Min.	
	Raw	30	45	Raw	30	45
I	7.6	7.2	6.7	9.9	9.5	9.2
II	8.5	6.1	5.9	10.2	9.5	8.5
III	8.9	6.5	6.3	10.6	9.7	8.6
IV	8.8	6.2	6.5	10.0	8.7	8.6
V	10.4	6.9	6.5	11.1	9.2	8.7
VI	9.2	6.5	6.7	9.8	9.3	9.3
VII	9.2	7.3	7.4	11.1	9.8	8.9
VIII	10.2	9.9	9.6	10.8	10.1	9.3
IX	11.1	10.0	9.9	11.1	9.6	9.6
X	11.8	10.3	9.9	10.4	9.9	9.1
Av.	9.6	7.7	7.5	10.5	9.5	9.0

[a] Per cent of total nitrogen.

Table II. Comparison, by *t* Test, of Collagen Nitrogen Values Obtained by Enzyme and Blender Methods

Measurements Compared	*t* Value	Conclusion
Enzyme *vs.* blender method		
Raw	4.50, 39 df[a,b]	Collagen nitrogen higher with blender method
Cooked 30 min.	5.20, 19 df[b]	
Cooked 45 min.	4.12, 19 df[b]	
30-minute *vs.* 45-minute		
Enzyme method	0.37, 38 df, ns[c]	No cooking time effect
Blender method	3.33, 38 df[b]	Collagen nitrogen greater for 30-min. cooking than for 45-min. (very low error variance)

[a] df degrees of freedom.
[b] Very highly significant ($P<0.001$).
[c] ns, nonsignificant.

Table III. Average of Mean Shear Values and Organoleptic Tenderness Scores[a]

Round No.	Shear Values, Lb.			Tenderness Scores	
	Raw	30 min.	45 min.	30 min.	45 min.
I	25.8	20.6	12.5	8.0	8.7
II	19.4	16.4	11.0	8.1	9.0
III	22.9	19.1	15.3	7.7	8.5
IV	20.5	11.3	10.9	6.4	7.8
V	27.5	11.6	14.4	7.5	8.2
VI	27.3	16.1	16.0	7.3	7.9
VII	27.5	16.4	11.9	7.6	7.8
VIII	24.6	21.3	17.0	6.6	8.4
IX	24.4	22.2	22.2	7.2	6.4
X	26.2	17.3	17.4	7.0	7.9
Av.	24.6	17.2	14.9	7.3	8.1

[a] 10, extremely tender. 1, extremely tough.

Table IV. Correlation Coefficients for Collagen Nitrogen Values with Shear Values and Collagen Nitrogen Values with Tenderness Scores

Measurements Correlated	Correlation Coefficient	Conclusion
Collagen nitrogen *vs.* shear values		
Raw		
Enzyme	0.10 ns[a]	No relationship
Blender	0.22 ns	No relationship
Cooked		
Enzyme 30 min.	0.39 near[b]	May be a weak relationship
Enzyme 45 min.	0.68[c]	Collagen nitrogen values related to shear values
Blender 30 min.	0.21 ns	No relationship
Blender 45 min.	0.28	No relationship
Collagen nitrogen *vs.* tenderness scores		
Enzyme 30 min.	−0.44[b]	Slight relationship
Enzyme 45 min.	−0.51[b]	Slight relationship
Blender 30 min.	0.04 ns	No relationship
Blender 45 min.	−0.28 ns	No relationship

[a] Nonsignificant.
[b] Significant ($P<0.05$).
[c] Very highly significant ($P<0.001$).

Statistical Analyses. The *t* test was used to compare collagen nitrogen values by the enzyme method (enzyme collagen nitrogen) *vs.* collagen nitrogen values by the blender method (blender collagen nitrogen), and collagen nitrogen values for samples cooked 30 minutes *vs.* the values for samples cooked 45 minutes for both the enzyme and blender methods of determining collagen. Correlation coefficients for raw meat and for meat cooked 30 and 45 minutes were obtained for enzyme collagen nitrogen values with shear values, blender collagen nitrogen values with shear values, enzyme collagen nitrogen values with tenderness scores, and blender collagen nitrogen values with tenderness scores.

Results and Discussion

Averages of mean values for collagen nitrogen in raw and cooked semimembranosus muscle from 10 top rounds, graded U.S. Good, as determined by the blender and enzyme methods are presented in Table I.

Although results in this investigation indicated lower blender collagen values in cooked than in raw beef, experience in this and other laboratories (*2*) has shown that this relation is often reversed. This reversal has been observed in samples in frozen storage more than a year in this laboratory (unpublished data). In the study reported here, samples were processed promptly after preparation. The possibility is suggested that collagen may be degraded by enzyme action in frozen, stored raw meat, whereas enzymes are inactivated by cooking the meat.

The *t* test showed that blender collagen nitrogen values were significantly ($P<0.001$) greater than enzyme collagen nitrogen values for both raw and cooked meat (Table II). Blender collagen nitrogen values were significantly ($P<0.001$) greater for samples cooked 30 minutes than for those cooked 45 minutes. The latter result may be explained by the greater opportunity for hydrolysis of collagen under moist heat when the meat was cooked for 45 minutes than when it was cooked 30 minutes. This is further reflected in the average shear values and tenderness scores given in Table III. Shear values generally were lower in the longer-cooked samples, and tenderness scores were slightly higher, with the exception of those for round IX. The enzyme method failed to show a significant difference in collagen nitrogen attributable to cooking time (Table II), but it indicated greater differences in collagen between raw and cooked samples in 7 of the 10 rounds for both cooking periods (Table I).

Of greater importance, enzyme collagen values (Table IV) gave a very highly significant ($P<0.001$) correlation ($r = 0.68$) with shear values for samples cooked 45 minutes, and a nearly significant correlation ($r = 0.39$) for those cooked 30 minutes. Also, enzyme collagen values were correlated significantly ($P<0.05$) with tenderness scores for samples both 30 ($r = -0.44$) and 45 ($r = -0.51$) minutes (Table IV). Obviously enzyme collagen values were correlated better with shear values and tenderness scores for the samples cooked 45 minutes than for those cooked 30 minutes. For the raw meat there was no apparent relationship between shear values and collagen nitrogen as determined by either method.

For these reasons it is believed that the use of proteolytic enzymes for collagen separation and estimation offers some promise. Since this investigation was made, the enzyme method has been improved by the use of 5 mg. of aureomycin hydrochloride in the incubation mixture (without toluene or chloroform) to suppress the activity of contaminating microorganisms. No putrefactive odor develops after the addition of aureomycin hydrochloride. Valenzuela (*11*) found that aureomycin hydrochloride (0.1 mg. per ml.) inhibited bacterial growth without interfering with growth of tissue in artificial media.

Also, the phosphate wash used in the blender method has been substituted for distilled water to remove water-soluble protein following incubation. The incubation temperature has been increased to 50° C., where Protease-15 appears more effective.

Acknowledgment

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The Separation of Organic and Inorganic Acid Anions in Filtered Tomato Purée by Partition Chromatography

DONALD B. BRADLEY
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A method, employing silicic acid partition chromatographic techniques, is described for the separation of those organic and inorganic anions which contribute to the acidity of tomatoes. Acids were converted to the hydrogen form by passing filtered purée through the cation exchange resin, Dowex 50. Titratable and total acidity were determined by titrating an aliquot of filtered tomato purée before and after resin treatment, respectively, with 0.1*N* sodium hydroxide to a phenol red end point. Separation of acids was quantitative and total recovery was approximately 94% of the total acidity. Ten acids (acetic, lactic, fumaric, malic, pyrrolidone carboxylic, citric, phosphoric, hydrochloric, sulfuric, and galacturonic) were found to be present in the filtered tomato purée. The phosphate, chloride, sulfate, and galacturonate ions had not previously been separated from tomato purée by partition chromatography or reported as constituents of the total acidity.

PROCEDURES REPORTED for the separation of the organic acids of tomato fruits (*2*, *3*, *7*) were adaptations of the methods outlined by Isherwood (*4*) and Marvel and Rands (*6*). The Isherwood method, as employed by Bulen, Varner, and Burell (*2*) and Rice and Pederson (*7*), to isolate the organic acids in tomatoes required the use of sulfuric acid in the initial extraction of the organic acids as well as in the preparation of the silicic acid partition column. The author found in preliminary studies that the sulfuric acid was eluted in sufficiently high concentrations to mask the presence of acids eluted after citric acid.

The purpose of this paper is to report analytical procedures found adaptable for the separation and identification of organic and inorganic acids present in tomatoes. The data as presented can be used as a general guide in determining the content of individual acids in other fruits. The ratio of chloroform to 1-butanol as well as the quantity of solvent may have to be modified to meet the specific requirement of the fruit.

Experimental

Preparation and Preservation of Tomato Purée Samples. Twenty-five pounds of freshly picked tomatoes were used for each sample. The fruits were washed, trimmed, macerated, and heated to 155° F. in a steam-jacketed kettle and held at this temperature for 4 minutes with constant stirring. This material was then passed through a Langsenkamp laboratory pulper operating at approximately 1600 r.p.m. and equipped with a 0.027-inch finishing screen. This operation removed skins and seeds and reduced the pulp to a purée. The resultant purée was heated to 200° F., placed in cans, sealed, processed in boiling water for 10 minutes, and then cooled immediately with water.

Removal of Cations by Ion Exchange. Approximately 150 ml. of canned purée were centrifuged to separate the red pulp from the clear amber liquid. The supernatant liquid was filtered to remove traces of pulp. Fifty milliliters of the filtrate were passed through a 30-ml. volume of the cation exchange resin, Dowex 50 in the hydrogen ion cycle, in a 17 × 0.75-inch glass tube. The eluent containing acids, sugars, and neutral material not attracted to cation exchange resin was collected in a 100-ml. volumetric flask at the bottom of the ion exchange tube. Five- to 10-ml. increments of distilled water were added to the top of the column to ensure a quantitative removal of the acids. Sugars present after cation exchange did not hinder the separation of acids on the silicic acid partition column.

The resin was regenerated by treating with four bed volumes of 2.5*N* hydrochloric acid and washing with distilled water (with occasional back flushing) until the eluent was free of chloride (silver nitrate test).

Separation of Acids by Partition Chromatography. The partition column was set up in a manner similar to that reported by Marvel and Rands (*6*). Only the coarser fraction of the silicic acid was used for the partition column. Six samples were run at one time using a battery of six ion exchange tubes and six partition chromatographic tubes.

Resin-treated filtrate, containing approximately 1 meq. of total acid (determined by titrating to phenol red end point) was pipetted into a 50-ml. beaker and concentrated on a water bath to 1.5 to 2 ml. at temperatures below 40° C. Two grams of oven-dried silicic acid were mixed thoroughly with the sample in a manner similar to that reported by Wise (*9*) until a free-flowing powder was obtained. The mixture was placed on top of the column in a dry form. Five milliliters of chloroform were used to remove any residue in the beaker and to suspend the sample at the top of the tube. A wad of cotton was placed on top of the sample to prevent disturbance of the sample or partition column as solvents were added.

The nine solvents used to elute the acids were composed of different concentrations of 1-butanol and chloroform

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The nine solvents used to elute the acids were composed of different concentrations of 1-butanol and chloroform

saturated with distilled water, with the exception of the last solvent which was methanol. Techniques employing a continuously changing mixture of solvents were not adopted because of problems involved in saturating the solvent mixture with water. It also appeared simpler to add the desired quantity of each specific solvent directly to each column. The sequence, composition, and quantity of each of the solvents used are shown in the following table.

Sequence	Volume, % Chloroform	1-Butanol	Total Solvent, Ml.
1	85	15	250
2	75	25	50
3	65	35	200
4	50	50	150
5	40	60	50
6	35	65	150
7	30	70	100
8	20	80	100
9	0	100	200
10	100% methanol		200

Air pressure up to 20 p.s.i. was required to force the solvents through the silicic acid column.

Eluted fractions were collected manually from the partition column in 10- or 50-ml. portions, into 50- or 125-ml. Erlenmeyer flasks, respectively. The 10-ml. fractions were collected between the elution of acids to determine completeness of separation. Preliminary studies had shown that each acid was eluted consistently within specific fractions of the total volume. The citric acid was eluted by this system at elution volumes from 540 to 850 ml. Peak elution of this acid occurred at 610 ml. (Figure 1). Pure samples of each acid were used for qualitative comparisons except for pyrrolidone carboxylic acid which was unavailable commercially. Identification of pyrrolidone carboxylic acid was assumed to be as reported by Rice and Pederson (*7*). The phosphate, chloride, and sulfate from the purée were identified by standard inorganic methods.

Eluted fractions were titrated with standard 0.02*N* sodium hydroxide using a phenol red indicator. Titer and indicator were blended with the nonaqueous organic solvent through addition of 10 to 25 ml. of acid-free methanol with vigorous stirring of the sample during titration. Blank corrections were required for each solution volume, because of impurities in the organic solvents.

Where the concentration of chloride exceeded 8 meq. per liter of filtered purée, there was danger of incomplete separation of the phosphate and chloride ions. When concentrations were too high, fractions containing these acids were combined and separated by other means. For routine samples, phosphorus was determined colorimetrically by the method of Sherman (*8*) and the chloride determined by difference.

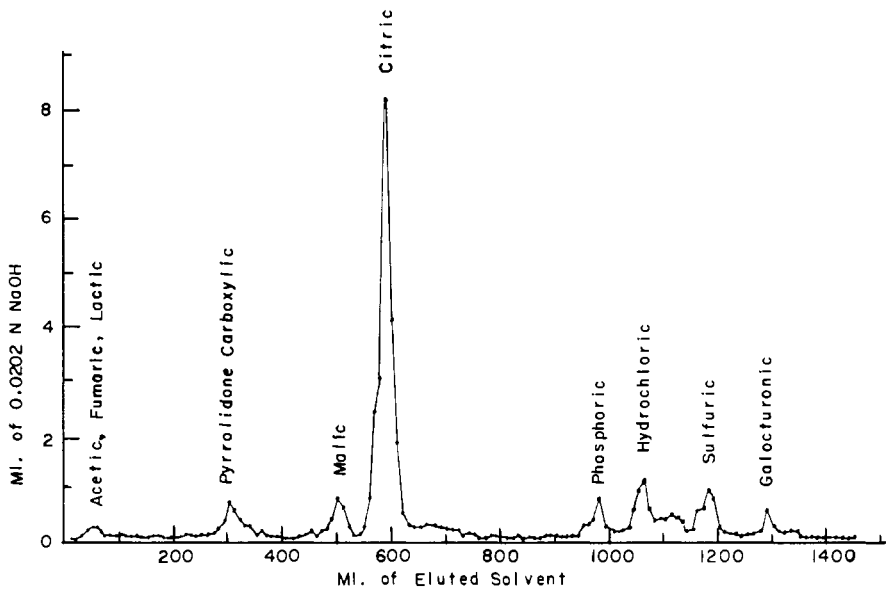

Figure 1. Titration of acids from tomato purée as eluted from the partition column

Identification of Acids by Paper Chromatography. The organic acids were separated and identified by paper chromatographic techniques employing the method described by Lugg and Overell (*5*) and Buch, Montgomery, and Porter (*1*). The developing solvent, 1-pentanol saturated with an equal volume of 5*M* formic acid, was used in the descending technique. The papers were sprayed with bromophenol blue, ammoniacal silver nitrate, acetic anhydride in pyridine, ammonium vanadate, or ceric ammonium nitrate. Papers were viewed both in daylight and ultraviolet light.

Results

The procedure as developed proved to have great utility. Acid constituents in over 100 samples of tomato purée were determined. Differences in the content of acid constituents between various tomato varieties were determined, as well as those induced in the content of individual acids by potassium fertilization. Recovery of acids averaged 94% of the total acidity.

Ten acids were determined qualitatively. The group included seven organic acids (lactic, fumaric, acetic, pyrrolidone carboxylic, malic, citric, and galacturonic) and three inorganic acids (phosphoric, hydrochloric, and

Table I. Volumes of Solvent Required to Elute Acid Constituents from Partition Column

Successive Volumes Required for Complete Elution of Acids, Ml.	Acid or Acids Contained in Eluted Fractions
0–250	Acetic, fumaric, lactic
250–400	Pyrrolidone carboxylic
400–540	Malic
540–850	Citric
850–1020	Phosphoric
1020–1135	Hydrochloric
1135–1250	Sulfuric
1250–1350	Galacturonic

Table II. Acid Composition Determined by Partition Chromatography, Titratable and Total Acidity, and Percentage Acid Recovery of Three Tomato Purée Samples

Acids	Concentration, Meq./Liter, Sample 1	Sample 2	Sample 3	Percentage of Total Acidity, Sample 1	Sample 2	Sample 3
Acetic, lactic, fumaric	1.1	3.2	2.1	1.2	2.7	1.7
Pyrrolidone carboxylic	6.4	6.5	8.6	6.7	5.5	6.7
Malic	8.3	6.3	5.6	8.8	5.2	4.4
Citric	52.5	62.7	65.8	55.5	52.9	51.5
Phosphoric	9.4	6.3	5.6	8.9	5.3	4.4
Hydrochloric	2.3	14.9	20.3	2.4	12.6	15.8
Sulfuric	4.4	7.3	7.3	4.6	6.2	5.7
Galacturonic	6.8	5.5	4.7	7.1	4.6	3.7
Titratable acidity	44.1[a]	54.1	58.1			
Total acidity	95.1[a]	118.5	127.8			
Recovery, %	96	95	94			

[a]Milliequivalents/liter of titratable and total acidity based on titration of 10 ml. of filtered purée to a phenol red end point before and after resin treatment, respectively.

sulfuric). All acids except lactic, fumaric, and acetic were separated quantitatively by silicic acid partition chromatography. These three acids were eluted and titrated as a group and together comprised 1 to 3% of the total acidity.

Analyses of some tomato purée samples not shown in this paper indicated that the sulfate ion was absent entirely, or present only in trace amounts.

The successive volumes of solvent required for the elution of each acid or acids are listed in Table I.

Figure 1 is a titration curve of a typical tomato purée sample showing the volume of 0.02*N* sodium hydroxide required to neutralize each fraction as it was eluted from the partition column.

The concentrations (milliequivalents per liter and percentage) of the individual acids from three typical tomato purée samples having a relatively wide range in titratable acidity are given in Table II. The titratable acidity, total acidity, and per cent recovery are also shown.

The data indicate that increases in the titratable and total acidity were accompanied by increases in the content of the citrate and chloride ions. Major increases in the titratable acidity are due to increases in the citric acid content. The pH of tomato purées is 4.5; therefore, the chloride ion would be present as a salt and would contribute little to the titratable acidity. In spite of the substantial differences in the titratable and total acidity, the content of citric acid for the three samples remained at approximately 53% of the total. The difference in the citric acid content between samples 1 and 3 was 13.3 meq. per liter. On a percentage basis, the difference was only 4.1%. The data indicate that regardless of changes in either the titratable acidity, total acidity, or in the concentration of the chloride ion, the citrate ion consistently made up approximately 53% of the total acidity. The difference in the chloride ion content between samples 1 and 3 was 17.9 meq. per liter.

This was the first time, as far as could be determined from the literature, that the three inorganic acids (phosphoric, hydrochloric, and sulfuric) and galacturonic acid have been separated from tomato purée by silicic acid partition chromatography. These acids were responsible for 20 to 30% of the total acidity of the tomato. As a result, a greater percentage of the total acidity was recovered than heretofore. The three tomato purée samples listed in Table II show a total recovery of 95.8, 95.0, and 93.9% of the total acidity.

A primary advantage of this procedure was the ability to convert the acid salts in the tomato purée to their respective acids through ion exchange techniques without the addition of foreign materials. Procedures employing adaptations of the Isherwood method (*4*) required the addition of sulfuric acid to the plant material in order to lower the pH value to 2 for conversion of the acid salts to acids. As the added sulfuric acid was eluted from the partition column it masked the presence of the inorganic acids normally present in tomatoes.

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MEASUREMENT OF PLANT ACIDS

Determination of Average Equivalent Weight and Total Weight of Plant Acids by Ion Exchange Resins Applied to Sugar Beet Molasses

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Ion exchange resins can be used to separate the anions in plant extracts and determine the average equivalent weight and total weight present. The equivalent weight of a California straight house sugar beet molasses was determined to be 82.1, with a standard deviation of 0.4.

As a contribution to increased knowledge of plant composition, a method is presented for the concomitant determination of average equivalent weight and total weight per cent of free and/or combined acids. The method has been applied to sugar beet molasses but should be applicable to other plant extracts with little or no modification. The procedure requires only the determination of the equivalents of anions as sodium or potassium salts.

Preparation of Ion Exchange Columns

The cation exchanger, Dowex 50–X8 (Dow Chemical Co., Midland, Mich.), is regenerated in large lots in a column with 3 liters of 5% hydrochloric acid per liter of wet resin. After regeneration, the resin is washed with distilled water until the effluent is chloride-free.

Initially the lot of anion exchanger, Duolite A-4 (Chemical Process Co., Redwood City, Calif.), is regenerated in a column with 3 liters of 4% sodium hydroxide per liter of wet resin and washed with distilled water until the effluent is colorless to phenolphthalein. The anion resin column used in the equivalent weight determinations is regenerated during use by the passage of ammonium hydroxide used to elute the acids and is ready for re-use after washing with 9 to 12 liters of distilled water.

Three different ion exchange columns are used for the separation of the acids in molasses from the remainder of the compounds. Two of the columns are glass tubes 60 cm. long and 4 cm. in diameter. One end is stoppered with a one-hole rubber stopper containing an outlet tube which can be closed with a screw clamp. A circle of fine-mesh nylon bolting cloth held in by the stopper is used to cover the outlet tube to prevent loss of resin. The columns are loaded to contain 400 ml. of wet resin, one col-

sulfuric). All acids except lactic, fumaric, and acetic were separated quantitatively by silicic acid partition chromatography. These three acids were eluted and titrated as a group and together comprised 1 to 3% of the total acidity.

Analyses of some tomato purée samples not shown in this paper indicated that the sulfate ion was absent entirely, or present only in trace amounts.

The successive volumes of solvent required for the elution of each acid or acids are listed in Table I.

Figure 1 is a titration curve of a typical tomato purée sample showing the volume of 0.02*N* sodium hydroxide required to neutralize each fraction as it was eluted from the partition column.

The concentrations (milliequivalents per liter and percentage) of the individual acids from three typical tomato purée samples having a relatively wide range in titratable acidity are given in Table II. The titratable acidity, total acidity, and per cent recovery are also shown.

The data indicate that increases in the titratable and total acidity were accompanied by increases in the content of the citrate and chloride ions. Major increases in the titratable acidity are due to increases in the citric acid content. The pH of tomato purées is 4.5; therefore, the chloride ion would be present as a salt and would contribute little to the titratable acidity. In spite of the substantial differences in the titratable and total acidity, the content of citric acid for the three samples remained at approximately 53% of the total. The difference in the citric acid content between samples 1 and 3 was 13.3 meq. per liter. On a percentage basis, the difference was only 4.1%. The data indicate that regardless of changes in either the titratable acidity, total acidity, or in the concentration of the chloride ion, the citrate ion consistently made up approximately 53% of the total acidity. The difference in the chloride ion content between samples 1 and 3 was 17.9 meq. per liter.

This was the first time, as far as could be determined from the literature, that the three inorganic acids (phosphoric, hydrochloric, and sulfuric) and galacturonic acid have been separated from tomato purée by silicic acid partition chromatography. These acids were responsible for 20 to 30% of the total acidity of the tomato. As a result, a greater percentage of the total acidity was recovered than heretofore. The three tomato purée samples listed in Table II show a total recovery of 95.8, 95.0, and 93.9% of the total acidity.

A primary advantage of this procedure was the ability to convert the acid salts in the tomato purée to their respective acids through ion exchange techniques without the addition of foreign materials. Procedures employing adaptations of the Isherwood method (*4*) required the addition of sulfuric acid to the plant material in order to lower the pH value to 2 for conversion of the acid salts to acids. As the added sulfuric acid was eluted from the partition column it masked the presence of the inorganic acids normally present in tomatoes.

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MEASUREMENT OF PLANT ACIDS

Determination of Average Equivalent Weight and Total Weight of Plant Acids by Ion Exchange Resins Applied to Sugar Beet Molasses

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Ion exchange resins can be used to separate the anions in plant extracts and determine the average equivalent weight and total weight present. The equivalent weight of a California straight house sugar beet molasses was determined to be 82.1, with a standard deviation of 0.4.

As a contribution to increased knowledge of plant composition, a method is presented for the concomitant determination of average equivalent weight and total weight per cent of free and/or combined acids. The method has been applied to sugar beet molasses but should be applicable to other plant extracts with little or no modification. The procedure requires only the determination of the equivalents of anions as sodium or potassium salts.

Preparation of Ion Exchange Columns

The cation exchanger, Dowex 50–X8 (Dow Chemical Co., Midland, Mich.), is regenerated in large lots in a column with 3 liters of 5% hydrochloric acid per liter of wet resin. After regeneration, the resin is washed with distilled water until the effluent is chloride-free.

Initially the lot of anion exchanger, Duolite A-4 (Chemical Process Co., Redwood City, Calif.), is regenerated in a column with 3 liters of 4% sodium hydroxide per liter of wet resin and washed with distilled water until the effluent is colorless to phenolphthalein. The anion resin column used in the equivalent weight determinations is regenerated during use by the passage of ammonium hydroxide used to elute the acids and is ready for re-use after washing with 9 to 12 liters of distilled water.

Three different ion exchange columns are used for the separation of the acids in molasses from the remainder of the compounds. Two of the columns are glass tubes 60 cm. long and 4 cm. in diameter. One end is stoppered with a one-hole rubber stopper containing an outlet tube which can be closed with a screw clamp. A circle of fine-mesh nylon bolting cloth held in by the stopper is used to cover the outlet tube to prevent loss of resin. The columns are loaded to contain 400 ml. of wet resin, one col-

umn with Dowex 50 and one with Duolite A-4. The third column, 30 cm. long and 3 cm. in diameter, is filled to contain 100 ml. of wet Dowex 50.

Procedure

Use carbon dioxide–free water for dilution of samples and washing solutions through the resin columns. Dilute 50 grams of molasses, containing about 50 to 100 meq. of anions, to 200 ml., and pass the solution through the column containing 400 ml. of wet, 20- to 50-mesh, Dowex 50 resin in the hydrogen form. This weight of molasses is greater than is necessary for the determination of equivalent weight and total weight of acids, but other analytical values are determined on the excess.

When the solution has completely passed into the resin, wash and collect 1500 ml. of effluent. Mix the effluent thoroughly and titrate 50-ml. aliquots to pH 8.3 with 0.1*N* sodium hydroxide. Calculate the equivalents of acid and use this value to calculate total weight of acids present (*1*). Pass 1000 ml. of the acidic effluent through the column of 400 ml. of Duolite A-4 resin (base form). Wash the column with 2 liters of distilled water. Discard the effluent. Elute the resin with 10% ammonium hydroxide (prepared by diluting 600 ml. of 28% ammonia to 3 liters with water). Add sodium hydroxide to the effluent equivalent to the anions present as calculated from the preceding titration and concentrate the solution on a hot plate to approximately 200 ml. Cool the solution and pass it through a column of Dowex 50(H) containing 100 ml. of resin. Wash and continue washing until the final volume of the effluent is 1000 ml. Mix the contents and measure three 50-ml. aliquots into tared 150-ml. beakers. Titrate each aliquot to pH 8.3 with 0.1*N* sodium hydroxide. Calculate the equivalents of acid present after subtracting the titration blank. Evaporate the titrated solution in a forced-draft air oven or by other suitable means, dry the sample at 105° C. for 2 hours, cool the beakers, weigh, and subtract the weight blank. From the corrected weight of the sodium salts and the equivalents of acid present, calculate the average equivalent weight. From this value and the equivalents of acid found after passage of the sample through the first column of Dowex 50, calculate the total weight of acid present in the sample.

Determination of Blank Values

The titration blank which arises from acidic resin decomposition products is obtained by using distilled water as the load in place of molasses and carrying out the regular procedure for equivalent weight determinations. In the determination of the titration blank, the final weight consists of the salts of acidic and neutral resin decomposition products. The weight blank consists of acidic and neutral resin decomposition products and neutral molasses substances. The solids due to neutral molasses constituents are determined by using molasses as the load on the cation exchange resin and carrying out the regular procedure up to the step of eluting the anion resin column with ammonium hydroxide. After the regular wash of 2 liters of water, 3 more liters of water are collected and treated as if it were the ammonium hydroxide solution obtained as the eluate from the anion exchange column. The final weight blank is the sum of weight due to these neutral constituents and that of acidic and neutral resin decomposition products obtained in the determination of the titration blank as outlined above. The titration blank was estimated at 0.13 ml. of 0.1*N* sodium hydroxide. The weight blank was determined to be 0.0023 gram. Blank values should be determined for each lot of resin.

Results and Discussion

Carbon dioxide–free distilled water was used to dilute samples and wash resin columns. Small amounts of carbonate salts introduced by the sodium and ammonium hydroxide solutions are mostly decomposed to sodium hydroxide, ammonia, and carbon dioxide during concentration of the ammonium hydroxide effluent (*2*). The carbon dioxide and ammonia will be volatilized while boiling during evaporation. Carbonate introduced in other steps of the procedure will interfere by giving acid titration values and increasing the weight of the salts.

Sodium hydroxide equivalent to the anions present is added to the ammonium hydroxide effluent to convert ammonium salts to sodium salts and thus decrease losses of acids during concentration.

Sodium sulfate and chloride are dried to constant weight over phosphorus pentoxide in vacuum in 3 days. Sodium salts of molasses acids could not be dried by this procedure and were dried to constant weight at 105° C. (in an air oven) in 2 hours. The difficulty in drying the salts of molasses acids is probably due to the presence of sodium lactate. Equivalent weight determinations of lactate derived from calcium lactate gave a value of 94.9 after the sodium salts were dried over phosphorus pentoxide for 16 days. Continued drying at 105° C. reduced the value to 90.6.

An error of 0.001 gram in 0.250 gram of salt equals 0.5 equivalent weight unit and a difference of 0.01 meq. of acid in a titration of 2.4 meq. in the 50-ml. aliquot equals 0.3 equivalent weight unit. The equivalent weight error is increased if either the weight of the salt or the titration is decreased by use of smaller samples.

Two experiments demonstrate the recovery of acid (added to molasses) and the determination of its equivalent weight. Approximately 50 meq. of oxalic acid were added to 50 grams of molasses, with acids of an average equivalent weight of 82.1, and the equivalent weight of the mixture was determined (found, 66.2, calculated, 66.6). The equivalent weight of oxalate was determined to be 42.9 (theoretical, 44.0). The experiment was repeated with acetic acid; the average equivalent weight was determined as 73.8 (calculated, 74.4). The equivalent weight of acetate was determined to be 57.2 (theoretical, 59.1).

Table I. Determination of Equivalent Weights of Anions by Ion Exchange Methods

Anion	Found	Equivalent Weight Av.	Equivalent Weight Calcd.
Chloride	35.6		
	35.9		
	37.0		
	36.3		
	36.1	36.2[a]	35.46
Acetate	59.4		
	59.4		
	58.7		
	59.6		
	59.3	59.3[a]	59.05
Sulfate	47.4		
	48.2	47.8	48.03
Pyrrolidone carboxylate	128.5		
	128.7	128.6	128.11

[a] Standard deviation. Chloride 0.53, acetate 0.35.

Five determinations of the equivalent weight of chloride and acetate were made with solutions of either the salts or the free acids as the load on the first Dowex 50 column. Two determinations each were made with solutions of sulfuric and pyrrolidone carboxylic acids. These results, presented in Table I, are slightly above theoretical, possibly because of the use of an insufficient weight blank.

The average equivalent weight of the acids present in a sample of California straight house sugar beet molasses from eight determinations was found to be 82.1, with a standard deviation of 0.4. Equivalent weight determinations of molasses acids made with potassium salts were 81.9 and 81.8, which compare favorably with the average of 82.1 found with sodium salts.

Compositional information in addition to the equivalent weight and total weight of acids can be obtained by further manipulation of the resins and their eluates (*3*, *4*). Basic and amphoteric nitrogen compounds relatively free from metallic cations can be obtained from the first column of Dowex 50 by elution with ammonium hydroxide. After evaporation of the ammonia, this fraction can be further fractionated into the more basic nitrogen compounds such as betaine and basic amino acids by passing the solution through a column of Dowex 1(OH) (Dow Chemical Co., Midland, Mich.). The neutral and acidic amino acids are retained on the resin, while the basic

amino acids and betaine pass into the effluent. The Dowex 1 column can be eluted with acetic or hydrochloric acid to recover the amino acids or can be eluted first with aqueous carbon dioxide to recover the neutral amino acids and other compounds having similar ion exchange properties.

The fraction containing acids can be used for identifying and determining specific acids. The effluent from the column of Duolite A-4 contains carbohydrates and other neutral substances.

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CITRUS BY-PRODUCTS

Lactic Acid Production by Fermentation of Citrus Peel Juice

JOHN J. KAGAN[1] and WALTER PILNIK[2]

Central Citrus Products Research Laboratory, Rehovot, Israel

M. DOREEN SMITH

Department of Food Chemistry, University of Toronto, Toronto, Canada

Citrus peel juice is produced in large quantities during the manufacture of canned citrus juices. A study was undertaken to determine the possibility of producing lactic acid by fermentation of this waste juice. Fermentations were conducted at 45° C. in the presence of excess calcium carbonate to neutralize continuously the acid formed. A naturally occurring lactobacillus isolated from a fermenting grapefruit juice was able to accomplish a 90%-efficient conversion of sugars to lactic acid in a period of 4½ to 5 days only in the presence of accessory nutrients. Growth factors supplied by yeast autolyzate or malt sprouts were particularly beneficial. Analyses of the fermentation runs were considerably simplified by the use of a cation exchange method for determining the lactic acid produced during fermentation in the presence of calcium carbonate.

IN THE CITRUS FRUIT processing industry, utilization of peel juice is becoming a major factor in maintaining economical plant operations. Preparation, composition, and current uses of peel juice have been adequately covered by Braverman (*2*). This report deals with the selection of a suitable lactobacillus organism which, in the presence of added growth factors, is capable of vigorous and efficient fermentation of peel juice. Furthermore, the need was felt for a simple and rapid method adaptable to routine determinations of lactic acid produced during fermentation in the presence of calcium carbonate. A cation exchange method was developed for this purpose; the principle involves the exchange of calcium ions from the calcium lactate in the clarified fermentor liquor for the hydrogen ions of the exchange resin. The acid thus formed is titrated with a standard alkali solution and calculated as lactic acid.

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[2] Present address, Obipektin Ltd., Bischofzell, Switzerland.

Materials

Peel Juice. Citrus peel juice was obtained directly from the canning line, adjusted to pH 6.0 by the addition of phosphoric acid and filtered to remove insolubles. During the off-season, citrus molasses was diluted with tap water to a Brix density of approximately 13°, adjusted to pH 6.0 with a 10% sodium phosphate solution, and filtered.

Yeast Autolyzate. Ground yeast cakes, 1 kg., were autolyzed with 100 ml. of ethyl acetate at room temperature. After complete liquefaction, the mixture was stirred mechanically for 1 hour, during which time it was maintained neutral to litmus by the addition of a 10% sodium phosphate solution. Toluene, 80 ml., was vigorously stirred into the mixture which was then incubated for 24 hours at 35° C. Neutralization and incubation were repeated always making certain of an excess of toluene to prevent putrefaction. The autolyzate was clarified by the addition of egg white and after being heated on a steam bath for 30 minutes, the mixture was filtered hot through a Büchner funnel with the aid of kieselguhr. The solids were extracted with an equal volume of water and filtered. The combined extracts were concentrated on a steam bath to a Brix density of 25° and stored under toluene in the refrigerator.

Tomato Extract. A quantity of tomatoes was macerated in the Waring Blendor and filtered through a Büchner funnel with the aid of kieselguhr. The extract was vacuum concentrated to a Brix density of 15° and stored, under toluene, in the refrigerator.

Culture Maintenance Medium. All cultures were maintained in a nutrient agar composed of glucose (2%), peptone (0.5%, Difco-Bacto), yeast autolyzate (10% v./v.), and agar (1%). Ten-milliliter portions were poured into lipless test tubes (150 × 15 mm.) containing a pinch of calcium carbonate, plugged with cotton, and sterilized at 15 pounds for 20 minutes. Before setting, the contents of the tubes were shaken to disperse the finely suspended calcium carbonate through the medium. In this way, growth of the lactobacillus cultures was easily detected by clearing of the medium. The cultures were maintained by weekly stab transfers followed by incubation at 45° C. until good growth oc-

amino acids and betaine pass into the effluent. The Dowex 1 column can be eluted with acetic or hydrochloric acid to recover the amino acids or can be eluted first with aqueous carbon dioxide to recover the neutral amino acids and other compounds having similar ion exchange properties.

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curred (18 to 24 hours) and refrigerated until the next transfer.

Peel Juice–Yeast Autolyzate Medium. This medium contained 230 ml. of peel juice, 50 ml. of yeast autolyzate, and tap water to make a total volume of 500 ml. Ten-milliliter portions of this solution were placed into test tubes (150 × 20 mm.) containing an excess of calcium carbonate, plugged with cotton, and sterilized at 15 pounds for 20 minutes. This medium was used to grow inoculum starters for all fermentation runs.

Isolation of Lactobacillus Culture. Of several lactic acid bacteria examined, including *Lactobacillus delbrückii* (ATCC 9649), a naturally occurring one was found to give best results. This organism was isolated from a sample of fermenting grapefruit juice as follows: 1 ml. of a spontaneously fermenting grapefruit juice was added to 10 ml. of a nutrient glucose solution (culture maintenance medium without agar) and incubated for 24 hours at 45° C. A stab transfer was made into the same medium with agar, followed by a similar incubation period. By repeated subculturing at 45° C. a typical lactobacillus organism was obtained showing no evidence of gas production. The microorganism was isolated as a pure culture by plate dilutions in Brewer-type anaerobic dishes (*3*). (No growth could be obtained aerobically.) It grew as white, round, slightly elevated colonies and was able to produce acid from glucose, fructose, sucrose, and lactose without evidence of gas production. Microscopic examination revealed typical lactobacillus rods occurring singly and in chains of four or five units. It was easily cultured on peel juice–yeast autolyzate medium (liquid or in agar stabs) and showed a catalase negative reaction.

Table I. Recoveries of Added Lactic Acid after Cation Exchange[a]

Lactic Acid Added, Mg.	Titer after Ion Exchange[b], Ml. 0.1N NaOH/Ml. Supernatant	Lactic Acid Recovered, Mg.	Recovery, %
0	5.24	...	...
106	6.42	106	100
212	7.66	218	103
530	11.1	530	100
530	10.5	461	87

[a] $CaCO_3$ added in all but last test.
[b] Average of two determinations which were within ±1% of each other.

Table II. Increase in Acidity during a Lactic Acid Fermentation of Citrus Peel Juice as Measured by the Cation Exchange Method

Fermentation Time, Hr.	Increase in Titer, Ml. 0.1N NaOH/Ml. Sample	Lactic Acid, G./100 Ml.
0	...	...
24	4.2	3.78
46	6.1	5.49
71	6.5	5.85
96	7.1	6.39
144	7.9	7.11

Total sugars originally present, 7.55 grams/100 ml.
Residual sugars, 0.20 gram/100 ml.

Methods

Columns of Dowex-50, each having a bed depth of 13 cm. and bed volume of 40 ml., were set up for downflow operations. A constant level device which prevented draining and possible channelling in the beds was attached to each of the columns. When exhausted, the beds were regenerated with 2% hydrochloric acid solution followed by thorough rinsing with distilled water.

The fermentation sample to be analyzed was treated with an excess of calcium carbonate and Seitz-filtered to remove insolubles and bacterial growth. (Alternatively, the sample was heated on a boiling water bath for 5 minutes and centrifuged.) An aliquot of the clarified sample—1 ml. was found to be convenient—was passed through the column followed by elution with distilled water. The effluent, approximately 150 ml., was titrated with 0.1*N* sodium hydroxide using phenolphthalein as an indicator. A zero determination was made immediately after inoculation, and the subsequent increase in acidity was calculated as lactic acid. Total acidity can be assumed to be lactic acid when homofermentative organisms are used. Duplicate determinations on each sample were made at the same time through two different columns, so that any irregularity in the behavior of the columns could be detected.

Extent of fermentation—lactic acid yield—was calculated as the ratio of lactic acid produced to the total sugars originally present in the medium and expressed on a percentage basis. Total sugars were estimated after inversion by the method of Shaffer and Hartmann (*8*).

A recovery experiment was carried out as follows: 0, 1, 2, and 5 ml. of a standard lactic acid solution (1.18*N*) were added to 5 ml. of a partially fermented peel juice and diluted to 10 ml. with distilled water. Calcium carbonate was added to all but one of the solutions. After neutralization and centrifugation, 1-ml. portions of the supernatants were analyzed by the cation exchange method as outlined above.

Volatile acids were determined (*7*) in the acidified samples after a 6-day fermentation period and found to be less than 0.15 gram per 100 ml. calculated as acetic acid.

Fermentation Procedure

An 18- to 20-hour stab culture of the lactobacillus was used to inoculate 10 ml. of peel juice–yeast autolyzate medium containing an excess of calcium carbonate. After 24 hours of incubation, approximately 2.6% of lactic acid formed, which is 50% of the theoretical maximum based on total sugars originally present. This vigorously growing starter was used to inoculate 100 ml. of the medium contained in a 200-ml. bottle to give a surface area of 0.27 sq. cm. per ml. Liquid accessory growth factors (yeast autolyzate and tomato extract) were added on a per cent by volume basis and dried malt sprouts on a per cent by weight basis. All fermentation media were sterilized at 15 pounds for 20 minutes, allowed to cool, and equilibrated at 45° C. before addition of inoculum starters.

The fermentations were run in the presence of excess calcium carbonate which was added to the mash before sterilization. Continuous agitation in these runs was not possible; however, the bottles were shaken intermittently throughout the day and the pH remained at 5.7 ± 0.1. After standing overnight the pH usually fell 0.3 to 0.5 unit.

Results and Discussion

Results of the recovery experiment on the cation exchange method are shown in Table I. They indicate that close to 100% recoveries are possible, except where the lactic acid is not neutralized with calcium carbonate. Complete neutralization of free lactic acid may therefore be an essential step in the procedure. The cation exchange method as used here has consistently given reproducible results with an accuracy of ±1%. Table II shows a typical set of results obtained when a peel juice medium undergoes a lactic acid fermentation. A good correlation is obtained when in-

Table III. Lactic Acid Fermentations of Citrus Peel Juice

Total sugars, G./100 Ml.	Accessory Nutrient	Time of Fermentation, Days	Residual Sugars, G./100 Ml.	Lactic Acid, G./100 Ml.	Fermentation, %
8.00	10% malt sprouts	4	0.52	7.02	87.8
7.60	10% yeast autolyzate	4	0.50	6.66	87.6
7.60	10% tomato extract	5	1.00	6.40	84.2
10.95	10% malt sprouts	4	1.88	8.92	81.5
10.83	5% malt sprouts	4	1.85	8.25	76.2
7.50	Nil	6	1.48	5.50	73.2

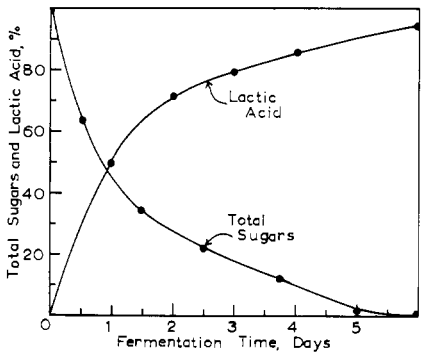

Figure 1. A peel juice–yeast autolyzate (10% v./v.) fermentation as measured by the cation exchange method

Total sugars at zero time was 7.60 grams/100 ml.

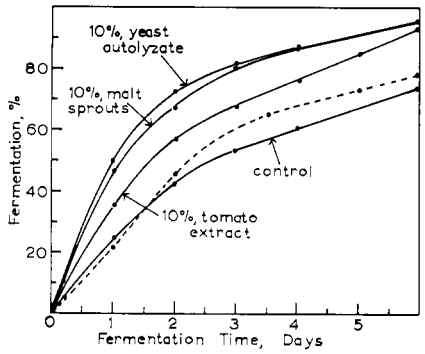

Figure 2. Effect of accessory nutrients on rate of peel juice fermentation using the selected lactobacillus

----A peel juice –yeast autolyzate (10% v./v.) fermentation using *Lactobacillus delbrückii* (ATCC 9649) under similar conditions

Table IV. Growth of Lactobacillus Cultures on Citrus Peel Juice Media after 24 Hours' Incubation at 45° C.

Medium	Selected Lactobacillus	*Lactobacillus delbrückii* (ATCC) 9649
Peel juice, 13° Brix		
Agar	++	+
Liquid	+++	−
Peel juice, 5° Brix		
Liquid	+++	++
Yeast autolyzate, agar	++++	++
Yeast autolyzate, peptone, agar	++++	+++

All media contained excess calcium carbonate.

crease in lactic acid is plotted *vs.* decrease in total sugars on a percentage basis (Figure 1).

The cation exchange method for determining acid production during fermentation in the presence of calcium carbonate considerably reduces the time required for analysis of fermentation runs. Using a single column, a determination can be obtained in 30 minutes, of which some 5 minutes are handling time. Furthermore, the acid produced is determined by a simple acid-base titration. The ease and rapidity of this method contrast with other analytical methods which have been employed for this type of study. The method used by Stiles and Pruess (*9*) requires a 5- to 7-hour extraction period and extreme care must be taken to ensure complete removal of sulfuric acid. The method of Friedmann and Graeser (*4*) involves a considerable outlay of apparatus and reagents, and the final titration of bound bisulfite is variable and influenced by several factors.

Previous investigations of lactic acid fermentation provide a sound basis for the study of a similar fermentation where a different source of fermentable carbohydrates is available (*5, 9, 10*). Those studies emphasized the importance of supplying accessory nutrients to achieve a high yield and rate of acid production. Table III shows that addition of yeast autolyzate or malt sprouts results in an efficient conversion of peel juice sugars to lactic acid. Tomato extract was not as beneficial as yeast autolyzate or malt sprouts, although a higher rate of lactic acid production was obtained than when no accessory nutrient was added. When peel juice containing a higher initial sugar concentration was fermented in the presence of malt sprouts, a corresponding increase in the rate of lactic acid production was obtained, at the expense, however, of a decreased efficiency.

Yeast autolyzate addition was consistently more effective than malt sprouts in stimulating the initial rate of fermentation; however, after 3 to 4 days the lactic acid yields in both cases were comparable (Figure 2). Tomato extract stimulated the initial fermentation rate, although not to the same extent as the yeast or malt adjuncts, but the lactic acid yield at the end of 6 days approached that attained by the latter additions. This result is of some interest, because preliminary experiments had shown that tomato extract was nearly equal to yeast autolyzate in stimulating the rate of acid production from a glucose basal medium. Citrus peel juice may possibly contain some of those factors present in tomato extract which are beneficial to the growth of lactobacillus organisms (*7*).

Residual sugars in the fermentor liquor after 6 days were reduced to 0.10 to 0.20 gram per 100 ml. in those runs where close to 95% of the theoretical maximum lactic acid was produced.

Lactobacillus delbrückii type of organism is usually selected for industrial lactic acid fermentations (*10*). When *Lactobacillus delbrückii* (ATCC 9649) was used to ferment peel juice with added yeast autolyzate (10% v./v.), the initial rate of fermentation and lactic acid yield at the end of 6 days were considerably lower than those given by the selected lactobacillus (Figure 2). Actually, this result was not unexpected because it was observed in a preliminary study that *Lactobacillus delbrückii* was not easily cultured on peel juice media (Table IV). On the other hand, the selected lactobacillus readily acclimatized to these media and grew vigorously whether or not they were fortified with nutrients.

The fermentation progress curves shown in Figure 2 indicate that citrus peel juice can undergo a 90%-efficient lactic acid fermentation in $4^1/_2$ to 5 days when suitable nutrients are added. Braverman (*2*) estimates that each ton of citrus fruit processed in the canning plant can give on the average 350 kg. of peel juice with about 8% of fermentable sugars. If this waste juice undergoes a 90%-efficient lactic acid fermentation, then each ton of fruit processed is capable of yielding 25 kg. of lactic acid. In the 1956–57 season, the state of Florida processed approximately 3,000,000 tons of citrus fruit which would be capable of yielding 150,000,000 pounds of crude lactic acid. Needle and Aries (*6*) predicted that the United States could utilize 200,000,000 pounds of plastic grade lactic acid if it were produced very cheaply. With improvements in refining methods and more efficient plants, citrus peel juice offers a potentially important raw material for producing lactic acid by fermentation.

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Mass Spectrometric Study of Nitrogenous Gases Produced by Silage

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The time course of production of nitrogenous gases from laboratory-scale and full-scale silos has been followed by mass spectrometric analysis. The concentration of NO rises rapidly, reaching a maximum about a day after the silo is filled; after 2 days the concentration of NO again is low. As aseptic soybean tissues ensiled in the laboratory produced NO and nitrogen, plant as well as microbial activity is important in the nitrogenous conversions accompanying silage production.

THE PRODUCTION OF TOXIC GASES in the early stage of silage making has been recognized recently as the cause of a mysterious pulmonary illness suffered by some farmers. Many plants—e.g., corn and oats—accumulate relatively high concentrations of nitrate, especially when a high dosage of nitrogenous fertilizer is applied. Photosynthetic oxygen production stops in ensiled plant materials, but aerobic respiration continues for a short time until it depletes the available oxygen from the silo gases. Under the anaerobic conditions produced, certain enzymes in the plant tissues and in the microorganisms can use nitrate as an electron acceptor. This reduction of nitrate yields the nitric oxide (NO) [nitrogen dioxide (NO_2) by its reoxidation], nitrous oxide (N_2O), and nitrogen observed in silage gases. Peterson and coworkers (*9*) briefly reviewed the pertinent literature on nitrate reduction.

In the current work, the accumulation of nitrogenous gases in silage has been examined under field and laboratory conditions. To aid in defining the respective roles of plant tissues and microorganisms in silage production, some tests have been made with aseptic soybean plants.

Material and Methods

Field Silo. Figure 1 shows a device which was installed in silos on the university farm. Holes were drilled in the pipe at the level of the sintered-glass dispersion tube to admit gases. The tubing assembly was pushed through the vertical pipe and through the horizontal drain pipe which led to a sump outside the silo.

To sample the silo gases, a previously evacuated 500-ml. round-bottomed flask equipped with a Y-stopcock was connected to the small bore plastic tube to withdraw all air from the tube. After removal of the air from the tube, the Y-stopcock was turned 120° to admit gas from the silo to the evacuated sampling bulb attached to the assembly with a 10/30 standard-taper joint; the stopcock on the sampling bulb then was closed. Gas samples were withdrawn from it into the mass spectrometer for analysis. In one experiment, NO_2 was determined by Saltzman's colorimetric method (*10*) to supplement the mass spectrometric analyses.

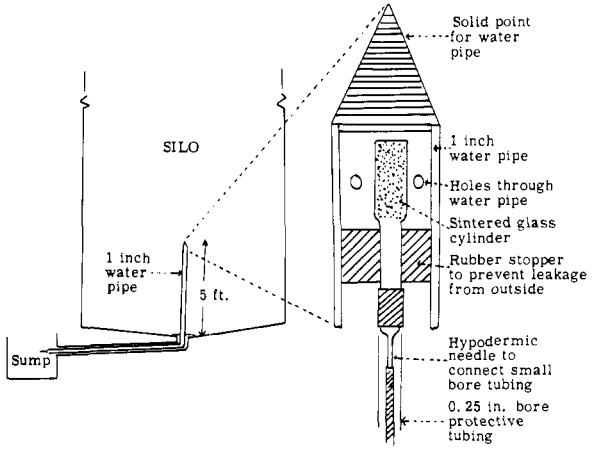

Figure 1. A sampling device installed in the silos used in this study

Laboratory Silo. For the generation of silo gases under controlled conditions, 100 grams of the tops of corn plants grown in the greenhouse were cut about 1 inch long and tightly packed into a 200 × 38 mm. borosilicate glass tube (Figure 2). Treatments were run in duplicate; 1.0 ml. of solution containing 0.166 gram of normal sodium nitrate or 0.166 gram of $NaN^{15}O_3$ (7.5 atom % nitrogen-15 excess) was added to each tube. The tubes then were immersed in a constant temperature water bath at 30° C.; each tube was connected to a T-stopcock. The combination manometers and measuring burets were made from 25-ml. graduated pipets; six burets of this type were connected through a manifold to a common mercury reservoir (Figure 2). The glass system was vacuum tight, but because the crushed plant material foamed, the vessels could not be completely evacuated. Approximately every 6 hours, gas

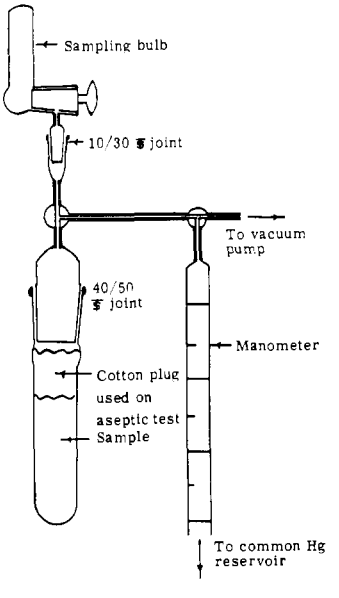

Figure 2. Apparatus to contain ensiled plant tissue to provide for evacuation and gas sampling

samples were taken from each unit, and the samples were analyzed with a Consolidated-Nier mass spectrometer for N_2, NO, oxygen, argon, carbon dioxide, NO_2, and in some cases for N_2O. N_2O was differentiated from carbon dioxide (each has a mass of 44) on the mass spectrometer by determining the proportion of the sum of the 44 and 45 peaks contributed by carbon dioxide and N_2O on the basis of the size of the 22 peak which is produced solely from doubly charged carbon dioxide ions; N_2O splits rather than acquiring a double charge. After the gas samples were withdrawn from the laboratory silos, the systems were re-evacuated to ensure that gas produced in the interval before the next sampling would not be contaminated by previously accumulated gas. Thus, the gases analyzed from the laboratory silos were those produced during the intervals between samples, whereas those from field silos were mixtures of gas originally present and not displaced plus the undisplaced fraction of gases formed after ensiling.

Aseptic Silo. For the aseptic experiments, soybean seeds in a Petri dish were vacuum infiltrated with 75% alcohol in a desiccator and allowed to stand for 10 minutes; they were washed three times with sterile distilled water. The seeds then were treated with sodium hypochlorite (about 0.75% available chlorine) for 10 minutes with vacuum infiltration and again were washed three times with sterile distilled water. The seeds were transferred aseptically into sterilized 15-liter wide-mouthed glass jars containing a 1.5-inch layer of vermiculite nearly saturated with Hoagland-Arnon nutrient solution number 1 (*5*); each jar contained a cotton plugged 200 × 38 mm. silo tube (Figure 2) with added nitrate solution. The jar mouths were covered with 5-ply Aeromat filter to remove microorganisms but to allow passage of air. After 14 days, the plant tops were harvested aseptically in a transfer room and packed into the silo tube in the jar. All manipulations were made inside the wide-mouthed jar, and the materials were handled with a sterilized surgical glove. The cotton plug was replaced in the silo tube above the plant tissue and below the standard taper joint to prevent subsequent contamination. The tubes were taken out of the jars, connected to the evacuation and sampling equipment, and the silage gas was collected and analyzed as described for the laboratory silos. At the end of the experiment, 1 ml. of silage liquid was added to a tube of glucose nutrient broth medium, and the medium was incubated at 37° C. for at least 36 hours. Sterility was checked by examining the medium for turbidity and by observing a stained sample microscopically.

Results

Field Silos. Results reported in Table I and Figure 3 were obtained from a silo on the university farm in 1957; the gas was sampled with the device shown in Figure 1. In general, the results were in good agreement with those obtained with laboratory silos. The sampling device was successful in eliminating contamination of the gas samples by air, for the levels of oxygen in samples were very low. The gradual decrease in the percentage of argon from 0.69% after 6 hours to 0.03% after 66 hours indicates that the air initially trapped inside the silo was gradually displaced by the silage gases. The rapid disappearance of oxygen, only 0.09% remained 6 hours after ensiling, indicates that anaerobic conditions were achieved quickly in the silo. During the experiment, the percentage of oxygen ranged from 0.05 to 0.1%. Carbon dioxide accumulated rather steadily throughout the sampling period and rose from 28.65 to 84.98% of the gas present.

In contrast to the increase of carbon dioxide, the percentage of nitrogen decreased gradually from 68.80 to 13.44%. Nitrogen must have been produced in the field silo, because its fivefold decrease in concentration was far less than the 23-fold decrease in argon concentration from 0.69 to 0.03%. The percentage of NO increased from 0.12% at 6 hours to a maximum of 9.71% at 23 hours and then decreased sharply to approximately the 6-hour level at 42 hours and thereafter. The higher peak values for NO in the laboratory tests than in the field silo can be accounted for on the basis that nitrate was added to plant tissues in the laboratory tests, and the laboratory silos were re-evacuated after each sampling.

The percentage of N_2O reached a peak of 4.35% (54 hours) after the virtual disappearance of NO. The colorimetric determination of NO_2 (Table I) showed about 0.1% NO_2 as a maximum level at 18 hours. Forty-six hours after ensiling, the NO_2 level decreased to 0.001% and remained low afterward. As NO_2 arises from the spontaneous oxidation of NO by oxygen, one would expect that the low level of oxygen in the silo would limit NO_2 formation to the low concentration actually found.

Figure 4 records the compositions of gas mixtures recovered from a silo filled with corn in 1958. The silo was filled over a period of 2.5 days, and the evolution of NO was not as great as in the silo filled rapidly in 1957; the peak concentration of NO did not appear until 30 hours after ensiling. In general, the gas production was much like that observed in 1957 (Figure 3).

Figure 5 indicates the results obtained from a 12 × 40 foot silo on the university farm filled with a mixture of soybeans and sudan grass treated at ensiling with 8 pounds of sodium pyrosulfite ($Na_2S_2O_5$) per ton of plant materials. The gas compositions were distinctly different from those in untreated silos. The air was displaced rather slowly as indicated by the fact that the percentage of argon decreased only from 0.85% at 18 hours to 0.59% at 70 hours. Similarly, the nitrogen decreased at an unusually slow

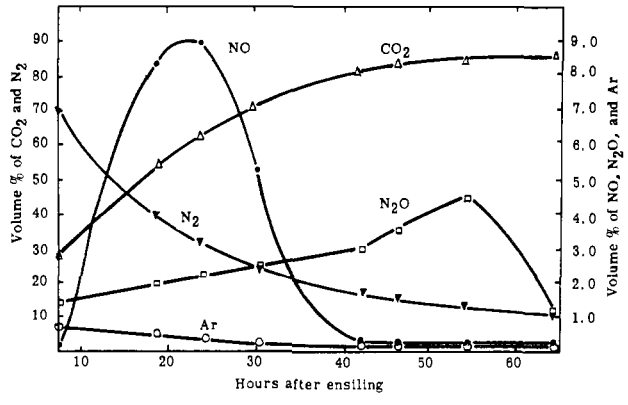

Figure 3. Composition of gas mixtures recovered from a 12 × 40 foot silo on the university farm; filled with corn in September 1957

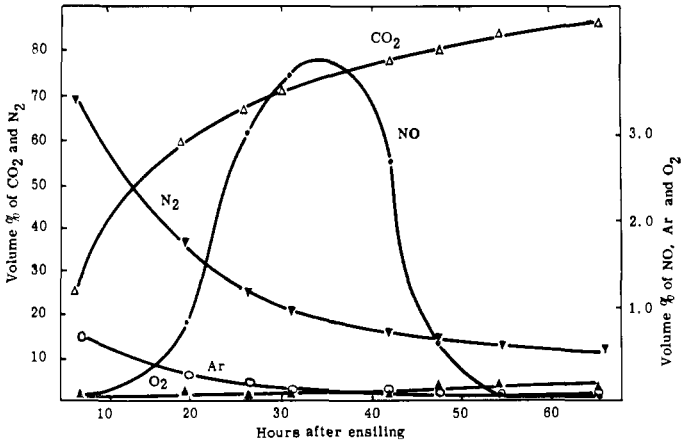

Figure 4. Composition of gas mixtures recovered from a 12 × 40 foot silo on the university farm; filled with corn in the fall of 1958

rate: from 73.7 to 55.5%. Throughout the sampling period, the oxygen concentration was reduced to less than 0.02%. The slow rate of accumulation of carbon dioxide indicated that the plant and microbial metabolism was strongly inhibited by sodium pyrosulfite. The level of NO was less than 0.1% in all but two samples. A normal silage fermentation did not occur in the presence of sodium pyrosulfite, but the plant material appeared to be well preserved.

Laboratory Silos. A number of preliminary experiments were conducted to find a satisfactory laboratory silo before the form described in the section on methods was adopted. The required conditions are that the silo can be operated conveniently on a laboratory scale under controlled conditions, and that it be gas tight to avoid contamination of the silage gases by air.

Table II presents the composition of gases evolved from corn silage in a laboratory experiment. Oxygen was depleted rapidly and remained at a reasonably low level throughout the experiment. As an inert gas, argon gives a good index of contamination of the samples with air. A comparison of the changes in percentages of nitrogen and argon indicates that the nitrogen gas arose partly from plant and microbial metabolism and partly from air contamination. For example, the level of 0.11% of argon in the 41-hour sample indicates that contaminating air constituted approximately 12% of the total gas sample. Such contamination with air should give only about 9.5% of nitrogen in the sample. However, the analysis showed 17.5% of nitrogen, so that nearly half of it was produced by denitrification in the silage.

Nitric oxide accumulated rapidly during the experiment. From 0.81% at 6 hours, NO rose to 47.22% of the total gas volume after 41 hours. This value is much higher than those reported by Peterson and coworkers (*9*) under approximately the same experimental conditions, except that the authors' experimental silos were evacuated initially whereas theirs were filled with air. In one experiment a peak value of 50.60% of NO was found in a 41-hour sample.

No attempt was made to separate N_2O from carbon dioxide in the laboratory silos, and the total gases at masses 44 and 45 were recorded as carbon dioxide; although observations with the field silos indicated that N_2O (mass of 44 like carbon dioxide) was a product of the silage fermentation. Percentages of NO_2 in this experiment were calculated from the peak in the spectrometer at mass 46. These values are rather inaccurate, because NO_2 reacts with the mercury of the pressure adjustor in the mass spectrometer, and the machine must be specially conditioned with NO_2 (*4*) to measure this gas accurately. $C^{12}O^{16}O^{18}$ also contributes to the mass 46 peak. The Saltzman method (Table I) is satisfactory for measuring NO_2 when the color reagent is added directly to the gas sample bulb and shaken vigorously therein.

To study the nitrogen-15-enrichment in each individual gas (nitric oxide, nitrogen dioxide, nitrous oxide, nitrogen), attempts were made to separate these compounds by gas chromatography; however, the method was not perfected and only results of the atom % of nitrogen-15 excess in the nitrogen evolved from corn silage treated with $NaN^{15}O_3$ containing 7.5 atom % of nitrogen-15 excess are given here. The level of nitrogen-15 was 0.157 atom % of nitrogen-15 excess after 6 hours and increased linearly with time to 0.298 after 18 hours, 0.476 after 29 hours, and 0.638 atom % nitrogen-15 excess at the terminal 40-hour sampling. These data give direct evidence that nitrogen-15–labeled nitrate added to ensiled plant material can be converted to molecular nitrogen and lost in this

Table I. Nitrogen Dioxide Found by Colorimetric Analysis of Samples from a Field Silo

Sample No.	Sampling Time, Hours after Ensiling	Volume % NO_2 in Sample
1	6	0.056
2	18	0.104
3	23	0.089
4	30	0.063
5	42	0.016
6	46	0.001
7	54	0.001
8	66	0.001

These samples analyzed by Saltzman's method were aliquots from samples whose analyses are recorded in Figure 3.

Table II. Composition of Silage Gases Produced at 30° C. under Vacuum from Corn Top Tissues in Laboratory Silos

Gas Components	Sampling, Hours after Ensiling: 6	16	22	29	41	49
	Volume % of Gases					
N_2	25.39	16.97	20.42	11.52	17.55	13.04
NO	0.81	2.21	12.99	38.23	47.22	38.84
O_2	0.64	1.39	2.08	0.74	2.14	0.67
Ar	0.16	0.07	0.14	0.03	0.11	0.03
CO_2	72.67	79.00	64.07	49.26	32.78	47.23
NO_2	0.32	0.35	0.28	0.22	0.19	0.19

0.166 gram of $NaN^{15}O_3$ in 1 ml. of solution added to 100 grams of wet weight of tissue.

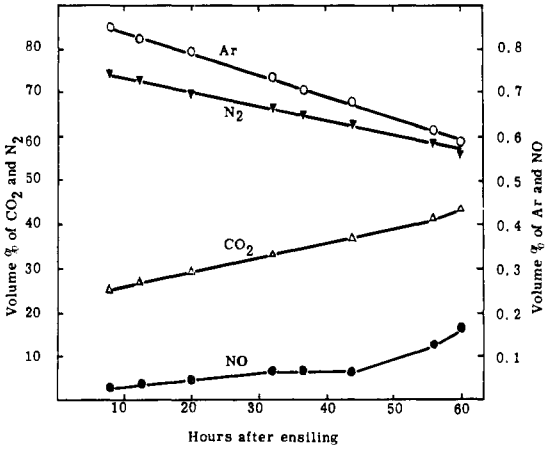

Figure 5. Gases present in a 12 × 40 foot silo filled with a mixture of soybean and sudan grass, October 1958, treated with sodium pyrosulfite

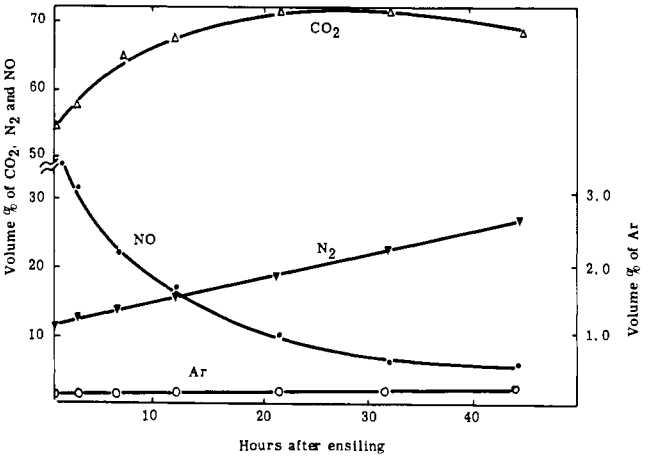

Figure 6. Composition of gas mixtures collected over aseptic soybean tissues

form from silage. These data, as well as the observation that nitrogen decreases in concentration much less rapidly than argon, prove that denitrification does occur in silage.

Aseptic Silage. As normal silage is a fermentation product, it seems improper to speak of aseptic silage, so the qualified term "silage" has been used to indicate that the plant material was packed in laboratory apparatus in the usual fashion except that it was kept aseptic. Exclusion of all microorganisms from the plants posed difficulties because of the necessity for extensive transfer and manipulations of the aseptic plant material during the process. After numerous trials, asepsis finally was achieved by performing all manipulations inside the wide-mouthed glass jar in which the plants were grown. Figure 6 indicates the compositions of the gas mixture collected over aseptic soybean tissues. The rapid rise of carbon dioxide from 54.2 to 71.7% resembled the pattern of carbon dioxide accumulation in a normal silage fermentation. The levels of nitrogen increased with time as the NO decreased. Thirty minutes after ensiling, the first sample contained NO at a concentration of 34.0 volume %. Thereafter the level of NO decreased to a low value of 4.3% at 45 hours. Apparently the plant tissue quickly reduced the added nitrate to NO and with time developed an increased ability to reduce the NO to N_2. These results agree with the finding of Chung and Najjar (*2*) that plant enzymes can reduce nitrate to form NO, but also indicate that plants have the ability to reduce this NO further to N_2.

The claim for the absence of contaminating microorganisms is supported by the pattern of NO formation. If contaminating microorganisms were present, their activity would increase with time as their population increased, and the formation of NO would be expected to increase as it did in many trials in which contaminants were present. The rapid formation of NO by the aseptic plant tissues suggests that, contrary to the general view expressed by Peterson and coworkers (*9*) "that bacteria and not plant enzymes are responsible for the formation of the nitrogen oxides," the plant tissues may have a major role in the production of gases in the silo.

Discussion

Nitrate present in or added to ensiled plant material can be reduced to nitrogen and various compounds intermediate in reduction level between nitrate and nitrogen. These studies have not covered compounds more reduced than nitrogen, but reduction of nitrate to ammonia is accomplished readily by plant tissues (*7*).

The experiments with nitrogen-15 as a tracer indicate that nitrate can be converted to NO, N_2O, and N_2 in silage. Nitrate reductase is widely distributed in plants and microorganisms and its formation of nitrite from nitrate would be the first logical step in NO production in silage. At the acid pH of silage, the next reaction of nitrite could proceed spontaneously as follows:

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$

The nitric acid formed could in turn be reduced by nitrate reductase.

Under the anaerobic conditions in the silo, the reduction of NO logically could continue in the following fashion:

$$2NO + 2H \rightarrow N_2O + H_2O$$

$$N_2O + 2H \rightarrow N_2 + H_2O$$

In the present study, there has been no attempt to establish the mechanism of nitrate reduction in silage, but the products NO, N_2O, and N_2 have been observed consistently. The data of Peterson *et al.* (*9*) indicated that nitrogen arose chiefly by reduction of nitrate, but that some nitrogen was liberated from nitrogen-15–labeled amino acids by their reaction with nitrous acid (Van Slyke reaction). Wijler and Delwiche (*11*) have demonstrated that NO, N_2O, and N_2 are products of denitrification in soil, and Najjar and Allen (*6*) have found that pure cultures of denitrifying bacteria produce these same gases. Evans and McAuliffe (*3*) have observed that anaerobically either ascorbate or DPNH can reduce nitrite to NO, N_2O, and N_2 nonenzymatically. Nommik (*8*) observed no nonenzymatic denitrification in soil but found that the balance among these gases was pH-dependent.

In addition to the denitrification route, undoubtedly a portion of the nitrate yielded ammonia by the usual process of nitrate reduction (*7*); the quantitative importance of the two routes was not established. Burström (*1*) has suggested that bound nitrite normally is reduced to ammonia, whereas free nitrite is denitrified; however, this possibility never has received substantial experimental support.

In the sequence of gas appearance in the silos, NO was produced first and the N_2O and N_2 followed. The appearance of the highest concentration of N_2O was clearly delayed, but the data do not establish the time of maximum nitrogen production. It would be informative to supply nitrogen-15–labeled NO and N_2O to silage and to determine their rate of conversion to nitrogen.

The toxic gas from silos, NO_2, is not a direct product of nitrate reduction but arises readily from the oxidation of NO by oxygen. Its formation is suppressed under the anaerobic conditions in the silo, and its level is low in uncontaminated samples of silo gases.

The data obtained with aseptic "silage" offer direct evidence which may be applicable in the interpretation of the controversy regarding the role of plant tissues *vs.* microorganisms in silage production. The importance of plant enzymes was stressed in many early studies of the changes occurring during silage making, but emphasis more recently seems to have been placed on the microorganisms. The current study indicates that the plant tissues themselves are capable of producing abundant NO and N_2 from nitrate. Although we have not studied the formation of organic acids and the other changes characteristic of silage production, the observed gas metabolism suggests that, under normal conditions, both the plant tissues and microorganisms likely probably contribute substantially in the process of silage production.

Acknowledgment

The authors are indebted to G. P. Barrington and R. P. Niedermeier for aid in designing and constructing apparatus for sampling gases from field silos. They thank W. H. Peterson for his stimulating discussions on the planning and interpretation of the experimental work and S. B. Crepea for his interest in and support of the project.

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Optimum Conditions of Hydrolysis for Microbiological Assay of Amino Acids, Methionine, and Cystine in Poultry Meat

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Acidimetric titrations are much more suitable for microbiological analyses of methionine and cystine in poultry meat than turbidimetric determinations. The use of a 4-hour sealed-tube hydrolyzate (3*N* hydrochloric acid) for cystine and an 8-hour sealed-tube hydrolyzate (3*N* hydrochloric acid) for methionine gave maximum values in microbiological analyses using titrations of the acid produced by the test organism *Leuconostoc mesenteroides*.

ALTHOUGH research has been conducted on the amino acid content of poultry meat, no reports could be found where the optimum time and method of hydrolysis of poultry meat had been studied. Studies on other products have indicated that long hydrolysis of the test sample causes some loss of cystine (*5*. A 2-hour hydrolysis was proposed fo cystine determinations. Balasubramanian and Ramachandran (*2*) recommended a hydrolysis period of $2^1/_2$ hours using 2.5*N* hydrochloric acid for wheat and rice. These studies were conducted on high carbohydrate-containing material and may have less applicability on foods such as meat. Cystine values obtained with short periods of hydrolysis may be in error due to peptide stimulation of the assay organisms (*1*, *4*).

This study was undertaken to determine the combinations of hydrolysis time and method which would result in maximum readings for methionine and cystine when assayed by microbiological means. Consideration was also given to a comparison of turbidimetric and acidimetric determinations of the growth of the test organism.

Experimental

Sample Preparation. Three eviscerated Hoosier White birds, each weighing approximately 1250 grams, were cooked in a reel-type oven at 325° F. (163° C.) for 1 minute per 10.5 grams of carcass weight. The birds were cooked in a shallow open pan, loosely covered with aluminum foil, and allowed to cool prior to taking samples. Breast and thigh muscles (minus skin) were combined and ground together three times. Portions for analysis were dried for 24 hours in a vacuum oven at 65° C. Fat was removed by Soxhlet extraction with petroleum ether for 15 hours. The moisture-free, fat-free samples were pulverized with a mortar and pestle, and 1 gram of this powder was used for each hydrolysis treatment. Thirty-milligram samples of the pulverized material were analyzed for nitrogen by the micro-Kjeldahl method.

Hydrolysis Procedure. The meat samples were hydrolyzed by the sealed-tube method or under reflux for 1, 2, 4, 8, 16, or 24 hours. For each combination of time and method, four samples (treated as two replicates, each containing two samples) were hydrolyzed and analyzed for methionine and cystine. The sealed-tube hydrolyses were done in borosilicate glass tubes 18 mm. in outside diameter and originally 16 inches long. Tubes became progressively shorter with each use and were discarded when the sample and acid filled more than one third of the tube. One gram of the pulverized moisture-free, fat-free sample was placed in the tube and 10 ml. of 3*N* hydrochloric acid were added. The sealed tubes, when cool, were shaken vigorously and placed in the autoclave in a horizontal position. The autoclave was then heated and maintained at 15 pounds pressure (approximately 120° C.) for the specified time. One-gram samples to be refluxed were placed in 300-ml. flat-bottomed round boiling flasks with 10 ml. of 6*N* hydrochloric acid and connected to condensers. Flasks were heated on a hot plate on high with a Powerstat setting of 70. Refluxing was timed from the fall of the first drop.

Hydrolyzates were rinsed from the hydrolysis container and brought to a pH of 4.0. One-half gram of activated charcoal was added, the solution filtered, and the residue washed until the volume of filtrate was almost 250 ml. The pH was then adjusted to 6.8, the volume made to 250 ml., and the solution frozen in 4-ounce polyethylene bottles until used for assay of amino acids.

Determinations. Methionine and cystine were determined both turbidimetrically and acidimetrically. From the standpoint of time, turbidimetric determinations are more desirable, as readings can be made more rapidly than titrating and the tubes need be held only 16 to 20 hours rather than 72. *Leuconostoc mesenteroides* P-60 ATCC No. 8042 was the test organism. The media used were Difco dehydrated Bacto-Methionine assay medium and Bacto-Cystine assay medium. Five milliliters of the rehydrated medium were placed in each 16 × 125 mm. screw-cap culture tube with additional test sample or standard and distilled water to make a volume of 10 ml. After inoculation, the tubes were incubated at 30° C.

After 16 to 20 hours of incubation, turbidimetric determinations were made in the test tube attachment of the Beckman DU spectrophotometer at a wave length of 660 microns as recommended by the Society of American Bacteriologists (*6*). Acidimetric titrations were made after 72 hours using 0.1*N* sodium hydroxide. The solutions were stirred with a magnetic stirrer while being brought to an end point pH of 7.0 as measured by a Beckman Model H pH meter. Standard amino acid and test material growth response curves were plotted on log-log paper as suggested by Block and Weiss (*3*), except that 11 × 16 inch, 1 × 2 cycles paper was used rather than $8^1/_2$ × 11 inch, 2 × 2 cycles paper.

[1] Present address, Poultry Department, Kansas State University, Manhattan, Kan.

Results and Discussion

Acidimetric *vs.* Turbidimetric Determinations. In general, the methionine values determined turbidimetrically were somewhat lower than those determined acidimetrically. Turbidimetric readings at the higher levels of methionine were difficult, as the growth curve failed to maintain a straight line when plotted on log-log paper. This was particularly true with the standard curve responses (Figure 1). The correlation coefficient was computed for the methionine values of 34 samples analyzed turbidimetrically and acidimetrically, and found to be 0.218. This correlation was not statistically significant at the 0.05 level, and it indicates that turbidimetric determinations are of questionable value for quantitative determination of methionine. Turbidimetric determinations were even less applicable to the determination of cystine. All methionine and cystine values in the discussion of the hydrolysis experiment are made acidimetrically with 0.1*N* sodium hydroxide.

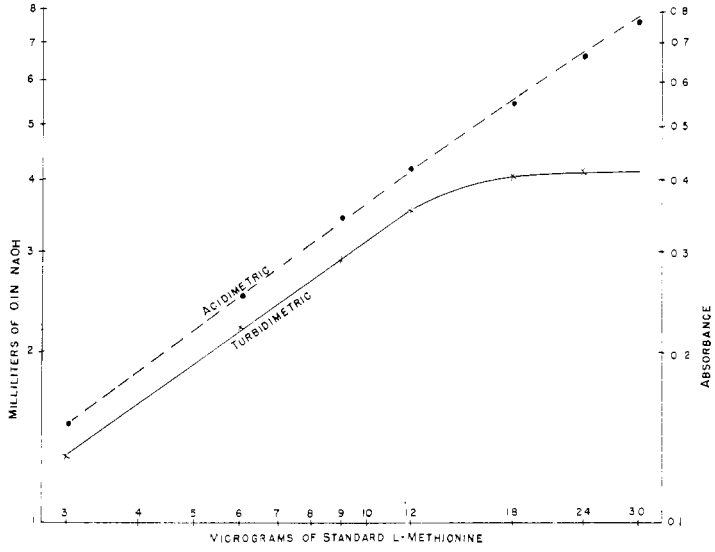

Figure 1. Typical growth curves for standard solutions of methionine

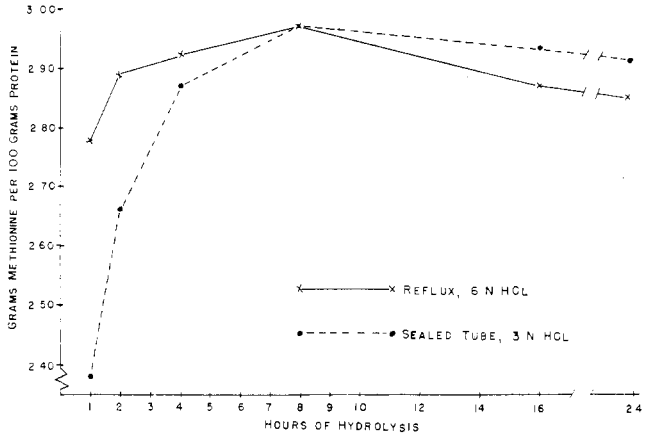

Figure 2. Effect of time and method of hydrolysis of methionine values of poultry meat

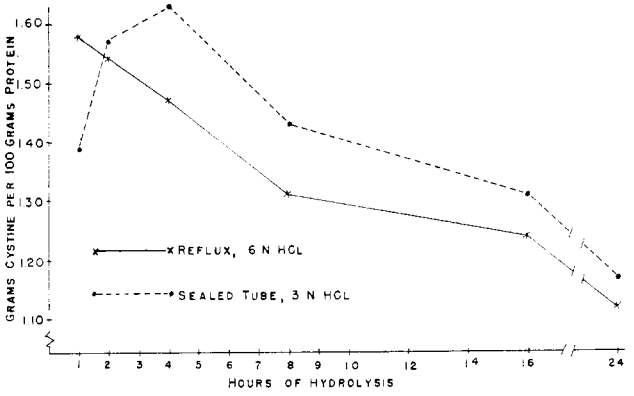

Figure 3. Effect of time and method of hydrolysis on cystine values of poultry meat

Optimum Hydrolysis Time and Method. The effects of time and method of hydrolysis are illustrated in Figures 2 and 3. It is readily apparent that sealed-tube hydrolysis with 3*N* hydrochloric acid requires a longer time than reflux with 6*N* hydrochloric acid to release the cystine from the protein; however, the sealed-tube method causes less destruction of the amino acids after the maximum has been reached. The time of reaching maximum values for the amino acids differs with each method and with each amino acid.

Statistical analysis by the analysis of variance method indicated that time and (time × method) interaction variations were highly significant for both cystine and methionine. Method differences were also significant in the methionine determinations. Replications were not significant at the 0.01 level for methionine or cystine. The standard deviation for subclass samples within replicates, as taken from the analysis of variance table, was 0.255 gram of methionine and 0.079 gram of cystine per 100 grams of protein.

Figures 2 and 3 show that the most suitable periods of sealed-tube hydrolysis are 4 hours for cystine and 8 hours for methionine. A sealed-tube hydrolysis for 6 hours might give near-maximum values for both methionine and cystine, with minimal destruction of either amino acid. The use of one 6-hour hydrolyzate would eliminate the need for a separate hydrolysis for each amino acid.

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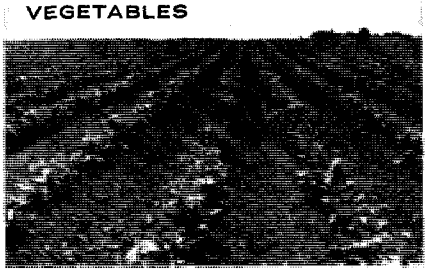

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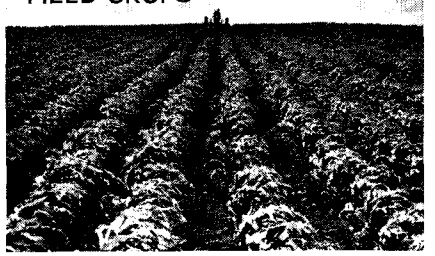

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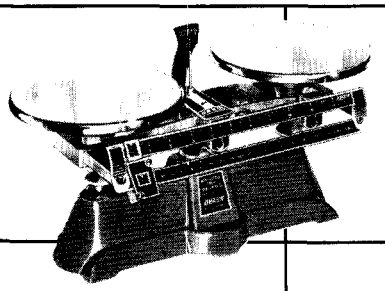

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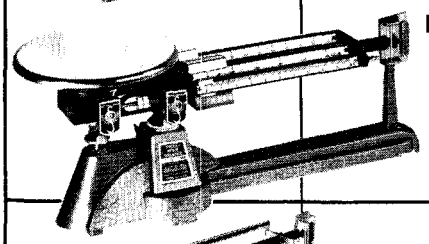

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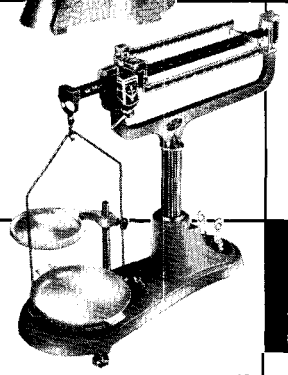

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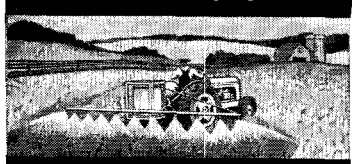

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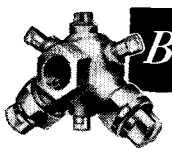

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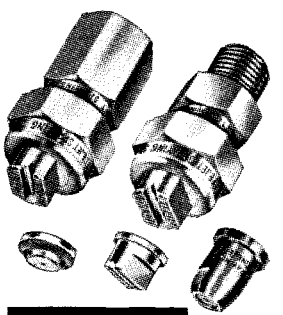

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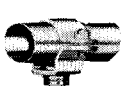

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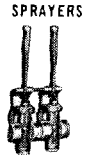

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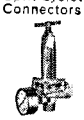

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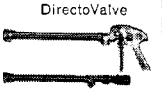

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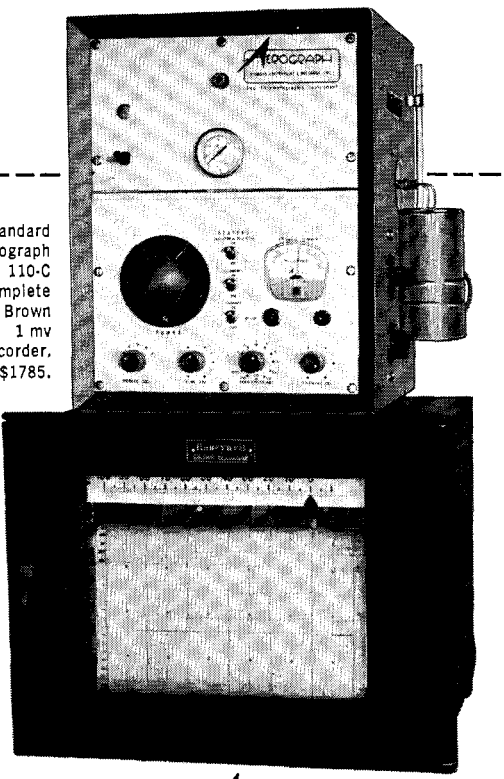

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Symposium on the Mechanism of Action of Pesticide Chemicals

In our knowledge concerning pesticides there exists a great hiatus where there should be an understanding of the intimate details of the mechanism of the toxic action of several important contact pesticides. The ability finally to overcome the defenses of the arthropod pests may have to await the filling in of this gap.

The Symposium on Research Progress on Insect Resistance, held in Washington, D.C., in October 1959 under the sponsorship of a committee of the National Agricultural Chemicals Association and the Entomological Society of America emphasized the growing need for a greater knowledge of resistance to pesticides.

The chief objective of the Symposium on the Mechanism of Action of Pesticide Chemicals, presented by the Division of Agricultural and Food Chemistry at the 137th meeting of the American Chemical Society in Cleveland, Ohio, in April 1960, was to stimulate efforts to find more effective and more lasting means of chemical pest control. Most of the papers from that symposium are printed in the following pages.

Some of the contributors present results of their recent investigations on the ability of certain chlorinated pesticides somehow to mobilize materials whose source is in and whose effect is on the nerve tissue. Another reviews our present limited knowledge of the metabolism of pesticides, a field whose development is of essential importance to a fuller understanding of both intoxication and detoxication, and hence of toxicity and resistance. The contribution which completes the symposium deals with the action of synergists as they may increase or decrease the toxicity of pesticide chemicals.

DOUGLAS J. HENNESSY, Symposium Chairman
Fordham University, New York, N. Y.

Approaches to Mechanisms of Insecticidal Action

E. H. COLHOUN

Pesticide Research Institute, Canada Department of Agriculture, London, Ontario, Canada

Insect species and vertebrates differ with respect to response to drug and insecticide action. The insect neuromuscular system appears to be unaffected by insecticides and drugs paralyzing vertebrates. In seeking an explanation for chlorinated hydrocarbon poisoning in the cockroach, no evidence of a biochemical lesion was obtained. Furthermore, biologically active substances found in cockroach blood appear to be a consequence, but not a cause of DDT or dieldrin intoxication.

Editor's Note

The four papers published here deal with mechanisms of action of pesticide chemicals in insects. A fifth paper in the symposium, by E. A. Hosein and P. Proulx, was concerned with some effects of pesticide chemicals in mammals. We hope to present the latter paper in an early issue.

INSECT NERVOUS SYSTEM TISSUE forms an essential integrated system which is a primary target for the action of many insecticides. Certain differences have been found between the insect and vertebrate in the comparative action of drugs and insecticides in muscles and in the nervous system. Differences were found when simple ions such as potassium were used, with pharmacological agents, including acetylcholine, eserine, and atropine, and finally with the organophosphates. Mechanisms of chlorinated hydrocarbon poisoning are discussed.

Potassium

Hoyle (*18*) showed that nerve conduction in the fifth leg nerve of the locust was not affected by concentrations of potassium high enough to block conduction in the vertebrate nerve. This observation led to a hypothesis that the insect nervous and muscle systems are protected by an ion-impermeable barrier. The data given in Table I show that when the effect of potassium upon nervous conduction in the locust, American cockroach, and toad is compared, only the locust nervous system is

resistant. This shows a difference between insect species rather than a general difference between the insect and vertebrate nervous systems. The locust muscle system is even more sensitive to potassium than the nervous system. Although no specific data are available for the cockroach, the muscles of the roach are probably no more insensitive to potassium than the nervous system.

An electron microphotograph of a section of the fifth leg nerve of the cockroach is given in Figure 1 (*15*). In structure the leg nerve is apparently similar to that of the locust (*1*). Hoyle (*18*) suggested that a tracheolated membrane is present around the locust leg nerve, but Ashhurst (*1*) has recently indicated that the tracheolated membrane is absent from the locust. So far no anatomical structure has been found to explain the difference in permeability to potassium in the locust and roach nervous systems. There may be a difference in active regulation of ion transport, which probably occurs in the mitochondrial-rich layer of glial cells underlying the connective tissue sheath. Interference with enzyme action (usually associated with mitochondria) in the nerve sheath may lead to self-intoxication of insects, but this would apply only if the irritable tissues of all insects were similarly protected against ion disruption.

Drug Action

A number of drugs produce different effects in the insect and in the vertebrate (Table II). Acetylcholine (ACh), which disrupts nervous activity in vertebrate ganglia and at the neuromuscular junction, has no apparent effect in the locust or cockroach. Unlike potassium, a difference has been found for ACh in the two insect species. In insects where it can be studied, the nervous system appears to be impervious to ACh and in this respect can be compared only with the vertebrate central nervous system. Although ionization of a drug may determine its ability to penetrate into a tissue, one must also consider effector sites

Figure 1. Electron micrograph of cross section of metathoracic fifth leg nerve of *Periplaneta*

Nerve is covered by a fibrillar neural sheath, *N.S.* Immediately below this is a highly basophilic layer, *G*, composed of dovetailed neuroglial cells containing numerous mitochondria and basophilic granular cytoplosm; these cells are bounded by glial cell membranes, *G.M.* The nerve consists of axons, *Ax*, ranging from a fraction of a micron to several microns in diameter. Each axon is covered by a membrane, *A.M.* The interstices between the axons are filled with neuroglial cells contoining mitochondria and an unknown compound, *U.* The neuroglial cells may surround one or several axons in the manner of Schwann cells. Fixed in permanganate and embedded in Shell Epon 812. × 33,000

Table I. Effect of Potassium Concentration on Nervous Conduction

Organism	K^+ Concn., mM	Connective Tissue Nerve Sheath: Intact — Time to Block, Min.	Connective Tissue Nerve Sheath: Removed — Time to Block, Min.
Locust (*18*), *L. migratoria*	50	No effect	Immediately (injected)
	140	240	
Cockroach (*33*), *P. americana*	180	12	10 sec.
	140	30	60 sec.
Toad (*12*), *R. tigrina*	120	15	3/4 to 1

Table II. Effect of Drugs on Nervous and Muscle Activity in Insects and Vertebrates

Insect: Drug concn.	Insect: Central ganglia	Insect: Axonic	Insect: Muscle	Vertebrate: Drug concn.	Vertebrate: Central nervous system	Vertebrate: Ganglio	Vertebrate: Axonic	Vertebrate: Muscle
ACh, 10^{-2} *M*, roach (*10*), locust (*16*)	None	None	None	ACh 10^{-4} *M*	None	Facilitation block	None	Excitation block
Eserine, 10^{-4} *M*, roach (*10*), locust (*16*)	Excitation block	None	None	Eserine 10^{-4} *M*		Block	Block	Excitation
Eserine, 10^{-3} *M*, locust (*16*)	Excitation block	None	Excitation					
Atropine, 10^{-3} *M*, locust (*16*)	None	None	Tremors	Atropine (therapeutic amounts)	Stimulates depresses	Excitation	None	None
Atropine, 10^{-3} *M* roach (*24*)	None	None						
Curare, 10^{-2} *M*, roach (*24*)	None	None	None	Curare		Blocks effect of ACh	None at ganglion concentration	Blocks effect of ACh

Table III. Effect of Organophosphorus Compounds on Nervous and Muscle Activity in Cockroach and Locust

Compound Concn., *P. americana*	Thoracic Ganglion	6th Abdominal Ganglion
TEPP[a], 10^{-4} to 10^{-5} *M* (*8*)	Block, cycling	Facilitation, block, cycling
DFP, 10^{-3} to 10^{-5} *M* (*24*)		Facilitation, block, cycling
L. migratoria (*16*)	Thoracic ganglion leg reflex	Leg muscle
TEPP, 10^{-3} *M*	...	No effect
TEPP, 10^{-5} *M*	Facilitated, blocked	...
Paraxon, 10^{-3} *M*	...	No effect
Paraxon, 10^{-5} *M*	Facilitated, blocked cycling	...

[a] Abdominal and thoracic connectives, motor and sensory fibers of fifth leg nerve and leg muscles. Not interfered with by TEPP at 10^{-4} to 10^{-5} *M*.

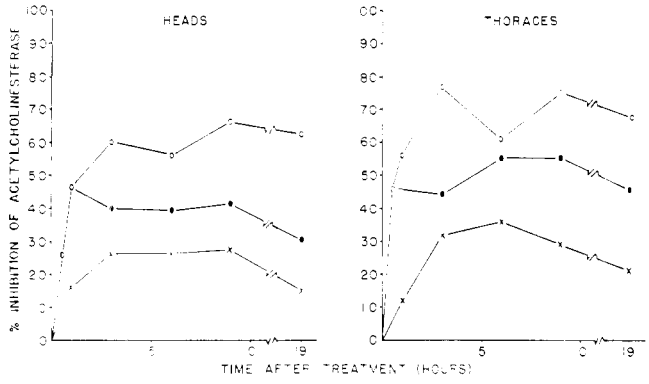

Figure 2. Acetylcholinesterase inhibition in heads and thoraxes of houseflies treated with sublethal dose of TEPP

O Prostrate flies
● Flies recovered from prostration
X Nonprostrate flies

of drug action. Very little is known about insect neuromuscular mechanisms, but it is evident that innervation of insect muscles (*19*) is different from those of vertebrates. Furthermore, the cholinergic system of the vertebrate neuromuscular system is absent in the roach, for Colhoun (*7*) was unable to demonstrate ACh, choline acetylase (ChA), or acetylcholinesterase (AChE) in leg muscles of the cockroach. The absence of the cholinergic system would explain the negative effect of ACh injected into insects better than the hypothesis that ACh is unable to penetrate into the neuromuscular junction.

It is more difficult to find a similar explanation for the different effect of atropine (Table II) in the locust and in man. Harlow (*16*) has shown that atropine stimulated the muscle contraction of the hind leg of the locust but at a higher concentration did not affect ganglionic nervous transmission. In man atropine affects central nervous activity at a concentration which does not interfere with neuromuscular transmission. The brain blood barrier of man is thought to be an effective barrier to certain ions which have easier access at the neuromuscular junction. Finally, eserine seems to have a similar effect in locust and cat. Although pH affects the ability of eserine to penetrate into tissues, eserine penetrates into the nervous system at physiological pH. In the cat eserine blocks ganglionic nervous conduction, but at the same concentration excites neuromuscular nervous transmission. Harlow (*16*) shows that at 2×10^{-4} *M* eserine blocked ganglionic nervous transmission in the locust, but did not affect muscle activity. At a higher concentration eserine affected muscle activity. Eserine may interfere with receptor sites apart from anticholinesterase action. On this basis it may be possible to explain the neuromuscular effect of eserine in the insect in the absence of cholinesterase at the neuromuscular junction.

Organophosphate Action

In the roach a number of organophosphates disrupt central nervous function (Table III) but have no effect upon muscle activity. The absence of the cholinergic system in the roach would explain this difference, as these substances have no apparent effect on muscle activity. The work of O'Brien (*22*) may point the way to devising good insecticides, as un-ionized Tetram has the ability both to penetrate into the nervous system and to inhibit AChE. Indeed, O'Brien's work appears to be further evidence of the importance of AChE in the insect nervous system. However, the introduction of nonionized insecticides would obviously increase their hazard to man, because it would enable the substance to penetrate more easily to all parts of the vertebrate nervous system. O'Brien (*21*) has pointed out the possibility of using insecticides with degrees of ionization such that useful insects with a good ion barrier would be protected. In extensive cropping practices this may be difficult to accomplish, for harmful and beneficial insects may have the same protection. The best insecticide with respect to low hazard to vertebrate life might be one which coupled the weak link theory of O'Brien (*21*) with good ability to penetrate into insect tissue. The weak link would allow the insecticide to be rapidly broken down in the vertebrate.

Recovery from Insecticide Poisoning

Insects are known to recover from prostration induced by insecticide treatment. Stegwee (*28*) showed that houseflies treated with a sublethal dose of tetraethyl pyrophosphate (TEPP) recovered from prostration with about the same degree of AChE inhibition as that determined when they first became prostrate. These results (Figure 2) might indicate that AChE was not involved in organophosphorus poisoning, but this question will best be resolved when the function of AChE in the insect nervous system is more fully understood.

The ability of an insect to recover without therapeutic aid from what appears to be a lethal dose of an insecticide may be explained by the ultimate evaluation of dependency of tissues and metabolic processes in the insect compared with a similar system in man. Although the insect may have less freedom than man with respect to environment, its internal processes may be less susceptible to the action of drugs and poisons. This argument is based on the known direct tracheal respiration of insects, which would eliminate blood as a necessary carrier of oxygen. An insect has the ability to withstand fluctuations of ions and metabolites in the blood and its individual tisseus may have aerobic and anaerobic metabolic mechanisms, enabling them to carry on under adverse conditions with adequate metabolic reserve. In these respects the vertebrate is a great deal more sensitive, for brain activity is intimately dependent upon the levels of sugar, ions, and oxygen in the blood; any interference with these factors by drug action—for instance, respiration in organophosphorus poisoning—results in far-reaching effects that may culminate in death.

The ability of an insect to survive after insecticide treatment for at least

a time without gross physiological disruption provides greater scope for any compensatory or adaptive mechanism to come into effect. For example, Stegwee (*28*) found that by the time the initial toxic effect of TEPP had disappeared, the housefly had been able to overcome the initial disruption of function. The fact that the insect may be able to tolerate adversity in the shape of a poison better than the vertebrate has at least two corollaries. An opportunity for detoxication mechanisms to operate is greater in the insects, and the wider range of individual tolerances among insects permits a more effective selection of resistant strains than in mammals.

Chlorinated Hydrocarbon Poisoning

The mode of action of chlorinated hydrocarbon poisoning in insects is not understood clearly. Recently it has been suggested (*29*) that a toxicant found in the blood of roaches treated with DDT may be the causal factor in DDT prostration. The term "toxicant" would imply that an intoxication agent has been found, but so far no precise quantitative evidence has been obtained that the intoxication of the DDT-treated roach is due to this or any other blood factor. The work of Sternburg, Chang, and Kearns (*29*) followed the earlier findings of Sternburg and Kearns (*31*), that the blood of DDT-prostrate roaches induced high nervous activity of the isolated nerve cord of a normal roach. Subsequently other investigators have reported active biological properties in insect blood. The data given in Table IV show that treatment of roaches with various insecticides of different molecular structure resulted in active blood factors. These were also found in roaches treated by electrical shock or in restrained roaches. The common effect of all these treatments would appear to be some form of stimulation of the roach, and this may in all instances affect the nervous and endocrine system. Because the toxicant of Sternburg, Chang, and Kearns (*30*) has been found in DDT, TEPP, or electrical stimulation, showing no specificity of action, it is reasonable to suppose that it may be found in other insecticide treatment.

While investigating the earlier report of Sternburg and Kearns (*31*) Colhoun (*6, 7, 9*) showed that the blood of roaches treated with DDT and TEPP contained a number of substances in abnormal amounts. Among these was a catechol amine which was biologically active when tested against the isolated heart and nerve cord preparation of a normal roach. The main source of the catechol amine in the roach appears to be the corpus cardiacum gland, although Gersch, Unger, and Fischer (*13*) reported its occurrence in the nerve cord. The presence of this active substance in the blood of DDT- and TEPP-treated roaches led to the correct supposition that more than one factor may be responsible for biological activity when blood is tested against various preparations. The work of Ozbas and Hodgson (*23*) and the very recent report of Milburn, Weiant, and Roeder (*20*) show that corpus cardiacum extracts are biologically active when tested in the roach.

There seems little merit in discussing the importance of one blood substance relative to that of another, for in the blood of a single treated roach all abnormal factors contribute to some degree of biochemical or physiological disturbance. It is important to resolve whether blood factors cause insecticide-like disturbances in roaches where the insecticide is not present. In this way it might be possible to separate primary from secondary insecticide action.

Without carrying out a single experiment it seemed that this was possible, for Spiller (*26*) showed that when a DDT-treated *Rhodnius* was joined in parabiosis to an untreated *Rhodnius*, the untreated insect was unaffected. This clearly showed that the blood of the treated insect was not toxic to the insect to which it was joined. This nontoxicity could scarcely be attributed to a poor interchange of blood, as both insects moulted; furthermore this type of preparation has been repeatedly used by Wigglesworth (*34*) to demonstrate the presence of hormones in *Rhodnius*. Bodenstein (*4*) has also successfully joined two cockroaches together to show hormone effects, and if it were possible for minute amounts of hormones to be translocated from one roach to another, it seemed possible to use this technique (Figure 3) to test the toxicity of blood factors in roaches treated with DDT, dieldrin, or TEPP.

Parabiosis Experiments with the American Cockroach. Roaches intoxicated by topical treatment with TEPP, DDT, or dieldrin were joined to untreated roaches. No toxic effect was observed in the untreated roaches in 48 hours, but they became prostrate at 72 to 120 hours. It is at once evident that the results are different from those found for *Rhodnius*, for after a few days all the untreated roaches died. The physiology of *Rhodnius* may be different from the roach, as *Rhodnius* appears to go into a state of akinesis when exposed to abnormal conditions. If this condition is induced by intoxication, the processes of tissue degeneration may be retarded in *Rhodnius* when they are attached. To learn the effects of the dying roach upon the normal roach, roaches treated with cyanide (which stops respiration and electrical nervous conduction) were joined to normal roaches. The normal roaches died, and their time of death was about the same as in the experiments with DDT, TEPP, or dieldrin.

The dying untreated roaches did not show the typical hyperactive symptoms of poisoning associated with insecticide treatment. They became sluggish, lethargic, and at the most uncoordinated, before they became prostrate. The presence of insecticides in the untreated roaches could not be detected, although DDT and dieldrin were recovered from the treated insects. Almost all of the dieldrin was recovered from treated roaches, showing that this insecticide was not metabolized at a detectable rate in the roach. The difference between symptoms of the treated and untreated

Table IV. Occurrence of Biologically Active Substances in Blood of Cockroaches

	Method of Determining Blood Activity[a]			
Treatment to Cockroach	Chromatogram	Roach isolated nerve cord, electrical activity	Roach isolated heart	Other insects or means
DDT (*5*)	..	..	..	Calliphora flies
DDT (*31*)	..	+	..	Sarcophagid flies
Pyrethrum (*3*)	..	..	..	Sarcophagid flies
Restrained (*3*)	..	..	..	Cockroach
Mechanical (*2*) stimulation (powder mill)	..	..	..	Cockroach
DDT (*7*)	+	+	+	...
TEPP (*7*)	+	+	+	Frog rectus abdominis muscle, Venus heart
Electrical (*10*) grid stimulation	+	+	+	...
Dieldrin (*9*)	..	+	..	...
DDT (*25*) + temp.	..	+	..	...
DDT (*30*)	+	+	..	...
TEPP (*30*)	+	+	..	...
Electrical stimulation (*30*)	+	+	..	...

[a] + Means of detecting biologically active substances.

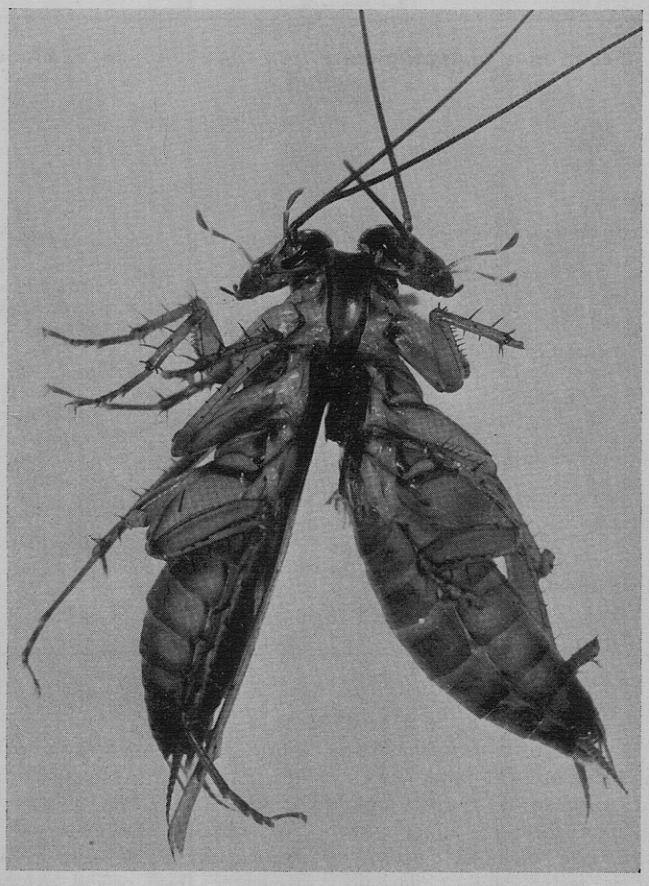

Figure 3. Technique of joining *Periplaneta* in parabiosis to determine toxicity of blood factors resulting from insecticide treatment

Note wax ring at prothoracic shield

roaches indicates that the primary hyperactive and convulsive effects of the insecticides cannot be due to blood factors. In experiments where DDT and TEPP were applied to one of a pair of joined roaches, in sufficient amounts to ensure intoxication of the untreated roach, the insecticidal effect was the same as in a roach treated alone, in both symptoms and the time to prostration. The lack of distinction between the effects, in the normal insect, of union with a dying insect and of union with one treated with either insecticide, makes it impossible to attribute the reaction of the normal insect only to blood factors resulting from insecticide treatment. In these experiments it was expected that a highly intoxicating blood factor would affect the untreated joined roach within the first few hours, for Beament (*2*) has shown that a paralysis factor induced in roaches when pinned to a block could affect a joined roach within 12 to 24 hours.

Central Nervous Effects of Chlorinated Hydrocarbon Poisoning

In seeking a possible explanation of the nervous effects of chlorinated hydrocarbon poisoning, it was established that the amount of acetylcholine increased in the nerve cords of roaches prostrate by DDT treatment (*6*) from the time of early prostration. The occurrence of free ACh in nerve cords would explain nervous excitation caused by DDT treatment, for ACh has this effect when applied to desheathed nerve cords (*32*). However, this explanation ignores the activity of acetylcholinesterase in nervous tissue, for this enzyme rapidly inactivates ACh when it and its substrate are brought together (*6*). Colhoun (*6*) concluded that ACh was a secondary factor in DDT intoxication, as in vivo evidence of free ACh or inhibition of AChE was not obtained. Increase of ACh in the nerve cords of roaches prostrate after treatment with dieldrin has been found, but not during the hyperactive early convulsive phase of intoxication. A common effect has been found in dieldrin and DDT intoxication.

Stegwee (*27*) showed inhibition of AChE in roaches treated with DDT. Until the recent finding of Sternburg, Chang, and Kearns (*30*), no other evidence was available that this enzyme was interfered with in DDT intoxication. These authors injected roaches with tetramethyl pyrophosphate (TMPP) and found that AChE of the nerve cords of DDT-prostrate roaches was only slightly inhibited, whereas the AChE of control roaches was entirely inhibited.

This work was repeated by injecting TMPP and TEPP into roaches prostrate by DDT, dieldrin, or cyanide treatment. The AChE of thoracic nerve cords of roaches prostrate by DDT, dieldrin, or cyanide was less inhibited by injection of TMPP or TEPP than the AChE of normal roaches. TMPP was a less effective inhibitor than TEPP. TMPP has a very short half life, and would be effective in the insect for only a short time after injection. The results confirmed the findings of Sternburg, Chang, and Kearns (*30*) but do they show that AChE is protected or inhibited by DDT treatment? An explanation seems to emerge from the experiments where cyanide was used, for TMPP was no more effective as an inhibitor of the AChE of the nerve cords of cyanide-treated roaches than of DDT-treated roaches. A factor produced by nervous activity in the nerve cord capable of occupying the hydrolysis sites of AChE would be absent in the roaches treated with cyanide, for cyanide blocked the electrical nervous activity of the nerve cords. It would appear more difficult to inhibit the AChE of a prostrate roach regardless of the treatment, and until an adequate explanation is found, it seems unwise to postulate a cholinesterase mechanism of chlorinated hydrocarbon poisoning.

The occurrence of high amounts of ACh in the nerve cords of DDT and dieldrin-treated roaches shows a biochemical abnormality in the nerve cord, for in the roach the ACh titer is remarkably constant under normal conditions. It seemed expedient to determine whether the increase in ACh was due to the peripheral action of DDT or to blood factors. Nerve cords were isolated from roaches treated with DDT or dieldrin at a time when the roaches showed signs of becoming prostrate, and washed and placed in saline. The ACh content of the cords and those left in intact treated roaches was examined at intervals. The ACh content of the isolated nerve cords increased with startling similarity to that of nerve cords of intact treated roaches. The results would appear to show a central nervous effect of chlorinated hydrocarbon poisoning which was not dependent upon the presence of the intact peripheral nervous system or blood factors. The nervous activity of the treated isolated nerve cords failed before that of the controls, which showed only slight reduction in nervous activity at 24 hours. Failure of nervous activity occurred first in the cords isolated from dieldrin-treated roaches and the highest increase of ACh was found in these cords.

Finally, in an attempt to learn the reason for the interference of nervous activity in the isolated nerve cords, tests

were made to determine the possible occurrence of γ-butyrobetaine which Hosein (*17*) concluded was a factor in dieldrin poisoning of the rat. While attempting to find this substance, Colhoun and Spencer (*11*) found that the esters of the betaine were more likely to cause toxic effects if found in the nervous system. However, neither γ-butyrobetaine nor its esters were present in the nerve cords of dieldrin-treated roaches or those of normal roaches. The occurrence of any one of these substances in the nerve cord of treated roaches would suggest that dieldrin is capable of inducing the metabolism of a substance not normally found. This principle, although exciting, has no factual foundation in chlorinated hydrocarbon poisoning in insects at present. However, the possibility suggests a biochemical approach to solving the mechanism of chlorinated hydrocarbon in the central nervous system of insects alongside the search for a physical effect thought to occur in the peripheral nervous system (*14*).

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Much of our understanding of the events occurring during neurotransmission in insects is based on a comparison of the properties of insect nerves with known properties of vertebrate nerves. In many ways, insect nerves respond to chemicals much as do the nerves of vertebrates. For example, physostigmine, a reversible inhibitor of cholinesterase, and the organophosphates, irreversible inhibitors of cholinesterase, disrupt synaptic transmission in insects. These synapses are located in the ganglia of the central nervous system and it is here that high concentrations of cholinesterase are located. Acetylcholine and choline acetylase, the other components required for cholinergic transmission, are also found for the most part within the ganglia (*3*). The presence of these substances clearly indicates that at least some phases of neurotransmission in insects are cholinergic. Disruption of normal neuroactivity by anticholinesterases confirms this. The intense stimulation of central nervous activity by pilocarpine in insects (*13, 16*) also lends support to the presence of cholinergic pathways, as pilocarpine is believed to act by stimulating cholinergic vertebrate effectors.

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But there are some disturbing differences. Curare and atropine are known in vertebrates to block neuromuscular junctions and sympathetic ganglia from the action of either

exogenous acetylcholine or electrical stimulation. These are cholinergic sites in vertebrates, and it is thought that curare and atropine desensitize these sites postsynaptically to either exogenous or endogenous acetylcholine. In insects, however, neither curare nor atropine has any effect when injected into cockroaches (*13*) nor do they affect synaptic transmission through the sixth abdominal ganglion of the cockroach (*14*). As it is known that insect neuromuscular junctions lack cholinesterase (*27*) and thus are presumably noncholinergic, it is not surprising that injected doses are ineffective here, but the failure to affect sensory-central synaptic transmission is difficult to explain, unless it is due to the failure of atropine or curare to penetrate to a critical site within the ganglion.

For ionized compounds failure to penetrate may be due to an ion-impermeable sheath around the nerve cord (*9*). As shown by O'Brien (*11*), ionized inhibitors of cholinesterase do not readily penetrate the extremely thin sheath that covers the nerves and ganglia of insects, whereas nonionized inhibitors penetrate rapidly. Acetylcholine itself is surprisingly ineffective when injected into insects, and several milligrams may be injected into a cockroach weighing 1 gram with no ill effects. When applied directly onto the last abdominal ganglion of the cockroach, acetylcholine is without consistent effect on synaptic transmission, even at concentrations of 1.0% (*14*). However, Twarog and Roeder (*23*) have shown that if the protective sheath surrounding the ganglion is ruptured, $10^{-3}M$ concentrations of acetylcholine will often cause a partial block of synaptic transmission, and that this is potentiated by physostigmine, with $10^{-4}M$ acetylcholine then causing bursts, after discharge and block. As Twarog and Roeder point out, this is still a relatively high concentration, and although the evidence favors interpreting these synapses to be cholinergic, the question cannot as yet be considered answered.

As insect ganglionic tissue is rich in both acetylcholine and cholinesterase, it is logical to conclude that neurotransmission is at least in part cholinergic. The site of action of the organophosphates appears to be those areas containing cholinesterase, localized within ganglia, whereas DDT has been shown to act primarily on sensory nerves and associated structures (*17*, *26*). Although these sites undoubtedly are distinct, certain characteristic effects produced by these two classes of toxicants suggest similar disruptions of normal nervous behavior. The visible symptoms of poisoning by DDT clearly indicate involvement of the nervous system. As shown by Roeder and Weiant (*17*), the sensory nerves are most sensitive to DDT, with little or no direct action occurring on central or motor nerves. Yet prostration and paralysis eventually do take place, indicating that more profound changes occur than mere excitation of afferent nerves.

Although the visible symptoms of DDT prostration and final paralysis are suggestive of cholinesterase inhibition, no inhibition of this enzyme by DDT can be shown, either in vivo or in vitro. Yet, during prostration bound acetylcholine within the central nervous system increases two to three times above normal (*2*, *21*). This increase is not great, nor does it occur during the earlier stages of poisoning, and may be of only secondary importance. However, the increase in free acetylcholine following complete inhibition of cholinesterase by an organophosphate is ultimately no greater (*2*).

Electrophysiological studies by Roeder and his associates have shown that multiple afterdischarges in giant fibers follow electrical stimulation of cercal nerves in DDT-prostrate cockroaches, and that similar discharges may occur during phosphate poisoning (*15*). Here again, the similarity is striking, and further indicates the impairment of a common system. This intriguing situation suggests that common factors may in some way be involved in a sequence of events initiated at different sites by DDT and an organophosphate antiesterase.

Release of a Neuroactive Substance

During DDT poisoning a toxic substance has been found to accumulate in the blood of American cockroaches [*Periplaneta americana* (L.)] (*18–20*). Chemical analysis indicated the absence of significant amounts of DDT in such blood. Yet, when injected into either susceptible or DDT-resistant houseflies, blood from DDT-prostrate cockroaches initiated typical symptoms of DDT poisoning. Furthermore, blood from DDT-prostrate cockroaches was found to cause an increase in the spontaneous nervous activity of an isolated central nerve cord obtained from a normal cockroach. After a brief period of high excitation, the activity usually decreased and blocked suddenly. As DDT itself has no such direct action on the central nervous system, it was concluded that something other than DDT was responsible for these effects.

Concomitant with instability of the sensory nerves, DDT-poisoned cockroaches become hyperexcitable. At this time, the first traces of a neuroactive substance appear in the blood. As the visible symptoms of poisoning by DDT progress through intoxication to prostration, the amount of the neuroactive substance in the blood increases. Conversely, if advantage is taken of the negative temperature coefficient of DDT (*25*), the amount of neuroactive substance present decreases rapidly and by the time recovery is complete—in a half hour or so—no neuroactive substance can be detected in the blood.

The neuroactive substance is not a metabolite of DDT, but is probably a natural component of the insect's body, as it can be produced without the use of DDT by electrical stimulation (*19*). Such treatment results in the appearance of a neuroactive substance in the blood similar chromatographically to that induced by the action of DDT. Under certain conditions, what appears to be the same neuroactive substance can be detected in the saline perfusing isolated central nerve cords. When a central nerve cord, with the cercal nerves and cerci still attached, is placed in physiological saline, and the cerci are suspended above the saline and treated topically with DDT, the perfusing saline gradually accumulates a neuroactive substance. Application of DDT suspensions or emulsions directly to isolated central nerve cords fails to lead to toxic perfusates, agreeing with Roeder's earlier observation that DDT has no direct action on the central nervous system.

Release of the neuroactive substance occurs concomitantly with excessively high central nervous activity (*19*). The initial effect of tetraethyl pyrophosphate (TEPP) on the electrical activity of an isolated cockroach nerve cord consists of a wild burst of spontaneous activity, varying in duration with the concentration of TEPP applied. Although TEPP rapidly inhibits the cholinesterase of isolated nerve cords, whereas the neuroactive substance has no known effect on cholinesterase, the similarity in the extremely high level of spontaneous activity following treatment with either substance suggested that neuroactive material might also be released following treatment with TEPP.

Under normal physiological conditions, an isolated thoracic and abdominal central nerve cord will maintain a continual rhythmic background of spontaneous nervous impulses for many hours. If the preparation includes an intact cercus, electrical stimulation of the cercal nerve will cause a normal postsynaptic response in the giant fibers of the abdominal region. If the abdominal region is stimulated directly, the response to axonal stimulation of the giant fibers can be observed. Application of $10^{-3}M$ TEPP to such a preparation results in a complete block of postsynaptic responses to cercal stimulation within 30 seconds. At the same time, spontaneous activity increases enormously for a brief period, then blocks suddenly, usually in less than 1 minute. Responses to direct axonal stimulation do not block, nor do they fail subsequently. If the nerve cord is

left in the initial TEPP saline, spontaneous activity remains more or less blocked, and only rarely returns within 15 minutes. However, if the saline containing the initial dosage of TEPP is removed, and the nerve washed four or five times with fresh $10^{-3}M$ TEPP, the spontaneous activity rapidly returns, generally within 2 to 5 minutes. The recovered spontaneous activity appears in general to be normal, and remains so for at least 5 hours, the longest time any have been monitored. The response to axonal stimulation continues during this time, and appears to be normal. Postsynaptic response to cercal stimulation, blocked by the first dosage of TEPP, does not return.

If the initial perfusate of TEPP is reapplied to the nerve cord, spontaneous activity again increases greatly above normal, then gradually declines to a low level or in some cases blocks again. As before, washing with fresh $10^{-3}M$ TEPP restores the spontaneous activity to a normal level.

During the entire experiment, the concentration of TEPP has been maintained at a nearly constant molarity ($10^{-3}M$). Calculated hydrolysis of TEPP during this time (2 hours) does not reduce the concentration below ca. $8 \times 10^{-4}M$ (*22*). No attempts were made to determine possible enzymatic hydrolysis of TEPP. However, replacement of the bathing solution with fresh TEPP saline caused no change in activity. The reappearance of spontaneous activity thus cannot be explained as due to hydrolytic destruction of the TEPP. The rapid return of spontaneous activity after removal of the initial dosage of TEPP, but with the concentration of TEPP maintained at a constant level, and the ability of the initial perfusate of TEPP to excite a cord again after recovery of spontaneous activity, indicate that a substance other than TEPP must be present in the initial TEPP perfusate. Because only nervous tissue is present, this must come from the nervous system, and presumably is liberated during the initial burst of high spontaneous activity following the application of TEPP.

The cholinesterase of nerve cords subjected to the treatment just described is completely inhibited within a few minutes after the start of the experiment and remains inhibited. Return of spontaneous activity to an essentially normal level, but with no recovery of cholinesterase activity, must mean that the acetylcholine-cholinesterase system is not necessary for the maintenance of spontaneous activity within the central nerve cord. Presumably the substance liberated during the initial burst of high activity is responsible for the block of activity. This does not mean that the cholinesterase system is not important. Normal synaptic transmission certainly depends on this system, and inhibition of cholinesterase ordinarily abolishes transynaptic responses.

Comparison with Other Active Substances

In recent years there have been other reports of physiologically active compounds present in various tissues of the cockroach. Some of these have cardiac activity. Unger (*24*) and Gersch, Unger, and Fischer (*6*) found several substances, active on isolated cockroach hearts, present in the blood, the central nervous system, and the corpora cardiaca. Colhoun (*2*) reported several active materials present in the blood of DDT- or TEPP-poisoned cockroaches, most with cardiac activity, but he also reported neuroactivity for blood from DDT-poisoned roaches. Ozbas and Hodgson (*12*) have reported that a neuroactive substance can be extracted from the corpora cardiaca of normal cockroaches, but that after electrical stimulation these glands are depleted of their substance (*8*). Because of differences in bioassay methods used by different workers, it is difficult to determine how many compounds are actually involved in these reports. By employing the procedures used by Unger for chromatographic separation of the cardiac active substances, it has been possible to compare them biologically with the neuroactive substance found during DDT poisoning. Briefly, it can be stated that the substances reported by Unger were located on the chromatograms and found to have cardiac activity but no detectable neuroactivity, nor did the neuroactive substance in the blood after DDT poisoning appear to have any effect on the heart. In regard to the neuroactive substance present in the corpora cardiaca, comparison of its R_f in a chromatographic system (1-butanol–acetic acid–water:4:1:5) for which the R_f is known for the DDT-induced neuroactive substance, has revealed that they are not the same compound. The corpus cardiacum substance has an R_f of about 0.3, whereas the DDT-induced substance has an R_f of 0.6 in this system.

Recently, Milburn, Weiant, and Roeder (*10*) have reported that the application of corpus cardiac extract to abdominal ganglia leads to increased rhythmic efferent activity in the phallic nerves, evidently because of the suppression of the inhibitory influence of the subesophageal ganglion. They found that the DDT-induced substance did not cause rhythmic bursts of activity to appear in efferent nerves, although both the DDT-induced substance and corpus cardiacum extracts caused increased central nervous activity in the abdominal cord. They concluded that the DDT-induced substance was not the same as the active material from the corpora cardiaca, thus confirming the difference found by chromatographic separation.

Partial Isolation and Identification

From Cockroaches. The neuroactive substance from blood of DDT-prostrate cockroaches has been partially isolated by chromatography (*19*). After development of the chromatograms, the active substance was located by extracting sections of the chromatogram with physiological saline and determining the effect of these extracts on the spontaneous activity of isolated cockroach nerve cords. By using several different chromatographic systems in sequence, each time rechromatographing only the biologically active section, good separation of the neuroactive substance from other components of the blood was obtained. Because of losses in biological activity entailed during the many manipulations involved, and because of the difficulty in obtaining large samples of cockroach blood, only a few chemical tests have been tried on this substance from roaches, and only one of these has been consistently positive. Diazotized *p*-nitraniline gave a red color coinciding with the biologically active section of chromatograms of toxic blood extracts. Normal blood extracts failed to give this color at the R_f for the active material.

Although little was learned concerning the chemical nature of the neuroactive substance, its R_f in various chromatographic systems was compared with the R_f's of a number of compounds believed to play a role in neurotransmission. It was immediately apparent that it was not acetylcholine, epinephrine, norepinephrine, serotonin, histamine, nor γ-aminobutyric acid. This was not surprising, as none of these compounds disrupt the normal spontaneous activity of isolated nerve cords from cockroaches.

From Crayfish. At best, cockroach blood is difficult to obtain in quantity. One hundred roaches will yield only 10 ml. of blood. As it was unlikely that more than a few micrograms were present per milliliter, it was necessary to find a better source. A search was made therefore for a DDT-susceptible arthropod easily obtained in quantity and possessing a larger volume of blood. The requirements were met by crayfish [*Orconectes virilis* (Hagen) and *O. propinquus* (Girard)], large individuals yielding 3 to 5 ml. of blood. Crayfish were poisoned by immersion for 15 minutes in water containing a suspension of 100 p.p.m. of DDT. They were then transferred to trays lined with wet paper towels, and held until prostrate (3 to 8 hours). This precaution was necessary to prevent premature death, presumably from anoxia after movements of the gills became erratic due to DDT poisoning.

Blood from DDT-prostrate crayfish, processed in the same manner as that

Table I. Organic Spot Test Analysis of Neuroactive Substance from DDT-Prostrate Crayfish Blood[a]

Test[b]	Result[c]	Remarks
Ignition with CaO	Positive	Contains nitrogen
Diazotized sulfanilic acid	Positive	Phenol or aromatic amine
Diazotized *p*-nitraniline	Positive	Phenol or aromatic amine
Concd. HCl + *p*-dimethylamino-benzaldehyde	Negative	Not pyrrole, unless lacking CH α or β to cyclic NH
EtOH + HCl + *p*-dimethylamino-benzaldehyde	Positive (yellow)	Primary aromatic or aliphatic amine
Ninhydrin	Negative	Not primary or secondary aliphatic amine
1,2-Naphthaquinone-4-sulfonate	Negative	No active CH_2 or NH_2 unless hindered by negative groups in ortho or para positions
Sodium nitroprusside + acetaldehyde	Negative	Not secondary aliphatic amine
Hydroxylamine (Hestrin test)	Positive	Ester of carboxylic acid
$NaN_3 + I_2$	Negative	Not thioketone or thiol
Millon test	Negative	Not phenol, unless di-*o*- or di-*m*-substituted
HNO_2 in concd. H_2SO_4	Negative	Not phenol, unless *p*-substituted

[a] Purified by chromatographing ethanol extract of lyophilized dialyzate of blood on paper with 1-butanol–acetic acid–H_2O::4:1:5, rechromatographing active substance found at R_f 0.6 on paper with benzene–H_2O–butanol–methanol::1:1:1:2, and using material found from R_f 0.90 to 0.95 for tests. Aliquots equivalent to 10 ml. of blood used for each test, replicated 3 times.
[b] According to procedures outlined by Feigl (*5*).
[c] Controls consisted of normal crayfish blood processed in same manner. Results all negative.

used for cockroach blood, was found to have neuroactive properties when applied to isolated nerve cords from either crayfish or cockroaches, causing first excitation and then depression of spontaneous activity (*7*). One difference was noted; the substance from crayfish was more active on nerves of crayfish than of cockroaches, and conversely, the substance from cockroaches was more active on the nerves of cockroaches than of crayfish. In other respects, their properties appeared to be identical. They had identical R_f's in all chromatographic systems tried. Both were soluble in water and ethanol, and insoluble in carbon tetrachloride and ether. Both were rapidly inactivated by mild alkaline conditions, and by exposure to acetone. For these reasons, it is believed that although they may not be identical, they are at least closely related chemically.

From 1 liter of blood from DDT-prostrate crayfish, enough neuroactive material was obtained to test for the presence of a number of functional groups. The tests used (*5*), the results obtained, and the interpretation of each test are summarized in Table I. Blood from nonpoisoned crayfish was processed in the same manner for controls, which were negative. Briefly, the chemical tests indicate that the neuroactive substance may be a primary aromatic amine, and possibly an ester.

It has not been possible to obtain enough of the substance liberated during the action of TEPP on an isolated nerve cord to run all of the tests listed. Chromatographically the TEPP-produced material appears to be the same substance as that found in blood from DDT-prostrate cockroaches. It is rapidly destroyed by weak alkali, and is biologically inactivated by acetone. After spraying with diazotized *p*-nitraniline, a faint red spot has appeared on the chromatograms coinciding with the biologically active section.

Discussion

To date, the evidence indicates that the DDT-induced substance is the same as that liberated by an isolated nerve cord following application of TEPP. The neuroactive substance seems to appear during periods of excessive nervous activity, whether initiated by disruption of the acetylcholine-cholinesterase system with an antiesterase or by excessive afferent bombardment of synaptic areas induced by DDT.

In the case of an antiesterase, the great increase of neuroactivity is presumably due to the sudden failure of cholinesterase to remove free acetylcholine released at the synapses, thus upsetting the normal balance at postsynaptic regions. But this explanation cannot apply to DDT, a compound that does not inhibit cholinesterase. Nevertheless, sensory-central synapses become unstable during DDT poisoning. It is unlikely that the instability is due to DDT itself. First, chemical analysis of even several grams of nerve cords from DDT-prostrate roaches fails to reveal the presence here of DDT. Second, the instability is reversible. By applying the proper dosage of DDT, it is possible to get a cockroach into a condition where it can be prostrated or recovered within a temperature range of a few degrees. Under these conditions, the instability at synaptic regions will be present or absent, depending on whether the roach is prostrate or recovered. Spontaneous trains of nerve impulses within the central nervous system are also present or absent, correlating with early prostration or recovery.

In a cockroach or crayfish that has advanced to a stage of DDT poisoning no longer reversible, the spontaneous activity of the central nervous system is depressed or nearly absent. Yet if the nerve cord from such an animal is carefully dissected out and washed with physiological saline, a higher level of spontaneous activity will return. Either washing removes whatever it is that caused depression of normal activity, or the cessation of abnormal afferent phenomena due to amputation of sensory nerves permits physiological recovery of the central nervous system.

In addition to reversal of physiological phenomena, certain biochemical events are known to occur reversibly. During DDT prostration, the free proline in the blood and central nerve cord is selectively depleted to about one fourth the normal level (*4*). By using C^{14}-proline it was found that as proline was depleted, there was a corresponding rise in C^{14}-glutamine. If the temperature was raised so that the roach recovered, the proline and glutamine contents were restored to a normal level. The significance of this observation is not known, but it emphasizes the ease with which recovery to a more or less normal state may take place.

The presence or absence of the neuroactive substance in the blood also correlates with DDT prostration and recovery (*19*). It first appears during the early hyperactive stage of poisoning, increases to a maximum level during prostration, and then rapidly disappears if the cockroach is placed at a higher temperature to permit recovery. A subsequent drop in temperature, leading to a second prostration, results in the reappearance of the neuroactive substance in the blood.

Under certain experimental conditions there is evidence that DDT-prostrate cockroaches are less sensitive than normal to injected dosages of an anticholinesterase (*1*, *19*). Tetramethyl pyrophosphate (TMPP) is an irreversible inhibitor of cholinesterase, nearly equal in potency to TEPP. Because of its rapid rate of hydrolysis in water [98% in 3 hours for an 0.02*M* solution (*21*)] excess TMPP that has not reacted with cholinesterase rapidly neutralizes itself. In normal cockroaches injected with 6 γ of TMPP in 2 μl. of physiological saline, about 90% of the cholinesterase within the central nervous system was found inhibited 3 hours later. When DDT-prostrate cockroaches were injected with 6 γ of TMPP, there was less than 10% inhibition of cholinesterase.

It is not known whether this protection is due to the accumulation of a substance that competes with TMPP for active sites on cholinesterase, to a change in membrane permeability barring the entrance of TMPP into esteratic sites, or to the failure of the circulatory system to transport TMPP through the body.

The in vivo protection against TMPP may also be cited as an example of a reversible phenomenon. DDT-prostrate cockroaches tolerated an injected dosage of TMPP that inhibited 90% of the cholinesterase of a normal cockroach, but if the DDT-prostrate cockroaches were allowed to recover even briefly by raising the temperature before injection of TMPP, no protection occurred.

The exact role in the mode of action of DDT for the events discussed is not known. Perhaps some of them are merely secondary, and are a result rather than a cause of poisoning. The primary action of DDT on sensory nerves still remains unexplained. Before there is a complete understanding of the mode of action of compounds affecting neurotransmission in insects, the basic biochemistry and physiology of insect excitatory tissue must be more thoroughly investigated. The chemical structures of compounds apparently involved with either cardiac or neuroactivity must be determined before their role under normal conditions can be understood. Only then can their part, if any, in the mode of action of pesticides be determined.

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Synergistic and Antagonistic Actions of Insecticide-Synergist Combinations and Their Mode of Action

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Synergistic and antagonistic actions of pyrethrin synergists in combination with many organophosphorus and chlorinated insecticides have been studied on several species of insects and mites. The highest increase in toxicity to houseflies was 38.7 times for a mixture containing 1% sesamex and methyl 3-[ethoxy(p-dimethylaminophenyl)phosphinyloxy]crotonate. Pyrethrin synergists also reduced the toxicity of some organic thionophosphorus and cyclodiene insecticides. Toxicity results as well as colorimetric and enzymic analyses indicate that synergistic or antagonistic action caused by a pyrethrin synergist appears to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the compounds.

In the study of joint action of insecticides, a true synergistic action resulting from a mixture of two or more insecticides is rarely found. Although high increases in toxicity to houseflies of pyrethrins, allethrin, etc., in combination with pyrethrin synergists (or activators) are well known, only small increases in toxicity have been reported on other species of insects.

Many pyrethrin synergists have been discovered, but little progress has been made on the study of their mode of action. This report includes not only the studies on the synergistic and antagonistic actions of pyrethrin synergists to vinyl phosphates, phosphonates, and other insecticides, but also the elucidation of a possible mode of action.

Materials and Methods

With the exception of common insecticides and synergists, chemical names

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Synergistic and antagonistic actions of pyrethrin synergists in combination with many organophosphorus and chlorinated insecticides have been studied on several species of insects and mites. The highest increase in toxicity to houseflies was 38.7 times for a mixture containing 1% sesamex and methyl 3-[ethoxy(p-dimethylaminophenyl)phosphinyloxy]crotonate. Pyrethrin synergists also reduced the toxicity of some organic thionophosphorus and cyclodiene insecticides. Toxicity results as well as colorimetric and enzymic analyses indicate that synergistic or antagonistic action caused by a pyrethrin synergist appears to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the compounds.

In the study of joint action of insecticides, a true synergistic action resulting from a mixture of two or more insecticides is rarely found. Although high increases in toxicity to houseflies of pyrethrins, allethrin, etc., in combination with pyrethrin synergists (or activators) are well known, only small increases in toxicity have been reported on other species of insects.

Many pyrethrin synergists have been discovered, but little progress has been made on the study of their mode of action. This report includes not only the studies on the synergistic and antagonistic actions of pyrethrin synergists to vinyl phosphates, phosphonates, and other insecticides, but also the elucidation of a possible mode of action.

Materials and Methods

With the exception of common insecticides and synergists, chemical names

of newer compounds are given in Table I. Purified samples of aldrin (m.p. 103–04° C.), dieldrin (m.p. 175–76° C.), endrin (m.p. 229–30° C.), isodrin (m.p. 234–37° C.), lindane (m.p. 110° C.), DDT (m.p. 107° C.), heptachlor (m.p. 95° C.), heptachlor epoxide (m.p. 163° C.), SD 3110 (m.p. 163–64° C.), SD 3450 (m.p. 256° C.), SD 2642 (m.p. 142.5–44° C.), SD 2648 (m.p. 221–22° C.), methyl parathion and methyl paraoxon, technical grades of DDVP, Amiton-oxalate, phosphamidon, Phosdrin, Diazinon, Guthion, malathion, EPN, parathion, Chlorthion, schradan, pyrethrins (20% concentrate), piperonyl butoxide, sulfoxide, propyl isome, sesamex, and distilled samples of other SD compounds were used for tests. In spray tests the percentage concentration of each toxicant and each synergist was expressed as grams of active ingredient per 100 ml. of solution.

Table I. Chemical Names of Newer Compounds

Compound	Chemical Name
SD 2244	3-(Diethoxyphosphinyloxy)-*N,N*-dimethylcrotonamide
SD 2409	Ethyl 3-[ethoxy(*p*-nitrophenoxy)phosphinyloxy]-crotonate
SD 2438	Ethyl 3-(dimethylamino)-phenylphosphinyloxy)-crotonate
SD 2642	5,6,7,8,9,9-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-epoxy-*exo,endo*-5,8-methanonaphthalene
SD 2648	5,6,7,8,9,9-Hexachloro-1,2,3,4,4a,5,8,8a-octahydro-1,2,3,4-diepoxy-*exo,endo*-5,8-methanonaphthalene
SD 2966	Methyl 3-[ethoxy(*p*-dimethylaminophenyl)phosphinyloxy]-crotonate
SD 3110	5,6,7,8,9,9-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanophthalazine
SD 3450	5,6,7,8,9,9-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanophthalazine 2-oxide
SD 3562	3-(Dimethoxyphosphinyloxy)-*N,N*-dimethylcrotonamide
SD 4092	Benzyl 3-(dimethoxyphosphinyloxy)crotonate
SD 4239	*p*-Chlorobenzyl 3-(dimethoxyphosphinyloxy)crotonate
SD 5567	4′-Chloro-3-(dimethoxyphosphinyloxy)crotonanilide
SD 5656	*N,N*-Diallyl-3-(dimethoxyphosphinyloxy)crotonamide
SD 5742	*N,N*-Diallyl-2-chloro-3-(dimethoxyphosphinyloxy)-crotonamide
Amiton-oxalate	*S*-(2-Diethylamino)ethyl *O,O*-diethyl phosphorothioate hydrogen oxalate

Table II. Toxicity to Houseflies of SD 3562, Methyl Parathion, and Their Mixtures

Material	LC_{50}, %	Cotoxicity Coefficient of Mixture
SD 3562	0.059	
SD 3562 + 1% sesamex[a]	0.0030[b]	19.7
Methyl parathion	0.0055	
Methyl parathion + 1% sesamex	0.015[c]	0.37

[a] 1% sesamex solution is not more toxic than kerosine alone.
[b] LC_{50} of SD 3562 in mixture.
[c] LC_{50} of methyl parathion in mixture.

In order to find the synergistic action of a mixture, the concentration of synergist should be so low that it would give little or no toxicity when applied alone. A 1% concentration was selected for most tests on the housefly because it is not more toxic than kerosine alone, because it is the concentration of pyrethrin synergists used in many commercial formulations, and because insecticides having a wide range of toxicity can be compared on equal weight of synergists by using a constant concentration of synergist instead of a definite ratio of insecticide and synergist. Other concentrations were selected for other tests because they were not more toxic than the control to the species tested.

The spray method used for testing houseflies, *Musca domestica* L. (National Association of Insecticide and Disinfectant Manufacturers 1948 strain), was essentially the same as that described by Sun (*10*). Houseflies were reared and tested at 80° ± 2° F. and at 40 to 50% relative humidity. Four-day-old flies of both sexes were thoroughly mixed and properly sampled before 100 individuals were counted into each cage. Three cages of flies were each sprayed with 0.6 ml. of refined kerosine (No. 10 oil) solutions. The average deposit was 41 μl. per 100 flies. For each toxicant and each mixture three concentrations were used. At least 1800 flies were sprayed for each pair of tests.

All stages of pea aphids, *Macrosiphum pisi* (Harr.), on broad bean plants were sprayed with aqueous suspensions containing 0.05% emulsifier (Atlox 1045A) and various amounts of insecticide with and without 0.05% sesamex [2-(2-ethoxyethoxy)ethyl 3,4-methylenedioxyphenyl acetal of acetaldehyde]. Immediately after spraying, the number of aphids on the plants was counted. After 18 to 20 hours living aphids were again counted. Results were then calculated as apparent mortality (*12*). Two-spotted spider mites, *Tetranychus telarius* (L.), were sprayed with aqueous suspensions with and without 1% sesamex. Only the mortality counts of adult mites were taken.

Before the determination of dosage-mortality curves, a range-finding test was made for each toxicant and its mixture. If the result did not agree with the preliminary finding, the test was repeated.

To evaluate the relative increase or decrease in toxicity on the same basis for a number of mixtures it was necessary to have a constant amount of synergist (or antagonist) as well as the same method for calculating the toxicity results. Several methods have been described by Sun and Johnson (*11*) for comparing the joint toxicity of insecticides in terms of cotoxicity coefficient. When one of the components in a mixture is not toxic at the test concentrations, the toxicity of that component in the mixture becomes insignificant. Then the cotoxicity coefficient, calculated by the following equation (*11*), is the number of times of increase (or decrease) in toxicity.

$$\text{Cotoxicity coefficient} = \frac{LC_{50} \text{ of toxicant alone}}{LC_{50} \text{ of toxicant in mixture}}$$

If the result of calculation is significantly higher than 1, the mixture indicates synergism; if less than 1, the result shows antagonism. Examples in Table II illustrate the data and results of calculation of synergistic combination of SD 3562 and sesamex and of antagonistic combination of methyl parathion and sesamex (*11*).

Because synergists and antagonists can increase and decrease, respectively,

Table III. Toxicity to Houseflies of Insecticide Mixtures Containing Pyrethrin Synergists

Material	LC_{50} of Toxicant, %	Cotoxicity Coefficient with 1% Synergist: Sesamex	Piperonyl butoxide	Sulfoxide	Propyl isome
Pyrethrins	0.0605	60.5	29.5	41.3	13.8
SD 2966	0.089	38.7(2)[a]	9.0	5.0	3.8
SD 5656	0.0425	30.4	4.0	7.7	2.5
SD 3562	0.059	19.7	4.1	1.7	1.6
Methyl parathion	0.0053	0.41(2)	0.86	0.91	0.84

[a] Number in parentheses indicates number of determinations.

the toxicity of insecticides, different causes of increase or decrease may influence the toxicity. The complexity of their function cannot be analyzed by a simple calculation. Therefore, the results of calculation may be considered as apparent increase or decrease in toxicity. When the results show a cotoxicity coefficient of slightly more or less than 1, the difference in toxicity may not be significant.

Results on Syngerism and Antagonism

Selection of Synergists. As is known, the methylenedioxyphenol group in pyrethrin synergists is related to their synergistic action on pyrethrins (*6*, pp. 77–89). Presumably they are common in their mode of action but differ in intensity of synergism. To simplify all possible combinations between synergists and insecticides the most active synergist should be selected.

Series of mixtures containing each of four common pyrethrin synergists, in combination with pyrethrins, SD 2966, SD 5656, SD 3562, and methyl parathion, were tested aginst houseflies. Results in Table III indicate that at 1% concentration sesamex is the best synergist for pyrethrins, SD 2966, SD 5656, and SD 3562 with increases in toxicity of 60.5, 38.7, 30.4, and 19.7 times, respectively. However, the same concentration reduced the cotoxicity coefficient of methyl parathion to 0.41. Piperonyl butoxide, sulfoxide, and propyl isome gave similar effects but were much less potent. Therefore, sesamex was selected as a representative of this group of synergists for further studies.

Phosphates and Phosphonates. In addition to SD 2966, SD 3562, and SD 5656, a number of other vinyl phosphates, vinyl phosphonates, and organophosphorus compounds were tested against houseflies with and without 1% sesamex. Results in Table IV showed that 1% sesamex increased the toxicity of vinyl phosphates and vinyl phosphonates 1.4 to 38.7 times. Higher increases in toxicity were found in compounds containing an amino or an amido group. In the nonvinyl organophosphorus compounds, a high increase (29.1 times) was found in Amiton-oxalate, which contains an amino group, but low (1.6 times) in schradan, which contains amido groups. The amino or amido group in almost all members of these three groups of organophosphorus compounds is related to unusually high synergistic action. Reasons for low increases in toxicity of SD 2438 and schradan in combination with synergist are discussed below.

In biological systems the primary and secondary amines are known to be susceptible to oxidation by amine oxidases with the liberation of ammonia (*5*). Schradan (amide) is oxidized in insects and plants to a monophosphoramide oxide (*2*), which is a strong cholinesterase inhibitor. It is reasonable to suspect that the amino or amido groups of the vinyl phosphate and phosphonates (Table IV) may also be susceptible to certain biological oxidations. The resulting products may be less or nontoxic. Therefore, the apparent high increases in toxicity may be largely due to the inhibition of such oxidation by pyrethrin synergists.

Table IV. Toxicity to Houseflies of Various Insecticides and Their Mixtures Containing Sesamex

Material		LC_{50} of Toxicant, %		Cotoxicity Coefficient with 1% Sesamex
		Alone	Plus 1% sesamex	
Vinyl Phosphates, $R_1 \diagdown N \diagup R_2$ Group				
SD 5656	$N(CH_2CH{=}CH_2)_2$ (amido)	0.0425	0.0014	30.4
SD 2244	$N(CH_3)_2$ (amido)	0.063	0.0025	25.2
SD 3562	$N(CH_3)_2$ (amido)	0.059	0.003	19.7
SD 5742	$N(CH_2CH{=}CH_2)_2$ (amido)	0.06	0.00335	17.9
Phosphamidon	$N(C_2H_5)_2$ (amido)	0.024	0.00165	14.6
SD 5567	NH—C$_6$H$_4$—Cl (amido)	0.034	0.0044	7.7
SD 4092	None	0.0223	0.0058	3.8
DDVP	None	0.0055	0.00245	2.2(2)[a]
SD 4239	None	0.023	0.0091	2.5
Phosdrin	None	0.0054	0.0022	2.5
SD 2409	None	0.0077	0.0055	1.4
Vinyl Phosphonates				
SD 2966	$N(CH_3)_2$ (amino)	0.089	0.0023	38.7(2)
SD 2438	$N(CH_3)_2$ (amido)	0.029	0.0068	4.3
Nonvinyl Phosphorus Compounds				
Amiton-oxalate	$N(C_2H_5)_2$ (amino)	0.77	0.0265	29.1
Schradan	$N(CH_3)_2$ (amido)	3.1	1.95	1.6
Phosphorodithioates				
Guthion		0.020	0.0113	1.8(2)
Malathion		0.0845	0.0725	1.1(2)
Phosphorothioates and One Oxygen Analog				
Chlorthion		0.058	0.198	0.30(2)
Parathion		0.0041	0.0065	0.63
Methyl parathion		0.0053	0.013	0.41(2)
Methyl paraoxon		0.0039	0.0022	1.8(2)
Phosphonothioate				
EPN		0.0103	0.0147	0.70
Cyclodiene Compounds				
Isodrin		0.016	0.0092	1.7(2)
Endrin[b]		0.0118	0.0031	3.8
Aldrin		0.0092	0.0153	0.60(2)
Dieldrin[b]		0.0059	0.00365	1.6
SD 3110		0.0014	0.0016	0.88
SD 3450[c]		0.00105	0.0006	1.8
Heptachlor		0.0064	0.0113	0.57
Heptachlor epoxide[b]		0.004	0.003	1.3
SD 2642		0.0103	0.0062	1.7(2)
SD 2648[b]		0.0072	0.0053	1.4
Other Chlorinated Insecticides				
Lindane		0.0067	0.0047	1.4
DDT		0.052	0.045	1.2(2)

[a] Number in parentheses indicates number of determinations.
[b] Epoxide.
[c] *N*-oxide of SD 3110.

Based on LC_{50}'s, Phosdrin is approximately 1.4 to 16.5 times more toxic to houseflies than its analogs (Table IV). When tested with 1% sesamex the toxicities of some of the vinyl phosphates (SD 5656, SD 2244, SD 3562, SD 2966, and phosphamidon) are raised to about that of Phosdrin. However, this is not true for all analogs tested. The mixtures of those compounds containing a phenyl, chlorophenyl, or nitrophenyl group (SD 4092, SD 4239, and SD 2409) are much less toxic to

Table V. Toxicity to Houseflies of Insecticide Mixtures Containing Various Concentrations of Sesamex

Material	LC_{50} of Toxicant, %	Cotoxicity Coefficient of Mixtures Containing Following Sesamex Concentrations					
		0.04%	0.1%	0.2%	0.5%	1%	2%
Methyl parathion	0.0053	...	0.93	...	0.53	0.41(2)[a]	0.36
Aldrin	0.0092	...	0.87	...	0.68	0.60(2)	0.69
SD 3562	0.059	2.1	...	10.3	...	19.7	...

[a] Number in parentheses indicates number of determinations.

Table VI. Toxicity of Organophosphorus Compounds and Their Mixtures Containing Sesamex to Various Insects

Species	Material	Sesamex in Mixture, %	LC_{50} of Toxicant, %		Cotoxicity Coefficient with Sesamex
			Alone	In mixt.	
Pea aphid	SD 2966	0.05	0.0032	0.0009	3.6
	SD 3562	0.05	0.000046	0.000031	1.5
	Parathion	0.05	0.00038	0.00062	0.61
Two-spotted spider mite	SD 2966	1.0	0.015	0.0074	2.0
	SD 3562	1.0	0.076	0.0165	4.6
Housefly					
Susceptible strain	SD 2966	1.0	0.099	0.0024	41.3(2)[a]
Resistant strain	SD 2966	1.0	0.314	0.0092	34.0(2)

[a] Number in parentheses indicates number of determinations.

houseflies than the sesamex-Phosdrin combination. This may be mainly because the decrease in water solubility by such substitutions changes the partition coefficient and, therefore, may affect the movement of toxicants in insects.

Thionophosphorus Compounds. It has been theorized that the increase in toxicity of several organophosphorus compounds (Tables III and IV) containing an amino or an amido group, when mixed with pyrethrin synergists, may be due to the inhibition of detoxification by certain biological oxidations. On the other hand, the activity of some organic thionophosphorus insecticides, such as parathion and methyl parathion (*7*), depends mainly upon their oxidation to oxygen analogs; their combinations with pyrethrin synergists should reduce their toxicity. To test this hypothesis two phosphorodithioates, three phosphorothioates and one oxygen analog, and one phosphonothioate were tested against houseflies with and without 1% sesamex. Results in Table IV indicate that sesamex significantly reduces the toxicity of some thionophosphorus insecticides, such as Chlorthion, parathion, methyl parathion, and EPN.

Although 1% sesamex reduced the cotoxicity coefficient of methyl parathion to 0.41, it increased the toxicity of methyl paraoxon, the oxygen analog of methyl parathion, 1.8 times. This low increase in toxicity indicated that sesamex may not only inhibit the oxidation of methyl parathion but also stabilize its oxygen analog. Other low increases in toxicity for sesamex mixtures containing Guthion, malathion, and some vinyl phosphates (Table IV) may be verified by the same reasoning.

Chlorinated Insecticides. It has been indicated that synergistic and antagonistic actions of sesamex on certain organophosphorus compounds were probably due to the inhibition of certain biological oxidations. The increase or decrease in toxicity depends upon the nature of compounds which may be either activated or detoxified by oxidation. Certain cyclodiene insecticides, such as aldrin (*1*) and heptachlor (*3*), are readily oxidized in animals to their respective epoxides, which are generally more toxic than their corresponding precursors. If sesamex inhibits certain biological oxidations, aldrin and heptachlor should be less toxic with than without pyrethrin synergist.

Twelve chlorinated insecticides were sprayed on houseflies with and without 1% sesamex. Their LC_{50}'s and cotoxicity coefficients are listed in Table IV. Results indicated that aldrin, heptachlor, and SD 3110 containing 1% sesamex gave lower toxicity than the toxicants alone, and that their corresponding oxides—dieldrin, heptachlor epoxide, and SD 3450—were higher in toxicity with 1% sesamex. However, such change of toxicity did not hold true for isodrin or SD 2642. Stabilization and slight joint toxicity may be related to the absence of apparent decrease in toxicity. Lindane and DDT containing 1% sesamex also showed slight increases in toxicity.

Concentration Effect. To estimate the effect of concentration of synergists on different types of compounds, several concentrations of sesamex were added to the kerosine solutions of methyl parathion, aldrin, and SD 3562. Each toxicant and its mixtures were sprayed on houseflies.

Results in Table V indicate that as the concentration of sesamex increased from 0.1 to 2% the toxicity of methyl parathion and aldrin decreased rather slowly. The largest change in cotoxicity coefficient was approximately 0.64 for methyl parathion and 0.40 for aldrin. However, the toxicity of SD 3562–sesamex mixtures increased very rapidly as the concentration of sesamex was increased from 0.04 to 1% to yield a toxicity increase of 19.7 times at this high sesamex level. The rapid increase in toxicity for synergistic action and the slow decrease for antagonistic action indicate that other stabilizing effects and/or some joint toxicity between synergist and toxicant may exist.

Resistant Houseflies. It was of interest to see whether synergists have the same effect on resistant as on susceptible strains of insects. Both susceptible and multiple resistant (highly resistant to chlorinated insecticides) houseflies were sprayed with SD 2966 with and without 1% sesamex. Results indicate that the low cross resistance to SD 2966 did not appreciably change the relative synergistic activity of sesamex (Table VI). One per cent sesamex increased the toxicity of SD 2966 41.3 and 34 times against susceptible and resistant strains of houseflies, respectively.

Aphids and Mites. To ascertain the extent of joint toxicity of some insecticide-synergist combinations against other species, both peak aphids and two-spotted spider mites were sprayed with aqueous suspensions of SD 2966, SD 3562, and parathion with and without sesamex. Sesamex increased the toxicity of SD 2966 and SD 3562 to pea aphids and two-spotted spider mites 1.5 to 4.6 times (Table VI), less than the increases obtained for the same compounds against houseflies (Table IV). However, 0.05% of sesamex gave a similar lowering of parathion toxicity to aphids as 1% sesamex to houseflies (Table IV). Under the test conditions 0.05 and 1.0% of sesamex were not toxic to aphids and mites, respectively.

Mode of Action

It has been suspected that the effect of pyrethrin synergists on some organophosphorus and chlorinated insecticides (Table IV) may be due to the inhibition of certain biological oxidations in houseflies in order to produce higher or lower mortalities. To explain their synergistic and antagonistic actions on various insecticides, the effect of SD 2966, SD 3562, and methyl parathion on the ChE activity in homogenates of treated flies was tested with and without

sesamex. If the inhibition of biological oxidation were an important factor affecting the toxicity of these compounds, there should be less conversion of methyl parathion to its active oxygen analog and probably less decomposition of SD 2966 and SD 3562. The results of such changes would not only give large differences in fly mortalities but also would produce different ChE inhibition values for fly extracts.

In each of the three series of experiments the concentrations of methyl parathion (0.005%), SD 2966 (0.02%), and SD 3652 (0.01%) were selected on the basis of previous data, so that the differences in fly mortality would be large between treatments with and without sesamex.

Five cages of flies each containing 100 four-day-old adults of mixed sexes were sprayed with kerosine solutions of each toxicant with and without 1% sesamex. They were then transferred to recovery cages or were at once homogenized with physiological saline at the rate of 10 flies per milliliter of solution in a Waring Blendor. Because of strong ChE inhibitory activity, houseflies treated with SD 2966 were washed with 2-propanol before homogenization. Two milliliters of filtered (through cheesecloth) homogenate were then incubated with a standard solution of bovine ChE using acetylcholine as the substrate. The inhibition was determined by the ΔpH method (*4*). For SD 2966 and SD 3562 the incubation period was 1 hour instead of 30 minutes.

Table VII summarizes the results of above experiments on toxicity and ChE inhibition. Four hours after spraying, 0.005% methyl parathion killed 90% of flies but only 5% with the same concentration of methyl parathion containing 1% sesamex. This indicates that sesamex may inhibit the oxidation of methyl parathion to its oxygen analog which is a potent ChE inhibitor and a more active insecticide. This explanation agrees with the ChE inhibitory data, which show 20.8% inhibition for methyl parathion alone but none for methyl parathion plus 1% sesamex. Sesamex alone did not inhibit the ChE activity in repeated tests under the same conditions. On the other hand 1% sesamex highly increased the toxicity of SD 2966 from 0 to 98% and SD 3562 from 2 to 99% at 1 and 1.75 hours, respectively, after spraying (Table VII), presumably because sesamex prevents detoxification caused by biological oxidation. Although the difference in ChE inhibition was not as sharp as that for methyl parathion, repeated tests indicated the same order of difference. Since SD 2966 and SD 3562 are strong ChE inhibitiors, the relatively high inhibition for the control samples may be due either to their rapid penetration into the flies or to incomplete washing of the toxicant from the surface. In general, the results on ChE inhibition tended to corroborate the theory of biological oxidation of these compounds.

Table VII. Effect of Sesamex on Toxicity of Organophosphorus Insecticides and on ChE Inhibition in Extracts of Treated Houseflies

Toxicant in Spray Solution, %	Housefly Mortality: Time interval after spray, hr.	Housefly Mortality: Mortality, %	ChE Inhibition of Fly Extracts: Incubation time, hr.	ChE Inhibition of Fly Extracts: Inhibition, %
0.005% methyl parathion	4	90	0.5	20.8
[a]	4	5	0.5	0
[b]	0	0	0.5	0
0.02% SD 2966	1	0	1	14.1
[a]	1	98	1	33.3
[b]	0	0	1	24.4
0.01% SD 3562	1.75	2	1	10.1
[a]	1.75	99	1	26.3
[b]	0	0	1	17.1

[a] Plus 1% sesamex.
[b] Control.

To test further the effect of sesamex on the biological oxidation in flies, aldrin was selected as an example for the cyclodiene group of insecticides, the epoxides of which are more toxic.

Twenty cages of houseflies (100 flies per cage) were sprayed with each of the following solutions: 0.01% aldrin, 0.01% aldrin plus 1% sesamex, 1% sesamex, and kerosine only. Immediately after spraying, the flies were transferred to recovery cages and mortality counts made 20 hours later. All the flies in the same treatment were ground with sand and anhydrous sodium sulfate, and extracted four times with a 2 to 1 *n*-hexane–2-propanol mixture. The extracts were then saponified with alcoholic potassium hydroxide and analyzed by colorimetric methods for aldrin (*9*) and dieldrin (*8*). In control samples, 0.0 and 0.6 γ of aldrin and 1.4 and 0.9 γ of dieldrin were obtained for 1% sesamex in kerosine and kerosine treatments, respectively. When known amounts of aldrin and dieldrin were added to the untreated extract, 86% of aldrin and 84% of dieldrin were recovered. All results were corrected for their corresponding blank values.

Results in Table VIII indicate that when flies were treated with aldrin alone more dieldrin (26.5 γ) was found than aldrin (7.4 γ). However, in the treatment with a mixture of aldrin and 1% sesamex as much as 20 γ of aldrin was recovered and only 4.8 γ of dieldrin were detected. This strongly suggests that sesamex inhibits certain biological oxidations. The higher mortality (42%) in the aldrin treatment and the lower mortality (26%) in the aldrin-sesamex treatment also indicate that aldrin is a less toxic form. There is no obvious indication that aldrin is nontoxic unless it is oxidized to dieldrin, because, based on the gross effect of toxicants, the amount of dieldrin (4.8 γ) found in the treatment with aldrin-sesamex is too low to produce a mortality of 26% without the joint action of aldrin.

Table VIII. Effect of Sesamex on Epoxidation of Aldrin in Treated Houseflies

Treatment	Average Mortality, %, 20 Hours	Toxicant Found γ: Aldrin	Toxicant Found γ: Dieldrin
0.01% aldrin	42	7.4	26.5
0.01% aldrin + 1% sesamex	26	20.0	4.8

General Discussion

Results on houseflies indicate that sesamex increased the toxicity of a number of insecticides. A general low increase was found for some organophosphorus and chlorinated insecticides (Table IV); high increases often occurred with organophosphorus compounds containing an amido or an amino group (Tables III, IV, and V). However, the same concentration of synergist significantly reduced the toxicity of several organic thionophosphorus compounds as well as several chlorinated insecticides (Tables III, IV, and V), the epoxides (or *N*-oxide) of which are generally more toxic than their corresponding precursors.

Results on ChE studies indicated that sesamex increased the ChE inhibition of extracts of flies treated with SD 2966 or SD 3562, but decreased the ChE inhibition of similar extracts treated with methyl parathion. Because methyl parathion is readily oxidized to its oxygen analog and SD 2966 and SD 3562 are probably decomposed to less or nontoxic compounds, it is reasonable to believe that by inhibiting certain biological oxidations sesamex acts as an antagonist to methyl parathion and related compounds, but as a synergist to SD 2966, SD 3562, and other organophosphorus compounds containing an amido or

an amino group. Data also indicated that methyl parathion, a phosphorothioate, was not only a poor ChE inhibitor but also almost nontoxic to houseflies at the test concentration without proper biological oxidation (Table VII).

Other pyrethrin synergists, such as piperonyl butoxide, sulfoxide, and propyl isome, also synergized the toxicity to houseflies of pyrethrins, SD 2966, SD 5656, and SD 3562, and reduced the toxicity of methyl parathion (Table III). The same mode of action on certain organophosphorus and chlorinated insecticides may occur to all four synergists, each of which has an active methylenedioxyphenyl group.

Some of the exceptional cases may be explained by the stabilizing effect of synergists on some insecticides and their joint action. For example, Phosdrin, methyl paraoxon, dieldrin, and DDT (Table IV), which are probably not affected by the mode of action of sesamex under discussion, have shown low increases in toxicity when mixed with sesamex. In other cases, Guthion, isodrin, and SD 2642 (Table IV) did not show decreases in toxicity in their sesamex mixtures. This may be explained by the degree of changes, the toxicity of oxidized products, and the effects of sesamex on the stabilization and penetration of toxicants and their oxidized products. In order to explain these factors more fully it is necessary to study each case individually.

Metcalf (*6*) made an excellent review on the synergism between pyrethrins and pyrethrin synergists. The general opinion was that synergists prevented the detoxification of pyrethrins in insects. Although the toxicity of pyrethrum would be greatly reduced under light and in air, and antioxidants have been widely used to prevent the deterioration of pyrethrum and its extracts, enzymic hydrolysis, rather than enzymic oxidation, was considered by most authors as a possible cause for detoxification. On the basis of the present study on synergistic and antagonistic action of pyrethrin synergists, a similar degree of synergistic action of four synergists to pyrethrins and three organophosphorus insecticides (Table III), their high synergistic action against houseflies, the inhibition of biological oxidation of methyl parathion, a phosphorothioate (Table VII), and of aldrin to dieldrin (Table VIII) lead to the speculation that pyrethrins may also be detoxified by biological oxidations and that the synergism produced by pyrethrins and synergists may be due to the inhibition of such oxidation.

Results on pea aphids and two-spotted spider mites were somewhat different from those on houseflies. Mixtures containing SD 2966–sesamex and SD 3562–sesamex gave only low increases in toxicity (Table VI). This relatively low order of increase was also reported in the literature on pyrethrins-synergist combinations against many species other than houseflies. However, the reduction in toxicity of methyl parathion or parathion was of a similar order for houseflies (Table IV) and pea aphids (Table VI). These indicated that aphids and/or mites, as compared to houseflies, reacted differently to SD 2966–sesamex or SD 3562-sesamex combinations but reacted similarly to parathion-sesamex mixtures. In other words, synergists may affect two or more biological oxidation systems which may be associated with oxidative enzymes. One may be associated with the oxidation of organic thionophosphorus compounds and possibly certain cyclodiene compounds, and others may act more specifically on compounds containing an amino or an amido group. The exceptions to this generalization are SD 2438 and schradan (Table IV), the amido group of which is attached to a phosphorus rather than a carbon atom. This difference may be related to the fact that contrary to the oxidation of schradan into a more active compound (*2*), other organophosphorus compounds containing an amino or an amido group are probably detoxified by certain biological oxidations.

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Metabolism of Insecticides by Various Insect Species

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A NUMBER of reviews on insect resistance to chemicals have appeared in the last decade (*4, 13, 14, 17, 27, 29, 40, 54, 73*). Several reviews have covered in detail the metabolic fate of insecticides in various insect species. This paper discusses only the highlights of this problem—i.e., what happens to the insecticide after it has penetrated the insect's tissues.

Like most other animals, insects must degrade or chemically alter a large variety of compounds to maintain their normal body functions. It is not surprising, therefore, that many foreign compounds, including poisons, are attacked in the metabolic process.

The fact that insects differ in their response to a chemical indicates the presence of inheritable variations arising from differences in the genetic constitution of individuals within a population or between populations of different origins. Our modern genetic theories postulate that genes, or units of inheritance, function in directing enzyme specificities, which, in turn, catalyze the innumerable biochemical reactions in the body. From a biochemical standpoint, it appears that resistance to insecticides results from the selection of those variants that can cope with the chemical more efficiently. The mechanisms by which insects accomplish these protective feats are discussed below.

Detoxication mechanisms involving

an amino group. Data also indicated that methyl parathion, a phosphorothioate, was not only a poor ChE inhibitor but also almost nontoxic to houseflies at the test concentration without proper biological oxidation (Table VII).

Other pyrethrin synergists, such as piperonyl butoxide, sulfoxide, and propyl isome, also synergized the toxicity to houseflies of pyrethrins, SD 2966, SD 5656, and SD 3562, and reduced the toxicity of methyl parathion (Table III). The same mode of action on certain organophosphorus and chlorinated insecticides may occur to all four synergists, each of which has an active methylenedioxyphenyl group.

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Like most other animals, insects must degrade or chemically alter a large variety of compounds to maintain their normal body functions. It is not surprising, therefore, that many foreign compounds, including poisons, are attacked in the metabolic process.

The fact that insects differ in their response to a chemical indicates the presence of inheritable variations arising from differences in the genetic constitution of individuals within a population or between populations of different origins. Our modern genetic theories postulate that genes, or units of inheritance, function in directing enzyme specificities, which, in turn, catalyze the innumerable biochemical reactions in the body. From a biochemical standpoint, it appears that resistance to insecticides results from the selection of those variants that can cope with the chemical more efficiently. The mechanisms by which insects accomplish these protective feats are discussed below.

Detoxication mechanisms involving

Most chlorinated hydrocarbon insecticides and many organophosphorus compounds are metabolized by insect species. The metabolic processes that bring about these chemical changes may be classified as "activating" and "detoxifying." Activating mechanisms usually involve epoxidation reactions, such as conversion of heptachlor to heptachlor epoxide and aldrin to dieldrin; or oxidation reactions, such as conversion of thionophosphates to phosphates, oxidation of thiol ethers to sulfoxides and sulfones, and oxidation of phosphoramides to more potent cholinesterase inhibitors. Detoxifying processes may convert insecticide to nontoxic metabolities, which are retained in the tissues or rapidly excreted. Detoxication of organophosphorus compounds in most cases involves hydrolytic reactions. The type of change is dependent on the chemical structure of the compound and the insect species—DDT is metabolized by the housefly, body louse, certain mosquitoes, American roach, Mexican bean beetle, boll weevil, milkweed bug, fruit fly, etc., but this process follows four or five metabolic pathways. Many of these reactions are enzymatically catalyzed.

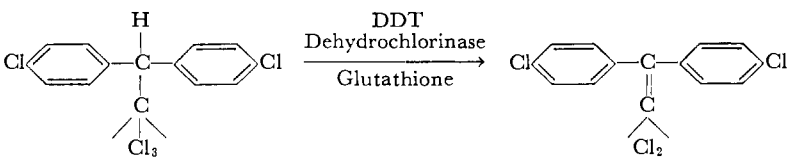
some insecticides and noninsecticidal compounds have been reviewed in detail by Smith (*60*). Prominent among them are (1) conjugation processes, such as the formation of hippuric acid from benzoic acid, formation of etheral sulfates and β-glucosides, acetylation, and methylation; (2) oxidation reactions, such as aliphatic oxidation, aromatic oxidation and epoxidation, hydroxylation of aromatic rings, and oxidation of thiophosphate and pyrophosphoramide insecticides; (3) reduction; (4) dehydrochlorination of insecticides; and (5) detoxication of heavy metal poisons by reaction with sulfur compounds. The present discussion is concerned primarily with the metabolism of chlorinated hydrocarbon insecticides and organophosphorus compounds.

Halogenated Hydrocarbon Insecticides

DDT. Since the appearance of DDT resistance in the housefly in 1946, numerous investigations have been undertaken to determine its cause. Among the many theories advanced, the most popular explanation was the finding that houseflies were able to convert DDT [1,1,1-trichloro - 2,2 - bis(*p* - chlorophenyl)-ethane] to the nontoxic derivative 1,1 - dichloro - 2,2 - bis - (*p* - chlorophenyl)-ethylene (DDE) (*34, 57, 63, 67, 76*).

Many investigators have confirmed this finding and have variously demonstrated that both susceptible and DDT-resistant houseflies are able to convert DDT to DDE, or that only the resistant strain is capable of accomplishing this conversion (*21, 57*).

Detoxication of DDT by houseflies has long been considered a major factor in the defense against the lethal action of this insecticide. This hypothesis was greatly strengthened by the isolation of the enzyme DDT-dehydrochlorinase (*64, 65*), which in the presence of glutathione catalyzes the dehydrochlorination of DDT in resistant houseflies according to the following reaction:



Recently, DDT-dehydrochlorinase has been isolated from susceptible houseflies also, but in much smaller titer (*33*). The enzyme has a pH optimum of 7.4 and a temperature optimum of 37° C. and requires glutathione for activation. It also catalyzes the dehydrochlorination of 1,1 - dichloro - 2,2 - bis - (*p* - chlorophenyl)-ethane (DDD) to 2,2-bis-(*p*-chlorophenyl)-chloroethylene. It does not attack *o,p'*-DDT, indicating some degree of specificity with regard to position and orientation of certain halogen groups in the molecule. DDT-dehydrochlorinase also has been isolated from Mexican bean beetle larvae and pupae (*19, 66*).

Enzymic breakdown of DDT has been demonstrated in the human body louse (*55*). The crude enzyme which has been isolated from susceptible and DDT-resistant body lice has a pH optimum of 9 to 9.5, is stable at high temperature, can withstand digestion by proteolytic enzymes without loss of activity, and may be activated with glutathione, cysteine, thioglycollic acid, ascorbic acid, or coenzyme A. The metabolite resulting from DDT breakdown in the body louse is a water-soluble acidic conjugate, which yields a pink complex when analyzed by the method of Schechter *et al.* (*59*).

Many other insects are capable of converting DDT to one or more metabolites. A fairly complete list showing quantitative data on the metabolism of DDT by various insects has been compiled (*53*). DDT metabolism follows at least five different metabolic pathways, as shown in Figure 1.

It is assumed that catalytic reactions involving the same substrate and yielding similar metabolic end products should be mediated by the same enzyme system. Several species of DDT-resistant mosquitoes, such as *Aedes aegypti*, *Aedes taeniorhynchus*, *Anopheles sacharovi*, and *Culex fatigans* have been shown to convert large amounts of DDT to DDE in vivo (*5, 15, 52*) but so far all attempts to isolate the mechanism in vitro have failed (*15, 52*). This indicates species specificity with regard to the enzymatic system involved.

Figure 1 shows that many intermediate or end products of DDT metabolism have not been identified yet. Judging

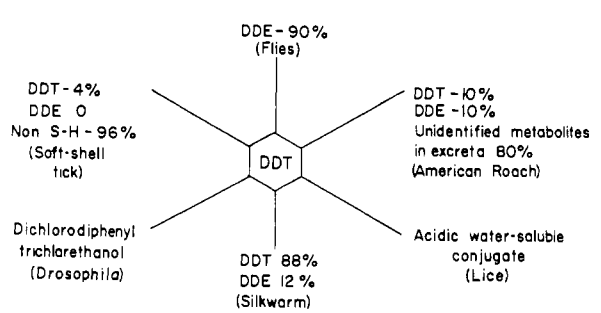


Figure 1. Pathways of DDT metabolism as illustrated in various insect species

from extraction procedures used, it would appear that many of the polar substances isolated are metabolite conjugates. The nature of the conjugation, however, has not been determined. The metabolites that have been identified include DDE in the housefly, certain mosquito species, the tent caterpillar, the European corn borer, the cabbage worm, the Mexican bean beetle, the red banded leafroller, and two grasshopper species (*53*). The metabolite *p,p'*-dichlorobenzophenone was detected in the excreta of DDT-treated American roaches (*28*), and 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)-ethanol was found in two species of *Drosophila* after they were exposed to DDT (*68*).

Benzene Hexachloride (BHC)

The weight of evidence and agreement that is nearly general among experimenters who have worked with this compound is that gamma-BHC (lindane) is metabolized by the housefly into polar metabolites, which are rapidly excreted.

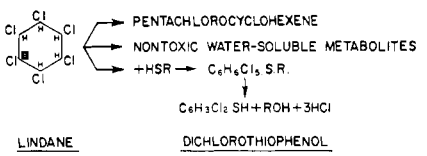
An early work (*7*) showed that pupae and adult houseflies originating from larvae reared in a gamma-BHC medium contained significant amounts of unchanged insecticide, which indicated the absence of an efficient detoxication mechanism. On the other hand, injection of gamma-BHC into resistant and susceptible houseflies (*49*) resulted in rapid breakdown of the chemical in the resistant strain and slower metabolism in the susceptible strain.

It has been demonstrated that metabolism of the alpha-, beta-, gamma-, and delta- isomers of BHC is faster in resistant than in susceptible houseflies (*8, 50*).

Using spectrophotometric and chromatographic methods, it was shown (*62*) that the first product of gamma-BHC metabolism was pentachlorocyclohexene. It was considered to be an intermediate metabolite, because the amount of gamma-BHC in the housefly decreased with an increased time interval but the metabolite did not increase in proportion. Pentachlorocyclohexene was shown to be metabolized further to unidentified products by resistant and susceptible houseflies. The enzyme DDT-dehydrochlorinase was not involved in lindane metabolism. Other studies using C^{14}-labeled alpha-, gamma-, and delta-BHC (*11*) showed that similar amounts of 1-chloro-2,4-dinitrobenzene occurred in houseflies treated with any of the three isomers, amounting to about 17% of the absorbed dose.

However, when a more specific isotope dilution technique was used, approximately 1.5% of the gamma-BHC absorbed by resistant and susceptible houseflies was detected as γ-pentachlorocyclohexene (*9*). The larger amount of 1-chloro-2,4-dinitrobenzene present was attributed to other possible metabolites giving rise to chlorobenzene on reduction. These and other experiments (*11*) suggested that chlorodinitrobenzene arises from three sources: chlorobenzene, occurring as a trace metabolite; some water-soluble metabolite which under reducing conditions gives rise to chlorobenzene; and pentachlorocyclohexene. The absence of larger amounts of pentachlorocyclohexene led to the conclusion that monodehydrochlorination is not the first step in a major pathway for the metabolism of gamma-BHC in houseflies. Quantitative data on the metabolism of gamma-BHC in several insect species (*6*) indicated that the housefly is unique in that respect, in that it possesses an efficient detoxifying mechanism before any selection pressure is applied.

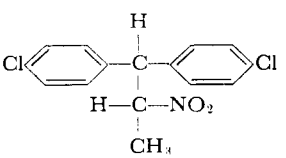
The latest experiments on the metabolism of gamma-BHC (*10*) have shown that alkaline hydrolysis of the metabolic products of alpha- or gamma-BHC yields dichlorothiophenols. It was inferred that the first step in the metabolism of BHC involves the removal of one chlorine atom and the formation of a C—S bond, followed by further dehydrochlorination and the subsequent formation of dichlorothiophenol according to the following scheme:



The nature of the RSH group has not been determined. However, when the procedure used for measurement of DDT-dehydrochlorinase in houseflies was followed in detail (*65*), it was shown that in vitro conversion of alpha- or gamma-BHC into water-soluble metabolites requires reduced glutathione for activation of the enzyme. Thus, gluthathione might be the source of sulfur for the C—S bond.

Prolan

Prolan [1,1-bis-(*p*-chlorophenyl)-2-nitropropane] is one of the two constituents of the insecticide Dilan, the other constituent being Bulan [1,1-bis-(*p*-chlorophenyl)-2-nitrobutane]. Although Prolan contains no chlorine atoms in the propane moiety, Prolan-resistant houseflies were shown to metabolize this insecticide readily into a neutral compound and an acidic derivative, both of which were excreted (*56*).



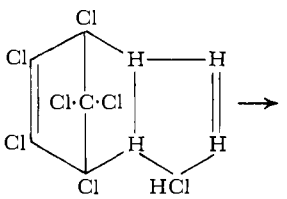
→ Neutral compound similar to Prolan

→ Acidic metabolite, possibly bis-(*p*-chlorophenyl) pyruvic acid

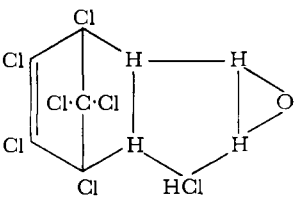
The neutral metabolite was soluble in common organic solvents, was similar to Prolan in infrared, ultraviolet, and colorimetric absorption spectra, and was almost as toxic to mosquito larvae as the parent compound. The acidic metabolite was extractable with dilute alkali, was different from Prolan in photometric absorption spectra and in chemical properties (showing loss of the NO_2 group in the propane moiety), and was much less toxic to mosquito larvae. Assessment of the relative rate of formation of the two metabolic products indicated that excretion of the toxic material was more important to the fly's survival in the initial stages of poisoning.

Cyclodiene Insecticides

Heptachlor. Topically applied, heptachlor was rapidly absorbed by resistant houseflies and converted to heptachlor epoxide (*58*).



Heptachlor

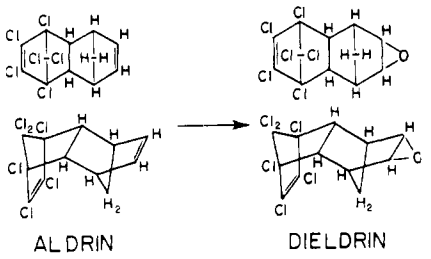


Heptachlor epoxide

Total recovery at the 24-hour interval after application, or beyond this period, did not exceed 53% of the amount applied. More recent information indicates that the missing portion is volatilized from the surface of the fly. The heptachlor epoxide extracted from houseflies was found to be identical in physical and chemical properties with an authentic sample of heptachlor epoxide melting at 159–60.5° C. It also was as toxic as heptachlor to susceptible houseflies and to mosquito larvae. Other experiments showed that the onset of symptoms of poisoning, following application of heptachlor to susceptible houseflies, coincides with the appearance of

heptachlor epoxide in the tissues. From these and other results, it was inferred that poisoning resulted from this conversion—a process which might be called "autointoxication." Neither the synthesized epoxide nor that recovered from houseflies was further metabolized.

Aldrin and Dieldrin. The metab-



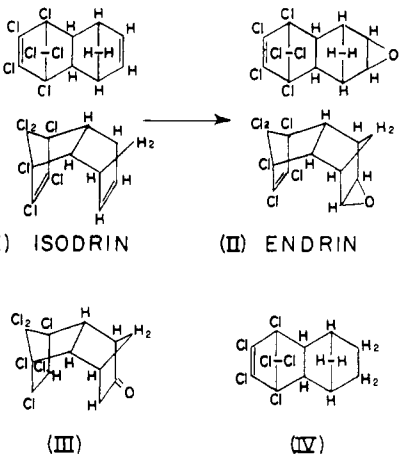
olism of aldrin in insects follows much the same pattern as that of heptachlor. Topical application or injection of aldrin into the American cockroach resulted in partial conversion of the aldrin to the corresponding epoxide, dieldrin (*24*). Tests with dieldrin-resistant houseflies (*70*) showed that more than 80% of the Cl^{38}- or C^{14}-labeled aldrin absorbed was converted to dieldrin within 24 hours following topical application of 5 to 10 γ of aldrin per fly. Conversion to dieldrin was virtually complete at the end of 96 hours. From 30 to 40% of the total radioactivity was lost by volatilization of the aldrin. This radioactivity was recovered by trapping the air from the holding container and assaying in a liquid scintillation counter.

Topical application of dieldrin to resistant or susceptible houseflies resulted in almost quantitative recovery of the unchanged chemical as long as 4 days after application. Eight to 12% of the dosage applied was found in the container, and this might be due to rub-off, egestion, and excretion.

Susceptible houseflies also convert aldrin to dieldrin, and the onset of symptoms of poisoning after a latent period of 2 to 4 hours coincides with the appearance of dieldrin in the tissues. From this observation, it might be inferred that toxicity is due to this conversion. On the other hand, it has been shown (*72*) that the reduction product (IV) of aldrin, which cannot be oxidized, is still toxic to houseflies. This finding suggests that the aldrin type of molecule has an intrinsic toxicity independent of its conversion to the epoxide.

The sulfur analog of dieldrin (S^{35}-labeled) was shown to be partly metabolized by resistant and susceptible houseflies (*74*). In addition, approximately 3% of the dosage applied was excreted unchanged, and 1 to 2% was excreted as water-soluble and insoluble metabolites. Twenty-seven to 34% of the radioactivity was lost by volatilization of the compound. A bromine analog of dieldrin (Br^{82}-labeled) was shown to be excreted unchanged in equal proportion by both resistant and susceptible houseflies (*74*). Small amounts of water-soluble metabolites were also produced.

Isodrin and Endrin. Topically



applied isodrin (I) and endrin (II) (C^{14}-labeled in the terminal unchlorinated ring) were found to be less toxic than aldrin and dieldrin to susceptible houseflies but more toxic to dieldrin-resistant houseflies. Both strains of houseflies converted isodrin to the corresponding epoxide endrin. Small amounts of endrin also were recovered in the external rinse. Endrin was not formed in the tissues of heat-killed insects, suggesting an enzymic epoxidation (*72*). Acetone extracts of live houseflies treated with isodrin or endrin contained small amounts of a nontoxic water-insoluble product, which behaved as a ketone (III) derivative of endrin. There was no evidence that radioactive material was excreted.

Chlordan and Toxaphene. Very little is known about the metabolic fate of chlordan and toxaphene in insects. The paucity of data might be due to lack of sensitive analytical methods (on a microgram scale) for toxaphene, and the fact that chlordan is not a pure chemical but is composed of at least five constituents, three of which are toxic to houseflies and other insects (*20, 35*).

Bioassay techniques for the estimation of chlordan and toxaphene have shown (*25*) that 74% of the absorbed toxaphene and 86% of the absorbed chlordan were metabolized to nontoxic derivatives within 24 hours following topical application of the chemicals.

Using Davidow's colorimetric procedure for chlordan (*20*), it was found (*69*) that 72 hours following topical application of chlordan to chlordan-resistant German roaches, 31% of the 100-γ dose per individual roach was unabsorbed, and 20% was recovered unchanged in the tissues. Forty-nine per cent remained unaccounted for and, hence, might be presumed to have been metabolized. No chlordan was found in the excreta.

Pyrethrins

The reversal of paralytic symptoms and the knockdown induced by pyrethrum suggest that insects may possess a detoxifying mechanism capable of attacking the compound at certain reactive sites. It was first suggested (*1*) that hydrolytic enzymes such as esterases might be involved in decomposing pyrethrins to nontoxic products.

Detoxication of pyrethrins was demonstrated first in the southern army worm by bioassaying tissue extracts of the treated insects against mosquito larvae (*77*). Greatest detoxication in vitro was brought about by fat body, followed by skin and muscle, digestive tract, and blood. Lipase extracts of roaches and houseflies readily hydrolyzed pyrethrin esters to nontoxic derivatives (*18*). Piperonyl butoxide (a pyrethrum synergist) inhibited lipase activity to some extent, and, consequently, detoxication of pyrethrins was diminished.

The American roach has been shown to hydrolyze C^{14}-labeled pyrethrins and cinerins to the corresponding keto alcohols and chrysanthemum monocarboxylic acids, plus unchanged esters, and several unidentified metabolites (*78*). Eight to 12% of the radioactivity was excreted as $C^{14}O_2$.

Houseflies were shown to metabolize significant amounts of C^{14}-pyrethrins and allethrins to nontoxic substances (nonpyrethroid derivatives) within 24 hours after application (*75*). Characterization of metabolites was shown by reversed phase paper chromatography (*72*). The synergist piperonyl cyclonene inhibited the detoxication of pyrethrins more than that of allethrin (*75*). This finding led to the conclusion that the mechanism of detoxication of pyrethrins and allethrins might differ in some respects. No C^{14} was lost by excretion or by expiration of $C^{14}O_2$.

An investigation of the metabolic fate of C^{14}-labeled allethrin (C^{14} on the chrysanthemum monocarboxylic acid moiety) in the housefly (*26*) showed that allethrin was rapidly absorbed, metabolized, and excreted. Twenty-four hours after treatment approximately 44% of the absorbed dose was excreted. Piperonyl butoxide depressed the rate of excretion, and this might account for the increase in mortality. Analysis of fecal and tissue extracts by paper chromatography demonstrated the presence of large amounts of a polar compound with the same R_f value as that of allethrolone. This indicates changes in the molecule other than those caused by hydrolysis of the ester linkage. If hydrolysis occurs, it indicates further degradation or conjugation of the free acids.

The above studies suggest somewhat differing metabolic pathways for pyrethrins and allethrins and differing detoxication mechanisms in houseflies and roaches.

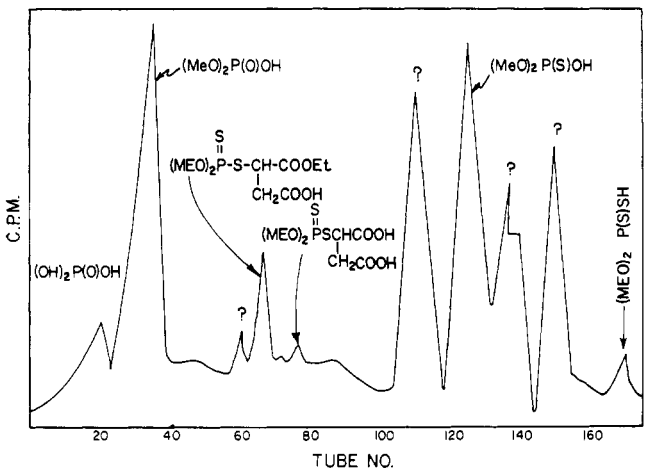


Figure 2. Ion exchange chromatography of metabolites 4 hours after application of P^{32}-malathion to housefly

Organophosphorus Insecticides

Several excellent reviews on the metabolism of organophosphorus compounds in insects, mammals, and plants have appeared in the last decade (*16, 22, 38, 39, 61*).

To be effective as an insecticide, an organophosphorus (OP) compound must first possess sufficient stability and suitable physiochemical properties to be absorbed and transported to the site of action. A second prerequisite is that the compound must be sufficiently unstable to act as a reactive phosphorylating agent. When these conditions are met, the OP compound will, at the site of action, combine with and inhibit a vital enzyme, cholinesterase, although other esterases have been implicated in the poisoning process and other modes of action have been suggested.

The metabolic fate of many of the OP compounds follow two general types of reactivity: metabolic intoxication of nontoxic or moderately toxic compounds to more active derivatives, and metabolic detoxication leading to destructive hydrolysis.

Metabolic intoxication may be brought about by oxidation of esters containing P=S groupings to P=O derivatives, such as occur in parathion, malathion, EPN, Thimet, diazinon; oxidation of thiol ether —S— to sulfoxides —S(=O)— and sulfones —S(=O)(=O)—, as in Systox, Di-Syston, and Trithion; and oxidation of —N—CH_3 to —N(=O)—CH_3 groups followed by rapid rearrangement to the methylol derivative —N—CH_2OH, as in the weakly cholinergic phosphoramides schradan and dimefox. These enzymatic oxidations result in the establishment in the molecule of an electrophilic center, which increases the instability and the consequent phosphorylating ability of the compound.

Other types of activation also occur. For example, *O,O*-dimethyl-2,2,2-trichloro - 1 - hydroxyethyl phosphonate (Dipterex) is dehydrochlorinated to the more active anticholinesterase, *O,O*-dimethyl-2,2-dichlorovinyl phosphate (DDVP). In fact, it has been demonstrated that the toxicity of Dipterex is due to its conversion to DDVP (*41*). This dehydrochlorination process does not involve the enzyme DDT-dehydrochlorinase (*61*).

It is evident that the toxicity of an OP compound to an organism will be markedly affected by the presence of enzymes capable of metabolizing the compound to inactive products. Metabolic detoxication is largely accomplished by base-catalyzed hydrolysis, which generally affects the labile bond broken during phosphorylation of the enzyme, leaving a dialkyl phosphoric acid ester and an alcohol or a phenol. In mammals, this hydrolytic attack is mediated by specific enzymes such as DFP-ase, which hydrolyzes diisopropyl fluorophosphate; tabunase, which splits CN^- from tabun; A-esterase, which hydrolyzes paraoxon; TEPP-esterase, etc. In insects, an aromatic esterase has been shown to hydrolyze parathion and paraoxon to liberate *p*-nitrophenate ion (*43*).

Malathion, *S*-[1,2-bis(ethoxycarbonyl)ethyl]*O,O*-dimethyl phosphorodithioate. Detoxication of OP compounds also may involve enzymatic hydrolysis of the carboxylic ester groups, either before or after oxidation. For instance, malathion appears to be degraded and rapidly excreted by the housefly through intermediates involving hydrolysis of the diethyl succinate nucleus and hydrolysis of the P—S and S—C bonds, as shown in Figure 2. In the American roach, this type of metabolism is less extensive, as judged by the limited number of metabolites resolved by paper chromatography (*36*).

This indicates that in the cockroach, activation (oxidation) is more rapid than hydrolysis, and the oxidation product malaoxon presumably accumulates to a lethal level (*44*). Later studies (*31*) have shown extensive degradation of malathion in the American roach, German roach, and housefly. This process involves at least two major degradative pathways: that due to phosphatases attacking P—S—C bonds, and that owing to carboxyesterases attacking the $COOC_2H_5$ moiety (cf. Figure 2). In the cockroach, both processes are about equal, but phosphatase attack predominates in the housefly.

With comparable doses of P^{32}-malathion, there seems to be little difference between susceptible and malathion-resistant houseflies in rate of excretion of nontoxic metabolites, but the tissues of susceptible houseflies contain a higher titer of a toxic compound that is not malathion (it is presumably malaoxon) (*71*).

Parathion, *O,O*-diethyl *O*-*p*-nitrophenylphosphorothioate. The toxicity of parathion to the American roach has been shown to be due to the conversion of parathion to paraoxon (*42*). The accumulation of paraoxon in susceptible houseflies but to a much lesser extent in parathion-resistant houseflies has been demonstrated in three strains of varying susceptibility (*51*). The failure of resistant houseflies to accumulate paraoxon might be attributable to slow rate of activation of parathion, rapid rate of degradation of parathion, and rapid rate of detoxication of malaoxon. As resistance to parathion extended also to paraoxon, it must be assumed that protection was afforded by the more rapid elimination of paraoxon.

Diazinon, *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate. Diazinon-resistant houseflies were shown to display a 40-fold resistance, as compared with a normal strain. Practically no difference was found in cuticle permeability from topical application of Diazinon. The level of Diazoxon (the oxidation product of Diazinon) was 2.5-fold higher, and the chloroform-extractable material 3-fold higher in normal than in resistant houseflies (*32*). These differences appear to be small in comparison with the magnitude of resistance demonstrated, and other factors than metabolism of the compound may play a vital part in the final toxicity.

After injection of Diazinon into the American roach at a rate of 30 γ per gram, the chloroform-extractables, which contain the toxic compounds, decreased slowly, indicating a slow degradative capacity. The P=O analog constituted

3 to 7% of the chloroform-extractables (*32*). In all probability the slow degradative process was due to the high dosage administered, as compared with the LD_{50} of only 0.75 γ per gram, for at a lower dosage, the degradative capacity was much more pronounced.

Dipterex, *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate. The metabolism of P^{32}-labeled Dipterex, its acetyl derivative (*O,O*-dimethyl 2,2,2-trichloro-1-acetoxyethyl phosphonate), and its vinyl derivative formed on dehydrochlorination (*O,O*-dimethyl 2,2-dichlorovinyl phosphate, DDVP) has been investigated in plants; in the dog and rat; and in the housefly, American roach, and several other insects (*2*). Of the three compounds, the vinyl derivative (DDVP) was generally the most toxic, but it was less selectively toxic than the two phosphonates. A large proportion of the Dipterex applied to the housefly was hydrolyzed, but no other P^{32}-containing metabolites could be detected by solubility characteristics, change in anticholinesterase activity, or permanganate-reducing characteristics. Rate of detoxication of DDVP by the housefly was slower than that of Dipterex. In vivo studies with two species of roaches failed again to demonstrate the presence of a vinyl derivative resulting from Dipterex metabolism. Furthermore, the anticholinesterase activity of Dipterex was not affected by incubation with whole roach intestines. Thus, no evidence was obtained that the toxicity of Dipterex was due to dehydrochlorination and rearrangement to the more active anticholinesterase DDVP, or that the in vivo detoxication rate can explain the relative susceptibility of houseflies to the two compounds.

Homogenates of housefly heads, imported cabbageworm heads, rat serum, rat liver, and whole pea aphids rapidly hydrolyzed the acetyl derivative of Dipterex, but failed to hydrolyze Dipterex or DDVP.

The low mammalian toxicity of Dipterex appears to be due to phosphonate hydrolysis by serum esterases and elimination in the urine of the trichloro portion of the molecule as trichloroethyl glucuronide (*2*).

More recent studies (*41*) using P^{32}-labeled Dipterex and DDVP showed that Dipterex is rapidly converted to DDVP under mild alkaline conditions. The reaction showed marked pH dependency ranging from 11% at pH 5.4 to 100% at pH 8.0. In vivo studies of the toxic action of the two compounds to the housefly showed that the rate of knockdown of houseflies feeding on Dipterex-sugar bait was much higher at pH 7.0 than at pH 5.4. This information together with the 4- to 7-fold greater toxicity of DDVP and the isolation by chromatography, anticholinesterase activity, and other techniques, of about 5% of P^{32}-DDVP from Dipterex-poisoned houseflies, strongly indicates that DDVP is responsible for the toxic action of Dipterex.

Delnav, 2,3-*p*-dioxanedithiol *S,S*-bis-(*O,O* - diethyl phosphorodithioate). P^{32}-labeled technical Delnav was separated by partition chromatography into eight fractions. The major components of interest were the cis- and trans-isomers and two minor components, 2-*p*-dioxenethiol *S*-(*O,O*-diethyl phosphorodithioate) and bis-(diethoxyphosphinothioyl) disulfide. The cis-isomer was the most toxic to houseflies and rats in vivo; but the in vitro anticholinesterase activity was least with the cis, trans, and dioxene derivatives, and greatest with the less toxic components. Two hours following topical application of the various fractions to the American roach at a rate of 200 γ per gram, the insects were sacrificed and the unchanged and hydrolyzed portions of the chemical were measured. The hydrolysis products accounted for 47.7% of the total radioactivity in fraction I, 26.2% in the trans- isomer, 17.6% in the cis- isomer, and 38.4% in the dioxene fraction. Thus, the greater toxicity of the cis- and trans- isomers correlates favorably with their greater stability to hydrolysis. Rats treated orally with technical Delnav excreted considerable amounts of hydrolytic metabolites in the urine and smaller amounts in the feces. The hydrolytic products recovered were identified as *O,O*-diethyl phosphoric, phosphorothioic, and phosphorodithioic acids (*3*).

Co-Ral, *O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate. Recently, the metabolism of Co-Ral has been investigated in cattle grubs and houseflies (*48*). Activation of Co-Ral to the oxygen analog, Coroxon, was pronounced in the housefly and less extensive but well evident in the cattle grub. Degradation to nontoxic derivatives was minimal for the insect species mentioned and for the mouse, but was fairly rapid for the ox and rat. Co-Ral is degraded primarily at the P—O—coumarinyl link and to a lesser extent at the ethoxy link (*30*).

Acethion, *O,O*-diethyl *S*-carboethoxymethyl phosphorodithioate. Compared with mouse liver slices, which degrade acethion extremely rapidly, minced housefly preparations showed some activating capacity. Minced cockroach preparations were effective in degrading acethion but were far less effective than mouse liver (*47*). Chromatographic separation of acethion and its metabolic products indicated that the principal metabolite was acethion acid. The degradative activity is reflected in the comparative toxicity of acethion to the three species: $LD_{50's}$ of 15, 375, and 1280 γ per gram for the housefly, the American roach, and the mouse, respectively (*32*). The levesl of the P=O analog of acethion, acetoxon, and the chloroform-extractable material were much higher in the housefly than in the cockroach 0.5 hour after treatment, but these levels reached almost the same value 2 hours after treatment. Throughout the experiment, these levels were lowest in the mouse. Thus, selective toxicity is apparent between insect and mammal but is not too convincing between insect species.

Dimethoate, *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate. Injection of 0.5 γ per gram of dimethoate in the housefly, cockroach, and mouse resulted in very rapid detoxication in the mouse (89% in 0.5 hour) and much slower degradation in the housefly and cockroach (32 and 16%, respectively). The P=O analog of dimethoate was approximately three times greater in the cockroach than in the mouse. A high degree of selectivity is indicated by the following $LD_{50's}$: 1.0, 1.0, and 140.0 γ per gram by injection for the housefly, the cockroach, and the mouse, respectively (*32*).

Schradan, octamethyl pyrophosphoramide. Scradan is a very weak anticholinesterase in vitro. It is practically nontoxic to certain insects but highly toxic to others (*39*). Schradan is converted by mammalian liver to a potent anticholinesterase. In susceptible insects, it completely inhibits cholinesterase. Tissues of both susceptible and nonsusceptible insect species contain an enzyme system capable of activating schradan. In the American roach—a nonsusceptible species—the fat body, alimentary canal, cuticle, and nerve cord are active in converting schradan to an anticholinesterase (*45*). It has been postulated that susceptibility of insects to schradan depends on activation of the compound within nerve tissue and that in nonsusceptible species the rate of conversion in the fat body is so rapid that little or no unconverted compound reaches the nerve tissue. Furthermore, the converted schradan is so unstable that it fails to reach and penetrate the lipoid sheath (Hoyle sheath) of insect nerve (*45*).

The active product of schradan metabolism in the American roach was found to be similar to one of the permanganate-oxidation products of schradan (*46*). Possible pathways for the hydrolytic breakdown of schradan that could account for its ineffectiveness against many insect species have not been found.

It was previously suggested (*45*) that the enzyme responsible for the conversion of schradan to its oxide might be trimethylamine oxidase. However, further studies showed evidence against the presence of this enzyme in insects (*46*).

Systox (Demeton), *O,O*-diethyl *O*-

(and *S*)-ethyl-2-thioethyl phosphorothioates. The sytemic insecticide Systox consists of two isomers, the thiono isomer (*O*-ethyl-2-mercaptoethyl moiety) and the thiol isomer (*S*-ethyl-2-mercaptoethyl moiety). Both isomers are rapidly metabolized, degraded, and eliminated in the mouse and cockroach by three different biochemical mechanisms (*37*). The biochemical system of primary importance from the toxicological standpoint involves the oxidation of the mercapto sulfur of the ethylthioethyl moiety of both isomers first to the sulfoxide and then to the sulfone. A second important mechanism, which is concerned only with the thiono isomer and its metabolites, involves the oxidation of the thiono sulfur to produce the respective phosphate and its sulfoxide and sulfone. Unlike the more toxic phosphate analogs produced by other OP compounds, such as parathion and malathion, the phosphate metabolites produced by thiono-Systox are of short duration, due perhaps to their rapid hydrolysis (*23*). They are, therefore, of no toxicological importance. The third biochemical mechanism is a degradative process and involves the hydrolysis of the P—O or P—S bonds of the ethylmercaptoethyl moiety to form the nontoxic ionic derivatives diethyl phosphoric acid or diethyl thiophosphoric acid and the respective alcohols.

These mechanisms follow the same pattern in mammals, insects, and plants; but the oxidation and hydrolytic rates are slower in insects than in mammals.

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End of Symposium on Mechanism of Action of Pesticide Chemicals

INSECTICIDE DETERMINATION

Colorimetric Determination of Dieldrin and Its Application to Animal Fat

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This method for determining microgram quantities of dieldrin is based on the color complex formed between dieldrin and diphenylamine in the presence of zinc chloride. The absorption at 650 mμ by color reaction products in an acetic acid solution follows Beer's law. The method is general for most chlorinated insecticides, but specificity is gained through alkaline hydrolysis and chromatography.

THE EXTENSIVE USE of insecticide commonly known as dieldrin or HEOD (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydro-1,4-*endo*-*exo*-5,8-dimethanonaphthalene) has created the need for a simple, sensitive method of analysis adaptable to routine determinations. Such a method is of prime importance in the study of the toxicity and hazards of dieldrin to humans, and might also find use in the determination of residues on food products and in the analysis of formulations.

An evaluation was made of the existing methods reported in technical literature for the colorimetric determination of microgram quantities of dieldrin. Bioassay and total organic chlorine techniques were not considered because of the lack of specificity. The only thoroughly described colorimetric procedure is that of O'Donnell, Johnson, and Weiss (*9*), which is long and tedious and subject to unduly high and variable blank values.

Davidow and Laug (*2*) reported a method involving the reaction of dieldrin with anhydrous hydrobromide in dioxane. The method is rather long, and the requirement of anhydrous hydrobromide limits its convenient use as a routine method.

Gunther, Kolbezen, and Blinn (*5*), and more recently Johnson (*6*), have reported on color reactions for the detection of dieldrin. These techniques have not as yet been expanded into quantitative methods of analysis. The color reaction of Gunther *et al.* appears to give inconsistent results, and that of Johnson lacks the desired sensitivity.

In 1945, Jones (*7*) and Lemley (*8*) reported on a color reaction of DDT with zinc chloride, phenol, and diphenylamine. Graupner has developed a method for toxaphene (*4*) based on the blue-green color reaction of this insecticide with diphenylamine and zinc chloride. On investigating the possibility that this color reaction under certain conditions is a more general one (*3*), it was noted that dieldrin gave a purple color of an intensity suitable for colorimetric determination.

The method described in this paper involves saponification, extraction, chromatography, and color development. In a final volume of 1.0 ml. of solution and using a semimicro absorption cell having a 10-mm. light path, a sensitivity of 5 γ with about 7% variation can be obtained. The method has been applied to the determination of dieldrin added to animal fat. Although all the possible variations have not been studied, it is believed that the following description will satisfy the immediate need for a sensitive, simple, routine method of determining dieldrin and will facilitate the expansion of the method to other biological materials and to metabolic and residue studies.

Apparatus

Beckman spectrophotometer, Model B, with red-sensitive phototube.

Aluminum heating block containing holes 22.5 mm. in diameter and 50 mm. deep. The block should be maintained at 205° ± 5° C.

Glass gas-drying unit charged with indicating Drierite.

Reagents

Diethyl ether, reagent grade. Ether containing peroxides is unsatisfactory and should be purified and redistilled before using. To test for peroxides, shake a small portion of the ether with an aqueous potassium iodide solution. The release of free iodine, as determined by the starch test, indicates the presence of peroxides. These are decomposed by washing the ether with a ferrous sulfate or sodium bisulfite solution until the potassium iodide test is negative. The ether is then washed with water, dried with sodium sulfate, and redistilled.

Alumina, adsorption grade, 80 to 200 mesh. Do not activate.

Diphenylamine solution, 0.25%. Dissolve 250 mg. of diphenylamine, recrystallized at least twice from hexane, in 100 ml. of redistilled *n*-hexane.

Zinc chloride reagent, 0.25%. Dissolve 250 mg. of zinc chloride in purified, redistilled ether. A slight suspension is formed which precipitates on standing. Resuspend before use. Do not filter through paper. This reagent is unstable and must be prepared each day.

Solvent for colored reaction products, 10% by volume of C.P. acetic anhydride in C.P. glacial acetic acid.

Dieldrin standard solutions. Dieldrin (at least 99.5% pure) is dissolved in purified redistilled *n*-hexane to give solutions containing 1, 10, and 20 γ per ml.

Procedure

Preparation of Standard Curves. Standard curves are prepared in three ranges of concentrations, depending upon the required sensitivity. In the 1- to 10-γ range, the colored products are dissolved in 1.0 ml. of solvent, and the absorbance is measured in a microabsorption cell having a 50-mm. light path. In the 5- to 50-γ range, the colored products are dissolved in 1.0 ml. of solvent, and a semimicrocell is used having an 0.8-ml. capacity and a light path of 10 mm. At the higher concentrations, 20 to 150 γ, a total volume of 3.0 ml. of solvent is used. The absorbance is measured in cells having a 10-mm. light path and a 3-ml. capacity.

Appropriate aliquots of the standard solution of dieldrin to give at least four points on each curve are pipetted into test tubes (22 × 175 mm.). The samples are evaporated just to dryness in a water bath at 40° C. with the aid of a

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gentle stream of dry, clean air filtered through a laboratory glass gas-drying unit. Two milliliters of the diphenylamine and zinc chloride reagents are added and the samples are again evaporated to complete dryness under the same conditions as before. A colorless, dry residual film should appear in each tube. The samples then are placed in the aluminum heating block at 205° ± 5° C. for exactly 3.0 minutes. The tubes are removed and cooled in running tap water. The colored reaction products are dissolved in either 1.0 or 3.0 ml. of the 10% acetic anhydride in glacial acetic acid solvent, depending upon the range of the curve being studied. The absorbance of the samples is read at 650 mμ within 10 minutes after the addition of the solvent; water or the acid solvent itself is used as reference. A reagent blank is carried through with the standards under exactly the same conditions. Plotting micrograms of dieldrin *vs.* absorbance on rectangular coordinate paper should give straight-line curves.

Analysis of Animal Fat. SAPONIFICATION AND EXTRACTION. To a weighed amount of animal fat (1.0 gram) in a 300-ml. ⅔ ground-glass-joint Erlenmeyer flask, 15 ml. of 95% ethanol (redistilled) and 3 ml. of 50% aqueous potassium hydroxide are added. The sample is refluxed gently for at least 1 hour or until a homogeneous solution is obtained. It is then removed from the source of heat and cooled, and 10 ml. of water and 50 ml. of redistilled *n*-hexane are added. The sample is shaken vigorously and the phases are allowed to separate. This operation is repeated three times, and then the lower phase is aspirated off. The organic solvent extract is washed once with 50 ml. of 1*N* hydrochloric acid, once with 25 ml. of water, and once with 25 ml. of 20% sodium chloride, the aqueous layer being aspirated off each time. The extract then is dried with sodium sulfate and a 40-ml. aliquot pipetted into a test tube. The sample is evaporated with a gentle stream of air in the 40° C. water bath to a volume of about 5 ml.

CHROMATOGRAPHY. The chromatographic columns are prepared by adding small amounts of dry adsorption alumina and tapping the tubes (10 × 33 mm.) gently to assure packing. This is repeated until a column of adsorbent 10 cm. in height is obtained. A plug of glass wool confines the alumina.

The *n*-hexane extract of the saponified fat is transferred to the column with the aid of a few milliliters of the 10% diethyl ether in hexane eluent. When the extract is completely taken up into the alumina, the column is eluted with 10% ether in hexane, and three 40-ml. samples of the eluate are collected in test tubes (22 × 175 mm.). The samples are evaporated just to dryness in a water bath at 40° C. with the aid of a stream of dry clean air. Two milliliters each of the diphenylamine and zinc chloride reagents are added, and the analysis is completed as for the preparation of the standard curves. A column blank is run through both the chromatographic and color reaction procedures. Its absorbance at 650 mμ is used as a correction for the absorbance value of the fat sample.

In the determination of unknown quantities of dieldrin, the amount of the colored reaction product formed after fusion offers a visual aid in determining whether this residue should be dissolved in 1.0 or 3.0 ml. of acetic acid solvent. This then establishes the absorption cells and the standard curve to be used. Alternatively the fusion products are dissolved in 1.0 ml. of the acid solvent. Then, if the color intensity of this solution warrants it, further appropriate dilutions are made.

It is important to run a blank analysis through the entire procedure on a sample of the same amount and type of material as that being analyzed. Further, a sample of the standard solution of dieldrin should be run through the chromatographic procedure to determine the quantitative recovery of dieldrin in the three eluates.

Results and Discussion

Standard Curves. Acetone, methanol, and ether were used as solvents for the two color reagents, diphenylamine and zinc chloride. The three solvents appeared to be satisfactory, but further work revealed that acetone and methanol gave higher reagent blank values than ether. This difference became significant when micro absorption cells were used. Care must be taken that the ether is pure and does not contain peroxides. These oxidation products cause high blank values and, depending upon their concentrations, may even cause extraneous color in the fused residue.

In the early work on this method, evaporation of the ether solution of the two color reagents, prior to fusion, occasionally gave a residue containing visible amounts of moisture. Furthermore, if a few milliliters of *n*-hexane were mixed with the ether solutions, the evaporation proceeded satisfactorily to complete dryness, and a colorless residual film was obtained at the bottom of the test tube. This same satisfactory effect was consistently achieved by preparing the diphenylamine reagent in *n*-hexane. The reagent blank values were not altered.

Varying the concentration of the color reagents between 0.25 and 1.0% did not increase the sensitivity of the method but increased the blank values. The effect of varying the reaction temperature on the absorbance of the color developed by dieldrin is shown in Table I. Reaction time was maintained constant at 3.0 minutes. The data show an increase in color intensity and in blank values with an increase in temperature. Varying the reaction time from 1 to 10 minutes and maintaining the temperature at 205° C. resulted in essentially the same effect—increased blank values and color intensity with some decomposition at the maximum time. These results indicate that the optimum time and temperature for the reaction of dieldrin with diphenylamine are the same as those reported for toxaphene (*4*)—e.g., 3 minutes at 205° C.

Table I. Effect of Reaction Temperature on Intensity of Color Developed by Dieldrin with Reference to Water

(Time of reaction, 3.0 minutes. Volume of colored solutions, 3.0 ml.)

Temp., ° C.	Absorbance at 650 Mμ: Reagent blanks	Absorbance at 650 Mμ: Dieldrin, 50 γ
125	0.003	0.007
160	0.014	0.124
205	0.020	0.337
225	0.093	0.420
250	0.170	Decomposed

Table II. Effect of Reaction Product Solvent on Intensity of Color Developed by 100 γ of Dieldrin

Solvent	Absorbance at 650 Mμ
Acetone	0.304
Methyl ethyl ketone	0.565
Methanol	0.158
Ethanol, 95%	0.160
2-Propanol	0.168
Ethyl acetate	0.200
Benzene	Insoluble
Glacial acetic acid	0.520
Acetic anhydride–acetic acid mixtures	
1:10	0.624
2:10	0.638
5:10	0.616

The effect of various solvents on the color intensity and thus the sensitivity of the method was studied using a reaction time of 3 minutes at 205° C. The representative data given in Table II indicate that, of the solvents studied, the acetic anhydride–acetic acid mixtures permitted the maximum development of color.

The color produced in all of the solvents studied faded rather rapidly. In the acetic anhydride–acetic acid solvent, the loss of color intensity is 15 to 20% in the first hour. It is therefore of prime importance that the absorbance of the samples be determined within a specified time. When the absorbance was measured at random, but within 10 minutes of the addition of the acid solvent, the variation between several standard curves prepared was not greater

Table III. Variables in Standardization of Colorimetric Determination of Dieldrin in Three Concentration Ranges

Variables	Range, γ 1–10	5–50	20–150
Volume of colored solution, ml.	1.0	1.0	3.0
Light path of cells, mm.	50	10	10
Maximum absorption, mμ	650	650	650
Reagent blank at λ_{max} abs.	0.100	0.020	0.010
Absorptivity	0.0869	0.0191	0.00641
Variation, %	7	7	5
Sensitivity, γ	1.0	5.0	15

Table IV. Recovery from Rabbit Fat of Dieldrin Added at Various Concentrations

Dieldrin, P.P.M. Added	Found	Recovery, %
20	18	90.0
40	39	97.5
80	75	93.8
100	94	94.0

Table V. Recovery of 100 γ of Dieldrin Added to 1 Gram of Animal Fat

Source of Fat	Dieldrin per Eluate, 40-Ml. Fractions, % 1	2	3	Total Recovery, %
Human A[a]	75	16	0	91
Human B[b]	68	24	0	92
Monkey	90	0	0	90
Rabbit	91	0	0	91
Rat	83	14	0	97
Dieldrin[c]	55	37	0	92

[a] Obtained in 1958.
[b] Obtained before advent of chlorinated insecticides.
[c] Standard dieldrin, no fat, subjected to complete analysis.

than 7%. This is satisfactory for most colorimetric determinations. Representative data pertinent to the standardization of the method are given in Table III.

Analysis of Animal Fat. SAPONIFICATION AND EXTRACTION. Davidow (*1*) has described a convenient technique for the isolation of the insecticide DDT from fat using a sulfuric acid–impregnated Celite column. However, this effective cleanup method is not applicable to dieldrin because the compound, unlike DDT, is not stable to the action of concentrated mineral acids but is stable to that of alkali. The latter property was utilized by O'Donnell *et al.* (*9*) in the isolation of dieldrin from glycerides.

In the present study, 1-gram samples of rat, rabbit, monkey, and human fat were saponified directly. Analysis of materials other than fat may require extraction prior to saponification. During the initial extraction of the saponified mixture, emulsion formation is avoided by adding only a minimum amount of water. No emulsions were formed in any of the samples analyzed when the alcohol content was reduced to 50 to 60% by the addition of water.

In the evaporation procedures, if a stream of air is used to aid in the complete removal of the solvent, the samples must be maintained at or below 40° C. to prevent loss of insecticide. If air is not used, hexane distillation temperatures may be utilized to concentrate extracts or eluates without loss of dieldrin. In any event, the evaporation should never be prolonged.

CHROMATOGRAPHY. O'Donnell *et al.* (*9*) described a magnesium oxide–Celite chromatographic column used in the isolation of dieldrin from extracts of saponified glycerides. This column is effective but requires vacuum or pressure to produce a flow of the eluents and necessitates collection of large volumes of eluate. A less time-consuming technique was desired.

After several adsorbents had been evaluated, a simple chromatographic procedure was developed capable of isolating dieldrin from an extract of saponified fat. Columns 10 mm. in diameter and packed 10 cm. high with adsorption grade alumina retained the impurities of an extract of 1 gram of saponified animal fat and permitted the quantitative recovery of dieldrin in about 80 ml. of eluate. A flow rate of 2 ml. per minute was used. If larger or different types of samples are to be analyzed, the column should be calibrated for its efficiency in cleanup. Column blank absorbance values were 0.015 to 0.030, using an absorption cell of 10-mm. light path and a 3.0-ml. final solution volume. Under the same conditions, control fat samples in general gave absorbance values of 0.020 to 0.050.

Table IV shows the recovery of known amounts of dieldrin added to rabbit fat. The eluates were collected in three 40-ml. fractions and analyzed individually and the results were totaled.

The recovery of 100 γ of dieldrin from 1 gram of different types of fat is shown in Table V. A fourth fraction of eluate, not shown in the table, was also collected and subjected to the dieldrin analysis. Although the added insecticide was recovered quantitatively in the first two fractions, the fourth fraction of eluates of some fat samples gave an extraneous violet color which faded rapidly. This was characteristic also of the human B fat sample, which was obtained from a patient before the advent of chlorinated

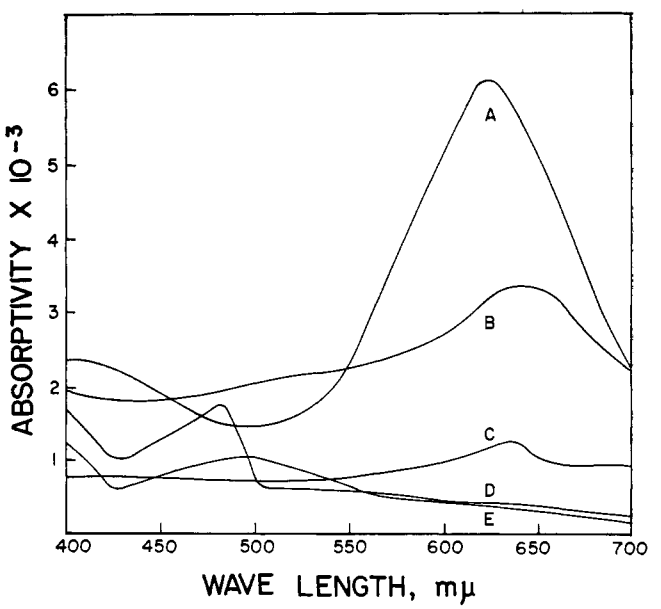

Figure 1. Spectra of chlorinated insecticides subjected to direct dieldrin colorimetric analysis

A. p,p'-DDD
B. Chlordan
C. Heptachlor
D. p,p'-DDT
E. o,p'-DDT

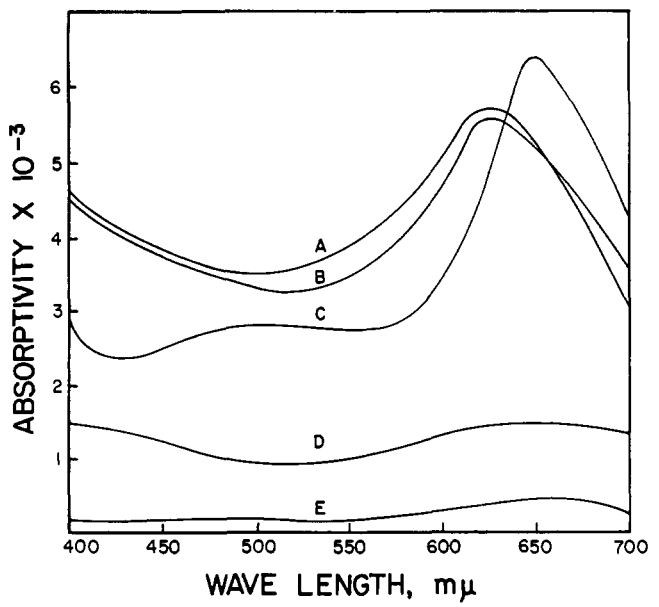

Figure 2. Spectra of chlorinated insecticides subjected to direct dieldrin colorimetric analysis

A. Strobane
B. Toxaphene
C. Dieldrin
D. Endrin
E. Aldrin

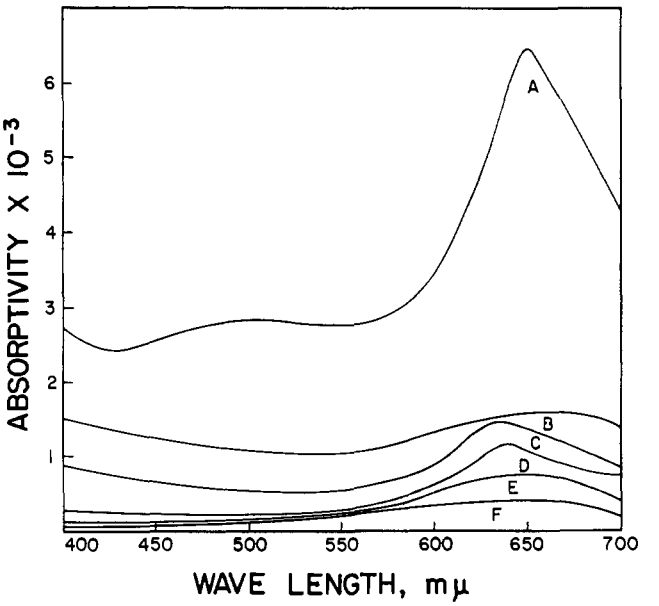

Figure 3. Spectra of chlorinated insecticides subjected to dieldrin colorimetric analysis after saponification and chromatography

A. Dieldrin
B. Endrin
C. Toxaphene
D. Strobane
E. Chlordan
F. Heptachlor

hydrocarbon insecticides. The findings indicate that continued elution of the chromatographic columns may result in recovery of a material of noninsecticidal origin. Therefore, adherence to the collection of eluates in fractions is desirable.

Reaction and Interference of Related Compounds. The nature of the color reaction is not thoroughly understood. Feigl (*3*) has reported that amines when melted with fluorescein chloride and anhydrous zinc chloride under conditions similar to those of this method produce a color complex. Fluorescein chloride is dehalogenated and the amine adds to the molecule. A similar reaction may be the basis of the colorimetric determination of dieldrin.

To determine the extent of this color reaction with related compounds, several chlorinated hydrocarbon insecticides were subjected directly to analysis. No saponification or chromatography was used.

Figures 1 and 2 give the spectra of the color complex formed with several insecticides and show that the wave length of maximum absorption and the intensity of color vary with most of the compounds. This may be due in part to variation of optimum conditions of color development for each material. For example, chlordan, when subjected to this analysis, gives an intense color without heating. Strobane also appears to be more reactive than toxaphene. DDE, DDA, dehydrochlorinated DDD, and lindane, not shown in the figures, gave no color reaction. From the spectra of some of the compounds certain possibilities are evident, such as the development of a simple method for the determination of DDD and its differentiation from DDT. This differentiation is not possible by the usual Schechter-Haller (*10*) procedure.

The general aspects of the reaction pose the problem of interference of related compounds on the determination of dieldrin. Some specificity is gained in the variations of color intensity and wave length of maximum absorption. To determine if further specificity could be gained through alkaline hydrolysis and chromatography, compounds shown in Figures 1 and 2 to offer maximum interference were subjected to the complete procedure described for the isolation of dieldrin from fat. Figure 3 shows both the spectra and the extent of interference of these insecticides after hydrolysis and chromatography. DDD on saponification is dehydrohalogenated to its ethylene derivative, which gives no characteristic color reaction. The instability of all of the compounds, except endrin, to alkaline hydrolysis is shown to be a means of limiting or of eliminating, by more intense saponification, their interferences in the dieldrin determination.

A confirmatory test for dieldrin was obtained by adding 0.2 ml. of concentrated sulfuric acid to the final colored solution. A characteristic color change occurred with the solution now having a maximum absorption at 540 mμ. The color is relatively stable but of low intensity. Oxidizing agents interfere with the test.

Acknowledgment

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Determination of Methoxychlor and/or Metabolites in Milk Following Topical Application to Dairy Cows

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Possible excretion of methoxychlor or its chlorine-containing metabolites in milk following topical application of this insecticide has been studied using two independent methods of analysis. Direct dusting at recommended levels of 10 grams of 50% powder per animal produced no detectable residues in milk. Spray applications at normal dosages resulted in traces of methoxychlor shortly after treatment, but the excretion level decreased rapidly with time. Repeated topical applications produced no increase in the excretion levels. Organic chlorine compounds other than methoxychlor were not detected.

Livestock ectoparasites such as biting or bloodsucking flies and lice can, if uncontrolled, cause annual losses of as much as half a billion dollars (*10*). These potential losses would result from damage to hides and meat, loss in weight and milk production, and ineffective utilization of feed. Methoxychlor, 1,1,1-trichloro - 2,2 - bis(*p* - methoxyphenyl)-ethane, applied directly to cattle is effective in combating certain of these pests. It controls hornflies and lice, and, in addition, affords protection against stable flies, horse flies, and mosquitos. Because it is well suited for the treatment of dairy cows, the excretion of methoxychlor in milk, following topical applications, has been the subject of considerable study.

Shortly after the introduction of methoxychlor, Carter *et al.* (*2*) studied the excretion of various chlorinated insecticides in milk following topical applications. Using an analytical procedure which was sensitive to a few tenths of a part per million of organic chlorine, he was unable to detect methoxychlor in milk following topical application of wettable-powder suspensions containing 0.5% methoxychlor. These results, coupled with the low mammalian toxicity of methoxychlor (*8, 11*), the even lower toxicity of its metabolites (*9*), and the low retention of methoxychlor in body tissues (*11*) indicated the safety of this pesticide for use on dairy cattle. Following this early work, somewhat conflicting results have appeared in the literature. Claborn and Wells (*5*) reported the detection of 0.7 p.p.m. of methoxychlor in the milk of a single cow, 1 day after spraying with a 0.5% emulsion of methoxychlor, and up to 0.4 p.p.m. after spraying with the same concentration of a wettable-powder suspension. Helrich *et al.* (7), working with herds of 4 to 6 animals, reported residues of a few tenths of a part per million of methoxychlor in milk shortly after spraying. On the other hand Birk and Dixon (*1*) and Cheng *et al.* (*3*) published data showing that less than 0.1 p.p.m. of methoxychlor is excreted in milk following topical applications.

The studies reported in this paper were conducted to re-examine the question of excretion of methoxychlor or its chlorine-containing metabolites in milk following topical application to cows. Methoxychlor was applied to cows by spraying and hand dusting at normal levels and recommended intervals, using commercial formulations. The effects of applying gross overdosages of methoxychlor were also investigated. To minimize variations caused by differences in individual animal responses, most of these tests were conducted on the same group of Guernsey-Jersey grade dairy cows, and composite milk samples were analyzed.

Two independent analytical methods were used to characterize the milk samples, one based on a selective colorimetric reaction, the other on total organic chlorine. Several sensitive colorimetric reactions have been employed for detecting traces of methoxychlor (*4*). The method used in this study is based on the reaction employed originally by Fairing (*6*). It is sensitive to a few micrograms of methoxychlor; DDT, lindane, toxaphene, and many other pesticidal chemicals do not respond. However, fats and oils must be carefully removed from methoxychlor residues to avoid interferences caused by the charring action of sulfuric acid on such substances.

Organic chlorine was chosen as the general index for metabolites because of its sensitivity, and because of the likelihood that such materials would contain chlorine. The procedure used in this investigation involves solvent extraction of whole milk, a Pregl catalytic combustion of the extract residue (*13*), and, finally, spectrophotometric determination of the resulting chloride (*15*).

Catalytic combustion was used because it requires only small amounts of a few reagents, an advantage for obtaining an over-all low chlorine blank. Using a platinum-silver preburner to purify the tank oxygen, the combustion procedure contributed only 2 γ of chloride to the blank in control milk.

Experimental

Animal Treatment In conducting these tests, detailed precautions were taken to avoid exogenous contamination. The cows were stanchioned in alternate stalls of a conventional dairy barn, and long hair was clipped from their udders and flanks. All applications of methoxychlor were made in a closed pen, remote from the dairy barn. During spraying, the udders were shrouded in cotton sacking and care was taken not to direct methoxychlor onto the shrouded areas. The cows remained tied until the spray had dried, after which the udder shrouds were removed and the cows returned to their stanchions. In the dusting tests, weighed amounts of powder were topically applied, using a shake-can, to the topline and sides of the cows. Working forward from the rump, the hair coat was raised by hand, and the dust was applied, gently rubbed into the hair and onto the skin. The cows were returned to their stanchions immediately after being dusted.

The cows were milked twice daily with a machine milker. Prior to each milking their udders were thoroughly sponged

and rinsed and the milking machines were cleaned with methanol, a good solvent for methoxychlor. After milking, the machine was removed to the laboratory where an appropriate aliquot was withdrawn. Butterfat content of composite milk samples, determined by the detergent method described by Sager and Sandars (*12*), averaged 5.0% during the test period, ranging from 4.6 to 5.4%.

Colorimetric Method for Determining Methoxychlor in Milk

Reagents and Apparatus. Dehydrohalogenation reagent, 4% (w./v.) of reagent grade potassium hydroxide in 95% ethanol, freshly prepared before use.

Colorimetric reagent, 85% sulfuric acid (82.5 to 88.0% is acceptable), containing 10 mg. of ferric chloride per liter.

Chromatographic column, 1-to-1 weight ratio of Celite No. 545 and heavy, adsorptive magnesia, U.S.P. grade. The composition is slurry mixed in redistilled hexane, dried, and dry-packed to a depth of 60 mm. in an all-glass, coarse, fritted-disk filter tube (100 × 20 mm.).

Procedure. One hundred grams of whole milk, 100 ml. of 95% ethanol, and 200 ml. of redistilled, commercial grade *n*-hexane are added to a 1-liter separatory funnel, and the mixture is shaken vigorously for at least 2 minutes. The resulting emulsion is divided into two equal portions, transferred to two 250-ml. centrifuge bottles, and centrifuged for approximately 5 minutes at 1800 to 2000 r.p.m. The upper (hexane) layers are removed, and transferred to a 600-ml. beaker.

An additional 100-ml. portion of hexane is added to each of the centrifuge bottles. The bottles are stoppered with polyethylene plugs and again shaken thoroughly. The contents are centrifuged, and the layers are separated as described above. The hexane layers are added to those previously collected, and the hexane extraction is repeated a third time.

The combined hexane extract is concentrated to approximately 125 ml. at room temperature, using a slow stream of oil-free air to hasten evaporation. The concentrated extract is transferred to a 500-ml. separatory funnel, 50 ml. of redistilled, technical grade nitromethane is added, and the contents are shaken vigorously for at least 2 minutes. When the layers have separated, the nitromethane is transferred to a second 500-ml. separatory funnel, containing about 50 ml. of hexane.

The original hexane is extracted with three additional 50-ml. portions of nitromethane as described above, each time draining the nitromethane layer into the second separatory funnel. The combined nitromethane extract is shaken with the 50 ml. of hexane to remove traces of interfering substances which had partitioned into the nitromethane. After phase separation, the nitromethane layer is drained into a 400-ml. beaker and carefully evaporated on a steam bath to a volume of approximately 25 ml. This residue is transferred quantitatively to a 250-ml. standard taper Erlenmeyer flask, using small portions of nitromethane for washing. The solvent is then carefully removed on a steam bath, using a current of air. To avoid losses of methoxychlor, solvent evaporation should be taken just to dryness.

Fifty milliliters of the alcoholic potassium hydroxide solution is added, the flask is attached to a water-cooled condenser, and the contents are refluxed gently for 30 minutes. The flask is cooled, the condenser is rinsed with 50 ml. of distilled water, and the entire contents of the flask are transferred to a 500-ml. separatory funnel with the aid of 100 ml. of petroleum ether (C.P. grade with boiling range of 35° to 60° C.) which has been passed through a column of chromatographic alumina. The contents of the separatory funnel are shaken thoroughly and allowed to separate, and the petroleum ether layer is removed and retained.

Extraction is repeated with 25 ml. of petroleum ether, and the combined extract is backwashed with 20 ml. of an equal-volume mixture of ethanol and water. The washed extract is transferred to a 100-ml. beaker and carefully evaporated to dryness, using a current of air. The residue is redissolved in 50 ml. of hexane and passed through a Celite-magnesia column by gravity flow. After the final incremental addition, the column is washed with an additional 25 ml. of hexane which is collected with the original effluent. This purified hexane solution is evaporated to dryness at room temperature in a 100-ml. beaker, using a current of air.

Twenty milliliters of the sulfuric acid color reagent is added to the residue, and the mixture is allowed to stand 1.5 hours with occasional swirling. The absorbance of the resultant pink solution is then determined in a 1-cm. cell at 550 mμ, using sulfuric acid reagent in the reference cell. The amount of methoxychlor in the sample is determined from a calibration curve prepared from known amounts of pure methoxychlor which have been subjected to the dehydrochlorination and color development steps described above.

Low blanks were obtained on untreated milk with the colorimetric method, averaging 0.02 p.p.m. with an average deviation of 0.01 (Table I). All methoxychlor values reported in this paper are corrected for this 0.02 average blank. In view of the level and variability of the blank, net methoxychlor values less than 0.02 cannot be considered

Table I. Apparent Methoxychlor Level in Pretreatment Milk

Designation[a]	Apparent Methoxychlor, P.P.M.
Delaware herd	
1	0.02
2	0.03
3	0.00
4	0.04
5	0.02
6	0.01
Texas herd A	
1	0.02
2	0.02
Texas herd B	
1	0.01
2	0.03
	Av. 0.02 ± 0.01

[a] Numbers indicate daily composites on different days.

Table II. Recovery of Known Amounts of Methoxychlor Added to 100 Grams of Whole Milk

Methoxychlor, γ		Recovery,	P.P.M.
Added	Found	%	Level
2.5	2.3	92	0.025
5.0	4.7	94	0.05
5.0	5.0	100	0.05
5.0	4.5	90	0.05
10.0	9.5	95	0.10
10.0	9.0	90	0.10
10.0	8.5	85	0.10
15.0	13.5	90	0.15
25.0	24.0	96	0.25
25.0	22.4	89	0.25
		Av. 92	

Table III. Stability of Methoxychlor in Whole Milk

Treatment	Methoxychlor, γ	
	Added	Found
None	20	17.0
Stored 3 days at room temperature	20	18.5
Stored 5 days at room temperature	20	17.5

significant. Satisfactory recoveries were obtained on known amounts of methoxychlor added to untreated milk (Table II). The stability of methoxychlor in milk at microgram levels was demonstrated (Table III); thus, the delays which necessarily occurred between milking and analysis were insignificant.

Method for Determining Total Organic Chlorine in Milk

Reagents and Apparatus. Diethyl ether, *n*-hexane, nitromethane, and methanol (reagent grade solvents) are redistilled through a 9-inch Fenske column or equivalent, discarding a 10% forecut and leaving a 15% bottom.

Deionized water with total electrolyte content preferably less than 0.1 p.p.m. as sodium chloride.

Reducing solution (prepared immedi-

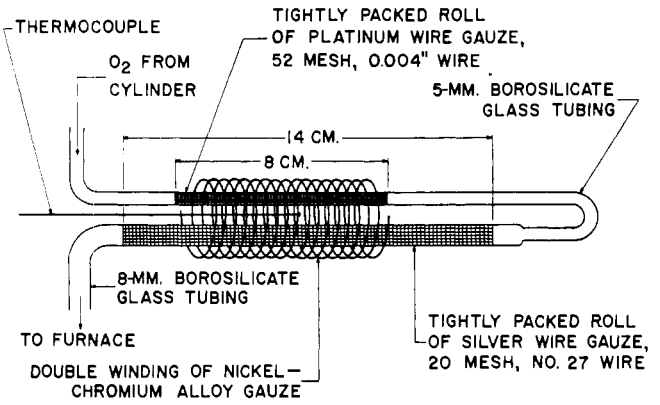

Figure 1. Oxygen preburner

ately before use) contains 5 drops of saturated hydrazine sulfate solution in 5 ml. of a 5% solution of reagent grade sodium carbonate. Deionized water is used throughout.

Urea solution (10%) is prepared in deionized water, using recrystallized urea. Recrystallization is accomplished by dissolving 60 grams of reagent grade urea in 50 ml. of deionized water at 30° C., filtering this solution through a fritted-glass funnel, and cooling to 5° C. The crystals are filtered off on a fritted-glass filter and dried overnight in a vacuum oven at 60° C.

Ferric perchlorate solution is prepared by extracting 20 to 30 grams of colorless ferric perchlorate (G. Frederick Smith Chemical Co.) with small volumes of 72% perchloric acid in a glass-stoppered flask. The acid is decanted and the extraction repeated until the acid layer no longer appears yellow. The final extraction mixture is filtered through a coarse fritted-glass funnel, and the crystals are pressed with a glass rod to remove as much of the acid as possible. Nine grams of purified ferric perchlorate is dissolved in 20 ml. of deionized water and 1 liter of 72% perchloric acid is added. The reagent is stored in a Machlett-type closed-system, all-glass buret. No lubricant is used on the stopcock and the drying tubes are filled with indicating anhydrous aluminum oxide.

Combustion Assembly. An oxygen cylinder, pressure reducing regulator, glass wool filter, flowrater, and preburner (Figure 1) are connected in that order by means of pure gum rubber tubing. The preburner is mounted horizontally and heated directly under the nickel-chromium alloy gauze with a Tirrell burner equipped with a fish-tail flame spreader. The flame is adjusted so that the temperature is between 680° and 700° C., as measured by the thermocouple. Neither end of the silver wire gauze should be covered with the nickel-chromium alloy gauze, nor should it receive any direct heat from the flame. The oxygen cylinder pressure is reduced to 20 pounds, and a flow rate of 4 to 5 ml. per minute through the flowrater is maintained by means of a needle valve. (The flowrater is calibrated with oxygen by means of a wet-test meter.) Oxygen must not be permitted to flow through the preburner unless the latter is heated to temperature. The free end of the preburner is connected by means of a section of gum rubber tubing to a small glass tube inserted in a one-holed cork stopper that fits the open end of the combustion tube.

The combustion tube is held vertically in a suitable clamp with the spiral end downward. The tip is immersed in 5 ml. of reducing solution contained in a shortened 15-cm. test tube. Gentle suction is applied to the open end of the combustion tube, so that the reducing solution is drawn up to cover only half the length of the spiral. The reducing solution is allowed to drain back into the test tube. The operation is repeated, again wetting one-half the length of the spiral and allowing the reducing solution to drain. The solution is poured from the test tube, leaving the latter moist. The procedure just described for wetting the spiral is repeated precisely from one analysis to another, in order to maintain a constant blank.

The combustion tube is then placed in the furnace rack, the entire spiral portion extending beyond the long burner, so that it will receive no heat during the combustion. The test tube, still moist with reducing solution, is placed over the tip of the combustion tube. Two clean platinum contact stars are placed in the section which is surrounded by the long burner. The contact stars are placed so that the wire loop of each is about 2 cm. inside the ends of the burner.

Procedure. A 100-gram sample of whole milk and 100 ml. of absolute ethanol are added to a 500-ml. separatory funnel (no lubrication on ground-glass joints). Five milliliters of 3.6*N* sulfuric acid in deionized water is added, and the contents of the funnel are mixed well. One hundred milliliters of 3-to-1 redistilled ether–redistilled hexane mixture is added and the separatory funnel is shaken gently for 3 minutes. The contents of the separatory funnel are poured into a 500-ml. borosilicate glass centrifuge bottle and the mouth of the bottle is covered with dental dam, held in place with an elastic band. The mixture is centrifuged for 5 minutes at about 1800 r.p.m. (explosion-proof centrifuge preferred).

The upper phase is transferred to a 250-ml. beaker with the aid of a 50-ml. pipet and suction bulb. Contamination with the lower phase is carefully avoided. The ether-hexane extraction is repeated three more times in the centrifuge bottles, using 50-ml. volumes of the solvent mixture, shaking vigorously for 3 minutes, and centrifuging as before. During these extractions, a rubber stopper covered with aluminum foil is used to seal the centrifuge bottle. The combined extract is evaporated to 150 to 175 ml. Contamination by dust, etc., is carefully avoided throughout the procedure.

The concentrated ether-hexane solution is transferred to a 500-ml. separatory funnel, and 100 ml. of 1-to-1 absolute ethanol–deionized water solution, adjusted to pH 2 to 3 with sulfuric acid, is added. The mixture is shaken for 3 minutes and the layers are allowed to separate. The lower aqueous layer is drained into a second separatory funnel and the ether-hexane layer is returned to the original beaker. The aqueous layer in the second separatory funnel is further extracted with three 50-ml. portions of 3-to-1 ether-hexane. Before each of these extractions, the first separatory funnel is washed with the solvent mixture. Following each extraction, the upper layer is transferred to the original beaker. The combined ether-hexane extract is evaporated to approximately 25 ml. and transferred to a 500-ml. separatory funnel with a total volume of 200 ml. of redistilled *n*-hexane. This solution is extracted successively with three 50-ml. volumes of redistilled nitromethane. The combined nitromethane extract is back-extracted with 50 ml. of redistilled hexane, transferred into a 250-ml. beaker, and evaporated on a steam bath to an oily residue. This residue, which is usually about 0.2 ml. in volume, is transferred to a 10-ml. beaker, using several small volumes of methanol, and evaporated again. Using a 0.1-ml. measuring pipet and small volumes of methanol, the oily residue is quantitatively transferred to an small platinum microcombustion boat. The boat is heated gently on a steam bath to evaporate the methanol continuously during the transfer.

The boat containing the sample is placed in the combustion tube about 5 cm. from the end of the long burner (*13*). The long burner is fixed to surround the combustion tube and heated to produce a temperature between 680° and 700° C. within the combustion tube. When this

Table IV. Chlorine Level in Pretreatment Milk

(100-gram samples analyzed)

Designation[a]	Chloride Found, P.P.M.
1a	0.17
1b	0.10
1c	0.10
1d	0.13
2a	0.10
2b	0.08
2c	0.08
Av.	0.11 ± 0.03

[a] Samples 1 and 2, milk from two different herds of cows; samples a, b, etc., composites of the daily productions of each herd on different days.

Table V. Recoveries of Methoxychlor and 1,1,1-Trichloro-2,2-bis-(p-hydroxyphenyl)ethane

(100-gram milk samples analyzed)

Chlorine Added, P.P.M.	Chloride Found[a], P.P.M.
As methoxychlor	
0.16	0.17
	0.12
	0.07
	0.09
	0.19
Av.	0.13
As 1,1,1-trichloro-2-bis-(*p*-hydroxyphenyl)ethane	
0.17	0.16
	0.12
	0.14
	0.19
Av.	0.15

[a] Corrected for Cl level in control milk (Table IV).

Table VI. Methoxychlor Excreted in Milk Following Topical Application[a]

Post-treatment Period	Amount of Methoxychlor Found in Raw, Whole Milk, P.P.M.							
	Hand Dusting			Spraying				
	Test 1[b]	Test 2[b]	Test 3	Test 4	Test 5	Test A	Test B	Test 6
6 hours	<0.02	...	...	0.02	...	...	...	...
1 day	<0.02	<0.02	0.03	0.06	0.10	0.08	0.05	0.23
2 days	<0.02	...	...	...	...	0.08	0.04	...
3 days	<0.02	<0.02	0.05	0.06	0.07	0.04	0.04	0.19
4 days	<0.02	...	...	...	...	0.02	<0.02	...
5 days	...	<0.02	...	0.04	0.03	0.02	<0.02	0.17
7 days	<0.02	...	0.04	0.02	0.02	<0.02	<0.02	0.13
14 days	...	...	...	...	...	<0.02	<0.02	...
21 days	<0.02	...	...	...	...	<0.02	<0.02	...

[a] Tests 1–6 conducted in Del. with 4 Guernsey-Jersey grade cows. Tests 1 and 2, 10 grams of Marlate 50; 3, 40 grams of Marlate 50; 4, 3 qt. of suspension containing 8 lb. of Marlate 50 per 100 gal. of water; 5, 3 qt. of emulsion containing 8 qt. of Marlate 2-MR per 100 gal. of water; 6, 3 qt. of suspension containing 32 lb. of Marlate 50 per 100 gal. of water.

Tests A and B. Separate herds of 30 cows sprayed in Tex. with 2 qt. of suspension containing 8 lb. of Marlate 50 per 100 gal. of water.

[b] Recommended treatment.

temperature is reached, the cork stopper which connects the oxygen supply is inserted into the open end of the combustion tube. The sample burner is then fixed to surround the combustion tube so that the end toward the long burner is in line with the trailing edge of the sample boat, and heated so that it produces a temperature between 680 and 700° C. in the combustion tube. Purified oxygen is allowed to flow through at the rate of 4 to 5 ml. per minute. The sample burner is moved slowly across the sample and up against the long burner, leaving it there for 10 minutes. (Caution: Heat the preburner to temperature before starting the oxygen flow.) A total of 45 minutes should be taken to complete the combustion, to ensure that a minimum of unburned material passes through to the spiral.

The burners are removed from the combustion tube and the latter is allowed to cool while oxygen is swept through. When cool, the platinum boat and contact stars are removed. The combustion tube is mounted vertically and the spiral is washed by drawing up twice, by means of mild suction, 5 ml. of deionized water contained in a 10-ml. platinum crucible. Three 1-ml. washes of deionized water are added from the top of the tube and collected in the platinum crucible. The test tube is washed with 2 or 3 small volumes of deionized water, and these washings are also added to the platinum crucible.

The sample is evaporated to dryness on a steam bath. The platinum crucible, with cover slightly displaced to allow gases to escape, is placed in a muffle furnace at 500° C. for 30 minutes; then it is removed and cooled. Deionized water (0.5 ml.) and 0.50 ml. of 10% urea solution are added to the crucible.

Ferric perchlorate reagent (7.4 ml.) is added to a 10-ml. volumetric flask, painted black nearly up to the mark. The 1-ml., urea-treated sample is transferred to the flask with the aid of a polyethylene dropping pipet, using three 0.5-ml. washes with deionized water to complete the transfer. After diluting to the mark, the flask is shaken to mix the solution, removing the stopper frequently to release pressure. The neck of the volumetric flask is covered with a small bag of opaque paper, and the solution is allowed to cool for 15 minutes. The absorbance of the sample solution is measured at 353 mμ in a 1-cm. cell against a blank which contains the same volume of hydrazine reducing solution, urea, ferric perchlorate, and deionized water. The micrograms of chloride present in the sample is determined from an appropriate calibration curve.

Calibration is carried out by adding 10, 30, 50, 70, and 100 γ of chloride (appropriate aliquots of a standard sodium chloride solution prepared to contain 100 γ of Cl^- per ml.) to a series of painted 10-ml. volumetric flasks, each containing 7.4 ml. of ferric perchlorate reagent. After dilution to the mark with deionized water, the flasks are shaken to mix the solutions, covered with opaque bags, and allowed to cool for 15 minutes. The absorbance of each solution is determined at 353 mμ in a 1-cm. cell *vs.* a blank containing only 7.4 ml. of the ferric perchlorate reagent and deionized water. Absorbance is plotted *vs.* micrograms of chloride. A new curve must be established each time a fresh ferric perchlorate reagent is prepared. If the reagent is kept over an extended period of time, occasional checks on the working curve should be made.

The average level of chlorine found in control milk was 0.11 p.p.m. with an average deviation of 0.03 p.p.m. (Table IV). This estimated precision of ±0.03 p.p.m. of organic chlorine includes both the variability of the method and possible variations in the actual chlorine content of milk from the same herd on different days and milk from different herds. Recoveries of known microgram amounts of organic chlorine as methoxychlor were excellent through the Pregl combustion procedure and the extra heating at 500° C. The recoveries obtained on milk samples "spiked" with known amounts of methoxychlor and 1,1,1-trichloro-2,2-bis(*p*-hydroxyphenyl)ethane are shown in Table V. The latter compound was included in the recovery work, because Weikel found that its polarity is similar to that of unidentified methoxychlor metabolites in rats (*14*).

Backwashing the original hexane extract with 1-to-1 ethanol–deionized water reduces the chloride blanks considerably. Without this step, control milk analyzes from 0.2 to 0.4 p.p.m. of chloride. A second cleanup of the ether-hexane was not used because fairly stable emulsions were formed. Small amounts of unidentified materials distilled through the catalytic combustion tube and interfered with the spectrophotometric determination of chloride. This interference is eliminated by heating the final chloride residue obtained from the final reducing solution for 30 minutes at 500° C. Treatment of the final perchloric acid

Table VII. Organic Chlorine Found in Milk Following Topical Applications of Methoxychlor

Treatment	Test 1, Spray with Wettable Suspension	Test 3, Hand Dusting with Overdosage	Emulsion Spray with Overdosage[a]
Formulation applied	8 lb. Marlate 50 per 100 gal. water (0.5% methoxychlor)	Marlate 50 powder	8 qt. of Marlate 2-MR per 100 gals. water (0.5% methoxychlor)
Dosage per animal	Single spray with 3 qt.	Single dusting with 40 g. powder (4 times recommended level)	18 1-qt. sprayings at about 3-day intervals
Animals used in test	4 Guernsey-Jersey grade cows	4 Guernsey-Jersey grade cows	4 Guernsey cows
	Total Amount of Organic Chlorine Found in Milk, P.P.M.		
Pretreatment (chloride blank)	0.11 ± 0.03	0.11 ± 0.03	0.11 ± 0.03
After treatment			
6 hrs.	0.14	...	...
12 hrs.	...	...	0.12
1 day	0.13	0.13	0.21
2 days	0.13	...	0.15
3 days	0.10	0.14	...
7 days	0.15	0.10	...

[a] Treatments and sample collection conducted by Helrich *et al.* (7).

solutions with urea is required to eliminate interferences from nitrite, which was identified as one of the combustion products.

Results and Discussion

Duplicate tests were run in which 10 grams of 50% methoxychlor powder was applied to each animal by direct dusting. The data under Tests 1 and 2 in Table VI show that no detectable residues of methoxychlor were present in any of the posttreatment samples analyzed, even those taken immediately following application. Direct dusting at 3-week intervals is the only method of application recommended by the manufacturer, and it is the only method of applying methoxychlor to dairy cows that is registered by the U. S. Department of Agriculture and sanctioned by the Food and Drug Administration.

Slight, but detectable, residues were present in milk shortly after spraying with aqueous suspensions or emulsions (Tests 4 and 5, Table VI). The maximum level encountered (0.10 p.p.m.) occurred 1 day after treatment, and detectable levels persisted for only 1 week. Similar results were obtained on samples of milk from two separate herds of cows sprayed by the U. S. Department of Agriculture in Kerrville, Tex. (Tests A and B, Table VI). This work showed a maximum level of 0.08 p.p.m. of methoxychlor the first day following treatment. It is interesting to note that even following spraying with an oil emulsion, the average level of methoxychlor present during the 3-week interval between treatments would amount to only a few hundredths of a part per million.

Application of four times the recommended quantity of methoxychlor by direct dusting resulted in minute, but detectable, amounts of methoxychlor in milk, attaining a maximum level of 0.05 p.p.m. (Test 3, Table VI). Wettable powder spraying at four times the normal dosage increased the level of methoxychlor in milk (Test 6, Table VI). However, even under these grossly exaggerated treatments, the level of methoxychlor in milk never exceeded 0.23 p.p.m.

The six numbered tests summarized in Table VI were all conducted on the same group of animals, over an 18-week period. At the beginning of each test, the pretreatment samples showed no methoxychlor, thus demonstrating that methoxychlor does not build up in milk following repeated treatments.

To investigate the presence of possible metabolites of methoxychlor which would not respond to the selective colorimetric method, total organic chlorine was determined on aliquots of selected samples. The samples examined were those which contained traces of methoxychlor, because these would be most likely to contain metabolites. The analytical data summarized in Table VII show only slight, if any, increase in the organic chlorine level in milk, following spraying or direct dusting with methoxychlor, even at exaggerated levels. In view of the precision and sensitivity of the organic chlorine method employed in this study, only one sample reported in Table VII contains sufficient chlorine to be of probable significance; however, the positive trend of all the net chlorine results suggests the presence of very small amounts of chlorine contaminants. These are, of course, accounted for by the known amounts of methoxychlor detected colorimetrically, thus establishing that milk from treated animals is not grossly contaminated with unidentified chlorine-containing metabolites.

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Colorimetric Determination of Guthion Residues in Crops

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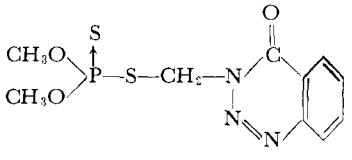
A colorimetric method for the determination of Guthion residues in crops is based on alkaline hydrolysis to anthranilic acid. The anthranilic acid is determined colorimetrically by diazotization and coupling with *N*-(1-naphthyl) ethylenediamine dihydrochloride. Cleanup procedures are described for cottonseed, cole crops, fruit, and milk. The method can be applied to the ethyl homolog of Guthion.

GUTHION, *O,O*-dimethyl *S*-[4-oxo-1,2,3 - benzotriazin - 3(4*H*) - ylmethyl] phosphorodithioate, is a broad-spectrum insecticide which has been introduced recently. It is particularly effective against a wide variety of insects which attack cotton, fruit, and vegetables. In the literature, this material has been referred to as Bayer 17147 and as "Gusathion."

Its structural formula is



The compound is soluble in many organic solvents. Solubility in water is approximately 1 part in 30,000.

In considering suitable means for determining Guthion (Chemagro Corp. trade-mark) residues in crops, several possibilities presented themselves. The compound can be hydrolyzed in alkali to form dimethyl phosphorodithioate (*4*). This compound forms a colored complex with copper similar to that used for the determination of malathion residues (*9*). A colorimetric procedure involving coupling with phenyl-1-naphthylamine in acid solution has been described by Wollenberg and Schrader (*11*). The alkaline hydrolysis of benzazimide to form anthranilic acid has been reported by Finger (*3*). The anthranilic acid formed on hydrolysis can be diazotized and coupled to form a colored complex by a procedure similar to that described by Bratton and Marshall (*2*).

The anthranilic acid method of analysis offered most promise, because the final colorimetric determination has been widely used and is well defined. The reaction involving the formation of a copper complex (*9*) has the disadvantage that the color is not stable. A preliminary study of the phenyl-1-naphthylamine method (*11*) showed that much development work would be required before this procedure would be applicable to routine analyses.

Recently a colorimetric procedure for Guthion residues in cottonseed has been reported by Giang and Schecter (*5*). The Guthion is hydrolyzed with acid and the liberated formaldehyde is distilled and determined colorimetrically with chromotropic acid.

In this paper, a colorimetric procedure based on hydrolysis of Guthion to form anthranilic acid is presented. Extraction and cleanup methods for cottonseed, fruits, cole crops, and milk are also described.

In the development of a satisfactory residue method, due consideration must be given to the possible presence of toxic metabolites and to interference from nontoxic decomposition products. The objective is to include the former compounds in the residue method while omitting the latter.

One would expect the principal toxic metabolite of Guthion to be its oxygen analog. Although metabolic studies (*10*) have shown that this compound is not formed in cotton, procedures have been developed which would include residues of this material if it should happen to be present.

Interference from Guthion hydrolysis products might be expected from 1,2,3-benzotriazin-4(3*H*)-one (I), 3-(hydroxymethyl) - 1,2,3 - benzotriazin - 4(3*H*)-one (II), 3-(mercaptomethyl)-1,2,3-benzotriazin-4(3*H*)-one (III), or *o*-aminobenzoic acid (IV). It has been shown that the first two compounds do not interfere with the method described. Compound III gives 70% of the theoretical amount of color and would interfere, if present. Compound IV would also interfere. The extent of interference due to IV can be determined by carrying a sample through the procedure, omitting the hydrolysis step.

Colorimetric Determination

Hydrolysis Conditions. In the initial studies on Guthion hydrolysis, the Averell and Norris (*1*) method was used without modification to determine the amount of anthranilic acid formed. Finger (*3*) reported that benzazimide is decomposed to nitrogen and anthranilic acid on heating with alkali, but did not describe the conditions which he used for hydrolysis.

At the outset, Guthion was dissolved in isopropyl alcohol and aqueous 1*N* potassium hydroxide was added. Under these conditions yields of anthranilic acid were approximately quantitative in about 10 minutes at room temperature; under the same conditions the Guthion oxygen analog produced only 50 to 70% of the expected color. Accordingly, the effect of the solvent system on this reaction was studied.

In this investigation all solutions were 0.5*N* with respect to potassium hydroxide. The solvents used were absolute alcohols and 1 to 1 mixtures of these with water. The results of this study are shown in Table I. The absorbances recorded were obtained using 100 γ of the appropriate compound. It is apparent that to obtain color from the oxygen analog equivalent to that formed from Guthion, one must use isopropyl alcohol as the hydrolysis solvent. Alcoholic potassium hydroxide is not a satisfactory solvent for the solution of waxy plant residues. Benzene was very useful in keeping these substances in solution during the hydrolysis. Further studies conducted using a 1 to 1 mixture of benzene and 0.5*N* potassium hydrox-

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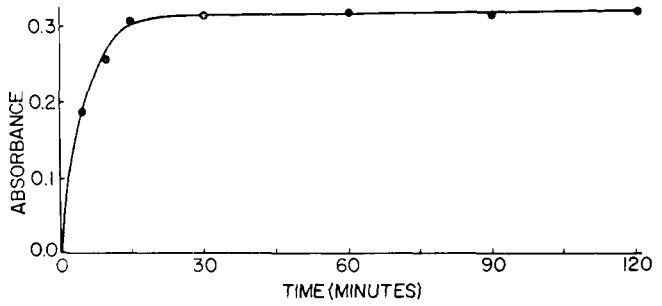

Figure 1. Effect of time of hydrolysis of Guthion on amount of color formed

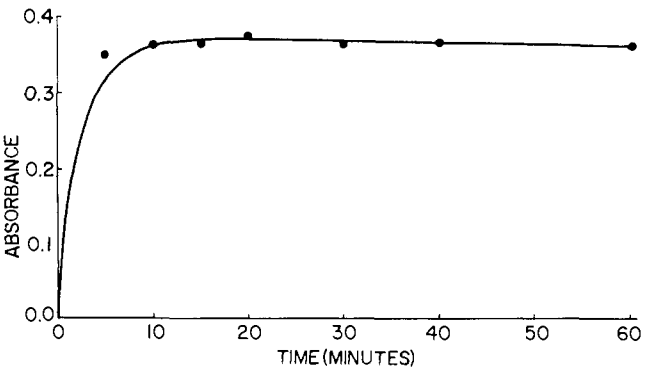

Figure 2. Rate of color formation

ide in absolute isopropyl alcohol showed that dilution with benzene had no effect on the amount of color developed by either Guthion or its oxygen analog.

The time required for completion of hydrolysis was investigated by conducting a series of determinations in which the hydrolysis time was varied from 5 to 60 minutes. In this study, 100-γ samples of Guthion and its oxygen analog were hydrolyzed in a 1 to 1 mixture of benzene and 0.5N potassium hydroxide in absolute isopropyl alcohol. The analytical results for Guthion are shown in Figure 1. Identical results were obtained with the oxygen analog. The hydrolysis is practically complete in 10 minutes at room temperature. To ensure complete hydrolysis, a hydrolysis time of 20 minutes was adopted for routine purposes.

Conditions for Color Development. To find the optimum final acid concentration, determinations were carried out in which 2, 4, 6, 7, 8, 10, and 12 ml. of 3N hydrochloric acid were added to a series of hydrolysis solutions. Maximum color development was obtained with 8 ml. of 3N acid. With less acid, less color was developed. When 10 or 12 ml. of acid were used, the same amount of color was developed as with 8 ml., but the rate of color formation was somewhat decreased.

The rate of color development was investigated using 8 ml. of 3N hydrochloric acid (see Figure 2). Color development is practically complete in 15 minutes. To be certain that small variations in time would have no effect on the amount of color formed, a standing time of 90 minutes was adopted for routine work.

The absorption spectrum of the color formed from Guthion was determined on a Beckman DU spectrophotometer. The absorption maximum is at 555 mμ and this wave length was adopted for routine purposes. When the conditions described above are used, the color obeys Beer's law.

The yield of anthranilic acid under the conditions described was shown to be 85% of the theoretical amount. Anthranilic acid in the hydrolyzate was positively identified by paper chromatography using *tert*-butyl alcohol containing 10% water. Co-chromatography and determination of the absorption spectra of the color complexes from Guthion and anthranilic acid also showed these to be identical.

Application to Crop Residues

Cottonseed. The separation of Guthion from cottonseed presents special problems because of the presence of a large amount of oil as well as gossypol and related pigments. Jones and Riddick (*6*) suggested extraction of a hexane solution with acetonitrile to separate pesticides from glycerides. Kolbezen and Reynolds (*7*) applied this approach to the determination of chlorthion in cottonseed. The efficacy of this system for Guthion was tested using refined cottonseed oil. In this experiment 250 γ of Guthion and 40 grams of refined cottonseed oil were dissolved in 350 ml. of petroleum ether. This approximates the amount of oil which would be obtained from 200 grams of cottonseed. Similar solutions were also prepared, omitting the Guthion and omitting the oil. The petroleum ether solutions were extracted with 50 ml. of acetonitrile for the first extraction and 25 ml. for each subsequent extraction. Each acetonitrile extract was evaporated and analyzed separately.

Four extractions gave a 98.1% recovery of Guthion when oil was omitted but only 90.9% recovery when oil was present. Three additional extractions of the solution containing oil increased the recovery to 94.1%.

At the outset it was observed that the acetonitrile extract contained pigments which interfered with the final color determination. Therefore, the acetonitrile solutions were chromatographed by passage through acid-washed alumina as suggested by Kolbezen and Reynolds (*7*). This was very effective in removal of the interfering materials. Experiment showed that neither Guthion nor its oxygen analog is retained by the column.

In the procedure as finally adopted, the acetonitrile is evaporated on the steam bath. A preliminary experiment with an acetonitrile solution of Guthion showed that no loss is incurred by this treatment.

Fruit. In the extraction of Guthion residues from fruit, two problems were encountered. The extraction of microgram quantities of oil-soluble insecticides from tissues which contain a large amount of water is difficult. In many cases this type of extraction has been carried out by maceration of the plant tissue with a water-immiscible fat solvent. However, fat is more completely extracted from such material by the use of a water-miscible solvent such as acetone or ethyl alcohol. In the procedure adopted, acetone was selected as the solvent for the initial extraction.

The second problem involves the interference of plant pigments which are extracted along with the Guthion. Extracted carotenoid pigments do not show appreciable absorption at 555 mμ and, therefore, do not interfere. However, the small amounts of chlorophyll extracted from green fruit interfere seriously in the final color determination. As separation of chlorophyll from Guthion in an aqueous acetone solution is difficult, it was decided to destroy the chlorophyll by treatment with a small amount of hydrochloric acid. The chlorophyll is rapidly converted to yellow-brown pheophytin-like pigments. In the case of fruit with small amounts of chlorophyll, these pigments cause only slight interference in the final determination.

The basic method, then, consists of the following steps. The tissue is macerated in an excess of acetone, and the insoluble material is removed by filtration. Following dilution with water, sufficient hydrochloric acid is added to make the solution about 0.06N. Guthion residues are extracted from the acetone-water phase with successive portions of chloroform.

The efficiency of chloroform in removing Guthion from aqueous acetone solutions was tested on an apple extract. Known amounts of Guthion were added to the aqueous acetone extract and this

was extracted with one 100-ml. and eight 50-ml. portions of chloroform. The extracts were analyzed separately. Seven extractions gave recoveries ranging from 97 to 114%. The eighth and ninth extractions had little or no effect on the over-all recovery.

Cole Crops. Vegetables and fruits containing high concentrations of plant pigments present a special cleanup problem. The use of solvent systems which have been found to be essential if the pesticide residue is to be recovered, results in extraction of large amounts of pigments from which the residues must be separated. Cole crops present an additional cleanup problem, in that they contain an extractable substance which, upon hydrolysis with alkali, produces a compound that can be diazotized and coupled to form a color similar to that formed by Guthion. The unknown substance apparently produces an aromatic amine on treatment with alkali. The color produced by this interfering material is usually equivalent to 0.25 to 1.5 p.p.m. of Guthion, but values equivalent to 10 or 12 p.p.m. are sometimes encountered. This material must be removed from plant extracts before Guthion residues can be determined.

After examination of many adsorbent-solvent systems, both Guthion and its oxygen analog were separated from all plant pigments except carotenoids by a chromatographic column composed of ascending layers of Super-Cel, a 1 to 2 mixture of Sea Sorb 43 and Super-Cel, alumina (acid-washed), and anhydrous sodium sulfate. Isopropyl alcohol was used as both the initial solvent and the eluting agent. This column was effective in removing the interfering substance from cole crops.

Carotenoid pigments remaining in the solution following hydrolysis of the Guthion to form anthranilic acid can be removed by a benzene extraction of the acidified hydrolyzate.

Milk. The Guthion residues were extracted from milk by blending the milk with acetone and removing the insoluble material by filtration. The acetone-insoluble fraction is re-extracted with benzene. The acetone and benzene extracts are combined and evaporated to dryness. The residue is partitioned between hexane and acetonitrile. The pesticide residue partitions into the acetonitrile, while the fat remains in the petroleum ether. The acetonitrile solution is treated as in the method for cottonseed.

Procedure

Reagents. Alumina, chromatographic grade, acid-washed (Merck).

Sea Sorb 43, chromatographic grade magnesium oxide (Westvaco Division, Food Machinery and Chemical Corp., Newark, Calif.).

Guthion, recrystallized (Chemagro Corp., Kansas City, Mo.).

Super-Cel (Celite Division, Johns Manville, Lompoc, Calif.).

Sample Preparation, Extraction, and Cleanup. COTTONSEED. Place a 200-gram sample of finely ground cottonseed in a large Soxhlet extractor and extract for 16 hours with petroleum ether (boiling range, 30° to 60° C.). Centrifuge or filter the petroleum ether extract to remove finely divided material carried over during the extraction. Wash the residue with fresh petroleum ether and combine washings and extract. Evaporate the extract to about 350-ml. volume on a steam bath and transfer to a 500-ml. separatory funnel. Extract the petroleum ether with 50 ml. of acetonitrile. Re-extract with six successive 25-ml. portions of acetonitrile. Wash the combined acetonitrile extracts with 300 ml. of petroleum ether and then wash this petroleum ether with four successive 10-ml. portions of acetonitrile. Combine the acetonitrile solutions and evaporate on a steam bath in a stream of air to a volume of approximately 10 ml. Pour the concentrated acetonitrile extract onto a column of 2.5 inches of acid-washed alumina. Apply gentle suction and elute with 100 ml. of acetonitrile. Evaporate the extract just to dryness on a steam bath under an air jet. Analyze the residue as instructed under Colorimetric Method.

FRUIT. Grind entire sample in a Hobart food cutter and mix thoroughly. Weigh out a 200-gram sample in a Waring Blendor jar. Blend at high speed with 200 ml. of acetone for 5 minutes. Add 200 ml. of acetone and mix for 5 minutes more at low speed. Transfer to a 1-quart screw-cap jar and dilute to 600-ml. volume with distilled water. Shake vigorously on a mechanical shaker for 30 minutes. Filter the mixture through a pledget of glass wool in a large funnel. If the first portion is turbid, refilter. The resultant filtrate should be crystal clear. Normally 400 ml. can be obtained easily and this is the volume usually taken for analysis. Place the extract in a 1000-ml. separatory funnel and add 3.3 ml. of concentrated hydrochloric acid and 300 ml. of water. Extract with seven successive portions of chloroform, using 100 ml. for the first and 50 ml. for each subsequent extraction. Shake gently during the first extraction, or a stable emulsion may be formed. Wash the combined chloroform extracts with 200 ml. of water. Remove the water and wash it with three successive 25-ml. portions of chloroform. Combine the chloroform extracts and dry by passing through anhydrous sodium sulfate. Evaporate the chloroform extract to dryness on a steam bath in a current of air. Analyze the residue as instructed under Colorimetric Method.

COLE CROPS. Prepare and extract samples according to the method described for fruit. Prepare a chromatographic column (20 × 400 mm.) by introducing successively 2 grams of Super-Cel, 5 grams of a 1 to 2 mixture of Sea Sorb 43 and Super-Cel, 10 grams of alumina (acid-washed), and 20 grams of anhydrous sodium sulfate. Maintain suction during packing of column and tap gently to assist packing after each addition. Dissolve the residue remaining after evaporation of the chloroform in 25 ml. of isopropyl alcohol. Boil gently, if necessary, to effect solution. Cool the solution and pour it onto the chromatographic column just as the last of the equilibrating isopropyl alcohol passes into the sodium sulfate layer. Wash with isopropyl alcohol until the total volume of effluent is 160 ml. Evaporate the column effluent to dryness on a steam bath under a current of air. Analyze the residue as instructed under Colorimetric Method.

MILK. Weigh 100 grams of milk into a Waring Blendor jar. Add 200 ml. of acetone and 5 grams of Super-Cel. Blend at high speed for 5 minutes. Filter with suction through a fine filter paper. Return the residue to the jar and add 200 ml. of benzene. Blend for 5 minutes at high speed. Filter as before. Discard the filter cake. Transfer the acetone and benzene extracts to a 1000-ml. separatory funnel. Shake vigorously. After separation, discard the lower aqueous layer. Clarify the acetone-benzene solution by adding 2.0 grams of Super-Cel and refilter. A crystal-clear solution should be obtained. Evaporate to dryness on a steam bath under a current of air. Dissolve the residue in 300 ml. of hexane and transfer to a 1000-ml. separatory funnel. Extract with acetonitrile as described for cottonseed. Evaporate the acetonitrile to dryness on a steam bath in a current of air. Analyze the residue as instructed under Colorimetric Method.

Colorimetric Method

Dissolve the residue obtained by one of the methods described above in 10 ml. of benzene. Add 10 ml. of 0.5*N* potassium hydroxide in absolute isopropyl alcohol and allow to stand for 20 minutes at room temperature. Add 8 ml. of 3*N* hydrochloric acid to acidify the solution. Add 15 ml. of water and 50 ml. of benzene. Transfer to a 250-ml. separatory funnel and shake vigorously. After separation of the phases, draw off the aqueous layer into a 50-ml. glass-stoppered graduated cylinder. Wash the benzene with 15 ml. of distilled water and add the washings to the cylinder. Discard the benzene phase. Dilute the aqueous solution to 50 ml. with distilled water and mix thoroughly. If the solution is turbid at this point, add Super-Cel and filter. Transfer 20-ml. aliquots of the solution to two 25-ml. glass-stoppered graduated cylinders. Add 1 ml. of 0.25% aqueous sodium nitrite solution to each cylinder. Mix and let stand for 10 minutes. Add 1 ml. of 2.5% aqueous sodium sulfamate solution to each cylinder. Rinse the stopper free of nitrite by inverting the cylinder and then, when the cylinder is returned to an upright position, remove the stopper. Repeat several times. Let the solution stand for 10 minutes. Add 2 ml. of a 1% aqueous solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride to only one of the cylinders. Dilute solution in both cylinders to 25

Table I. Effect of Solvent System on Hydrolysis of Guthion and Its Oxygen Analog

Alcohol	Analog	Absorbance[a] 0.5N alcoholic KOH	Absorbance[a] Alcohol + 1N KOH (aqueous)
Methanol	P--O	0.017	0.210
	P--S	0.066	0.359
Ethyl alcohol	P--O	0.162	0.325
	P--S	0.444	0.428
Isopropyl alcohol	P--O	0.432	0.341
	P--S	0.427	0.427
n-Amyl alcohol	P--O	0.140	0.334
	P--S	0.182	0.484

[a] Absorbance values obtained using 100 γ of appropriate compound.

Table II. Recovery of Guthion and Its Oxygen Analog from Crops

Crop	Compound Added	Added, P.P.M.	Found, P.P.M.[a]	Mean Recovery, %
Cottonseed		0.05	0.05 ± 0.01 (5)	100
		0.10	0.9 ± 0.01 (2)	90
		0.20	0.18	90
		0.40	0.32	80
Apples		0.25	0.23	92
		0.50	0.51 ± 0.01 (2)	102
		0.75	0.75 ± 0.04 (2)	100
		1.00	0.91	91
Peaches		0.50	0.50 ± 0.00 (2)	100
Pears		0.50	0.44 ± 0.02 (2)	88
Apricots		0.50	0.44	88
Cabbage[b]		0.20	0.21 ± 0.03 (2)	105
		0.30	0.31 ± 0.01 (2)	103
		0.40	0.43 ± 0.02 (2)	108
		0.50	0.48	96
	Oxygen analog	0.20	0.15	75
		0.30	0.21	70
		0.40	0.36	90
		0.50	0.48	96
Cauliflower[b]		0.20	0.20	100
		0.30	0.28	94
		0.40	0.40	100
	Oxygen analog	0.20	0.22	110
		0.30	0.40	133
		0.40	0.43	107
Broccoli[b]		0.20	0.21	105
		0.30	0.27	90
		0.40	0.34	85
		0.50	0.49 ± 0.03 (2)	98
	Oxygen analog	0.50	0.37 ± 0.02 (2)	74

[a] Values followed by average deviation from mean and in parentheses by number of determinations.
[b] Zinc reduction step included in analytical method used for these samples.

ml. with distilled water and mix thoroughly. Allow to stand for 90 minutes at room temperature and compare the absorbances at 555 mμ in a suitable spectrophotometer or colorimeter. Read both solutions against a solvent blank. The solution from which the coupling reagent is omitted will permit correction for residual color in the extract.

Determine the concentration of Guthion in the samples by comparison with a calibration curve prepared using highly purified Guthion.

In the case of cole crops, the recovery of Guthion and its oxygen analog can be improved by introduction of a zinc reduction step following the hydrolysis and extraction with benzene. When this modification is desired, add 0.2 gram of zinc dust to the solution after dilution to 50 ml. Shake the sample vigorously two or three times during a 15-minute reaction period at room temperature. Filter through a Whatman No. 12 fluted filter paper. Place 20-ml. aliquots of the filtrate in 25-ml. graduated cylinders and proceed according to the instructions above.

Discussion

Recovery Experiments. Recovery experiments were conducted on each of the methods described above (Table II), in which known amounts of Guthion were added to the raw agricultural commodity. While such experiments do not indicate the efficiency of the initial extraction system, they show whether or not the material is lost in the steps subsequent to extraction. Approximately 75% of the recovery values shown fall between 90 and 110% and are considered excellent for this type of procedure. In only three cases were such values less than 80%. It is apparent that the recovery values for the Guthion oxygen analog are not quite as good as those obtained for Guthion itself.

Sensitivity and Precision. Using the method as described above, 8 γ of Guthion in the final 25 ml. of solution will give an absorbance of 0.063 with the standard tubes (approximately 2 cm.) in an Evelyn photoelectric colorimeter. This is considered the limit of sensitivity of this method. This corresponds to a concentration of 0.15 p.p.m. when a 200-gram sample and the dilutions and aliquots described are used. Sensitivity can be increased to 0.05 p.p.m. by using a cell of 5-cm. path length in a Beckman DU spectrophotometer.

A study of the precision of the method showed that in the concentration range up to 1 p.p.m. an average deviation of about 5% of the mean can easily be obtained. With larger residues, the precision is greater.

That compounds I and II are not hydrolyzed under the conditions used for Guthion is surprising. The stability of compound I is attributed to enol-keto tautomerism involving a shift of hydrogen from the nitrogen in position three to the carbonyl oxygen group in position four. This does not explain the stability of compound II. It may be that, in this case, very rapid release of formaldehyde results in the formation of I.

A study showed that approximately 80% of compound III is removed by the cleanup procedures. On this basis, compound III would produce interfering color equivalent to 15 to 20% of the amount present.

The variations in the rates of hydrolysis of Guthion and its oxygen analog in various solvents merit some consideration. The oxygen analog of Guthion is more rapidly hydrolyzed than Guthion (*8*). Because Guthion is hydrolyzed in alkali to form the *O,O*-dimethyl phosphorodithioate, it must be assumed that compound II is also formed. If the rate of formation of compound II is greater for the oxygen analog than for Guthion, it would explain the lower color yield from the oxygen analog in a partially aqueous system. This would require the assumption that the hydrolysis rate of the oxygen analog is greatly decreased in absolute isopropyl alcohol. No explanation is proposed for the fact that the absolute isopropyl alcohol is more effective than absolute methyl or ethyl alcohol.

The methods described can be applied directly to the determination of residues of the ethyl homolog of Guthion.

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INSECTICIDE MEASUREMENT

Determination of Toxaphene by a Spectrophotometric Diphenylamine Procedure

ALDA JOHNSON GRAUPNER and CHARLES L. DUNN

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A confirmatory method for the positive identification of toxaphene in small residues has not previously been reported. A sensitive method for the qualitative and quantitative determination of toxaphene in residues and in formulations is based on fusion with diphenylamine in the presence of zinc chloride. The greenish blue reaction product, dissolved in acetone or glacial acetic acid, has an absorbance maximum at 640 mμ. Toxaphene in the range of 20 to 700 γ can be readily determined by spectrophotometric measurement. When combined with cleanup procedures, the method has been applied to extracts of crops and foods and to various formulations, including mixtures with sulfur or DDT.

THE need for an accurate method for the specific determination of micro quantities of toxaphene in food and forage crops and in milk and beef has become increasingly apparent with the more widespread use of this insecticide. Toxaphene is chlorinated camphene containing 67 to 69% chlorine. Microdetermination by a sensitive method for organic chlorine (*5*) has been widely used for determining toxaphene residues, but this method lacks specificity. Macrodetermination by an infrared method (*4*) is specific, but has not been employed as a residue method because of low sensitivity. A qualitative colorimetric test with pyridine and methanolic potassium hydroxide has been reported (*3*). A spectrophotometric method based upon the reaction of toxaphene with thiourea in the presence of alkali, developed by Hornstein (*2*), is quantitative in the 0.5- to 5.0-mg. range. However, there is need for a more sensitive method.

The reaction of DDT with diphenylamine in the presence of zinc chloride has been reported to give a reddish orange color with an absorbance maximum at 490 mμ (*1*). Toxaphene reacts with these reagents at high temperature and in the absence of solvents to give a greenish blue complex which is soluble in acetone or glacial acetic acid and exhibits an absorbance maximum at 640 mμ; the absorbance–wave length curve is shown in Figure 1 and the spectrophotometric constants are given in Table I. This reaction has been made the basis of a sensitive quantitative method for both residue and assay analyses. Toxaphene in the range of 100 to 700 γ can be readily determined with an accuracy and precision to ±2%; in the 20- to 100-γ range, ±7% is usual. Naturally occurring organic chlorine and most chlorinated insecticides do not interfere; however,

Table I. Sensitivity Constants for Toxaphene Spectrophotometric Procedure

Absorptivity, a, liter/gram cm.	17.0
Molar absorptivity, ϵ, liter/mole cm.	7.0×10^3
Absorbance concentration, γ/ml. (γ/ml. required to give absorbance of 1.00 in 1-cm. cell)	58.8

Measurements made at 640 mμ

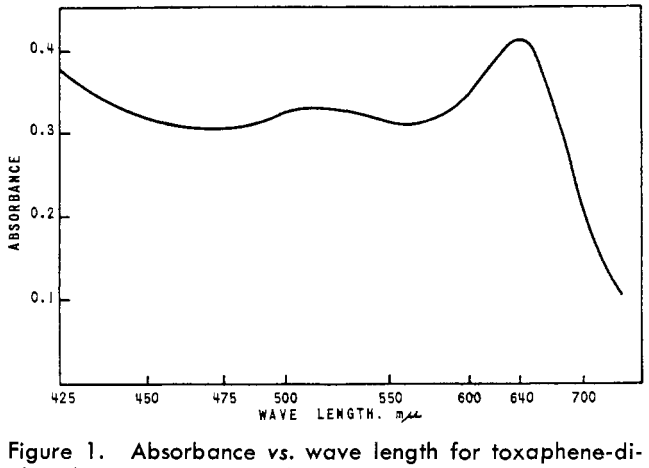

Figure 1. Absorbance *vs.* wave length for toxaphene-diphenylamine reaction product

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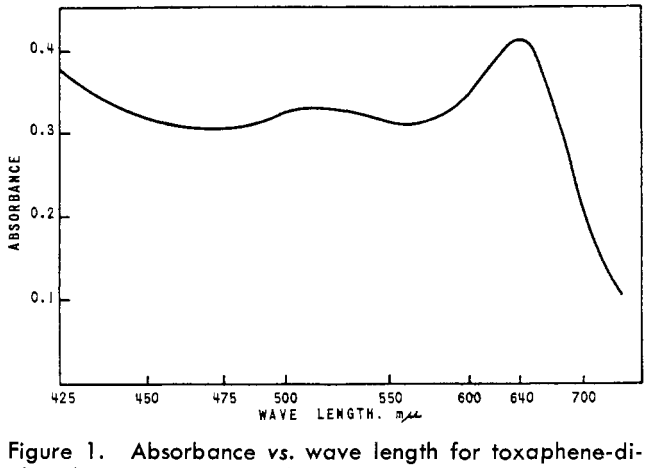

Figure 1. Absorbance *vs.* wave length for toxaphene-diphenylamine reaction product

Table II. Recovery of Toxaphene from Fortified Extracts[a]

Extract	Fortification Level, P.P.M.	Av. Control, P.P.M.[b]	Cleanup Procedure	Detns.	Av. Recovery, %	Std. Dev. of % Recovery
Alfalfa, green	1–5	0.2	A	2	69	..
	10–50	0.3	A	5	91	5
Barley, grain[c]	1–7	0.5	A	7	74	6
Beef fat	7	1.3	CA	5	72	10
Milk[d]	0–1	0.1	B	11	74	10
Milk	0–1	0.06	CA	7	78	7
Oats, green	7	0.6	A	2	75	..
Pears	1–4	0.2	BA	3	98	8
Rice, grain	1–7	0.4	A	7	76	7
Rice, straw	7–20	3.5	A	5	93	12
Tobacco, cured	10–1000	3.1	A	19	78	12
Tomatoes	3–10	0.5	A	6	100	6
Wheat, grain	0–3	0.2	A	6	83	1

[a] Range for colorimetric determination 0 to 700 γ generally.
[b] Not corrected for solvent blank.
[c] 0 to 100 γ. [d] Both ranges.

plant and animal extractives must be eliminated when the method is applied to residue analysis. For assay of toxaphene formulations, an aliquot of a methanol solution of the active ingredients may be used directly.

Experimental

Reagents. Diphenylamine, recrystallized from *n*-hexane, 0.5% solution in acetone.

Zinc chloride, reagent grade, 0.5% solution in acetone, filtered, prepared fresh daily.

Acetone, reagent grade.

Methanol, absolute.

n-Hexane (Skellysolve B, Solvents Division, Skelly Oil Co., St. Louis, Mo., or redistilled).

n-Hexane, acid-treated with fuming sulfuric acid supported on dry Celite 545, stirred for an hour, filtered through glass wool, washed with cold water, dried over anhydrous sodium sulfate, and redistilled.

Methylene chloride, redistilled.

Sulfuric acid, concentrated, 96%.

Sulfuric acid, fuming, 20 to 23% SO_3.

Florisil, 60 to 100 mesh (Floridin Co., Tallahassee, Fla.), conditioned by heating at 125° to 130° C. for 24 hours.

Celite 545 (Johns-Manville Corp., 4th and Chestnut Sts., Philadelphia 6, Pa.), dried at 110° C. for at least 2 hours.

Sodium sulfate, anhydrous, granular, reagent grade.

Apparatus. Chromatographic tubes, 28 mm. (inside diameter) × 340 mm., tapered sharply to a stem 8 mm. (outside diameter) × 50 mm.

Chromatographic tubes, No. 2 (20 mm.), Scientific Glass Co., Rahway, N. J., or equivalent.

Glass packing rod, 8 × 500 mm. glass rod, with flattened disk at one end 20 mm. in diameter.

Fritted-glass crucibles, medium porosity, and crucible holders.

Fisher Filtrator or other vacuum filtering apparatus.

Test tubes, 150 × 16 mm.

Oil bath, 205° C.

Spectrophotometer, Beckman Model B with red phototube or equivalent, and 1-cm. covered cells.

Preparation of Standard Curves

0- to 700-γ Range. An acetone or *n*-hexane solution of toxaphene containing 100 γ per ml. is prepared. Aliquots (1, 3, 5, and 7 ml.) of the solution are pipetted into separate test tubes, and 1 ml. each of the diphenylamine and zinc chloride solutions are added. A reagent blank is also prepared. The solutions in the test tubes are evaporated to dryness in a 60° to 70° C. water bath; a rapid stream of dry air is used to speed evaporation. The residue should be deposited in a thin film along the bottom inch of the test tube; rinsing down the side walls with a little acetone near the end of the evaporation may be necessary.

The test tubes are then immersed in a 205° C. oil bath for 3 minutes. After cooling, the greenish blue complex is dissolved in a little acetone and transferred quantitatively to a 25-ml. volumetric flask. The volume is adjusted to 25 ml. with acetone. This color is stable after a 15-minute interval for about 1 hour, then gradually fades. The fading is not due to a wave length shift of the peak.

The absorbance is measured at 640 mμ in covered 1-cm. cells; acetone is used to zero the instrument. The standard curve for this range is obtained by plotting absorbance *vs.* micrograms of toxaphene; Beer's law is obeyed.

0- to 100-γ Range. The same procedure is used, except that 0.2-, 0.4-, 0.6-, 0.8-, and 1.0-ml. aliquots of the standard solution are measured (a more dilute standard solution appears to degenerate on standing), and the colored complex is diluted to 5 ml. with acetone.

Removal of Interfering Substances

As with nearly all pesticide residue methods, the plant pigments and waxes and animal fats extracted with the pesticide interfere in its subsequent determination, and suitable cleanup procedures must be applied. The presence of more than 5 mg. of extraneous material affects the fused reaction mixture significantly and toxaphene recoveries are low. Three separation techniques have been used. Either alone or in combination they have been adequate for the preliminary treatment of all the crop extracts and fats shown in Table II.

Procedure A. Florisil Column. This procedure is used for the isolation of toxaphene from crop extracts which contain relatively small amounts of waxes, fats, or oils. It serves as the final cleanup step for extracts treated initially with sulfuric acid to remove large amounts of waxes, fats, or oils. Toxaphene is adsorbed weakly by Florisil, and is held just long enough to permit elution of most waxy or oily materials in a preliminary *n*-hexane cut.

A large tube (28-mm. inside diameter) is plugged with glass wool and packed with conditioned Florisil to a height of 15 cm. A circle of coarse filter paper prevents disturbance of the packing when solvent is added. An aliquot of *n*-hexane extract of a control sample is concentrated to 5 to 10 ml. and fortified with a known amount of toxaphene. Usually 300 to 500 γ of toxaphene in an extract from 50 to 100 grams of sample is used. The fortified extract is rinsed onto the dry column with small amounts of *n*-hexane and elution with this solvent is continued until 20 ml. of eluate has been collected. This cut, which contains the bulk of the wax, is discarded. Then the toxaphene is eluted with 125 ml. of 1 to 1 methylene chloride–*n*-hexane. This cut is evaporated to a small volume and transferred to a test tube with *n*-hexane, and the insecticide is determined by the spectrophotometric method. As the adsorptive capacity of Florisil seems to vary slightly from batch to batch and the interfering materials in crop extracts vary, a check on the recovery from a fortified control is necessary. If recovery is low, variations of the wax-cut volume must be tried—wax cuts from 10 to 30 ml. have been used in this laboratory. When the Florisil column is used in conjunction with Procedure B or C, a 10-ml. wax cut is generally satisfactory.

Procedure B. Sulfuric Acid Column. Toxaphene is stable in concentrated sulfuric acid–fuming sulfuric acid mixtures. Procedure B is a partitioning system between sulfuric acid and *n*-hexane for use with small fat samples (2 to 5 grams) or *n*-hexane crop extracts containing acid-sensitive material.

For fat samples, the large (28-mm. inside diameter) tube is used. After insertion of a glass wool plug, 10 grams of dry Celite 545 is packed in the bottom of the tube and tamped down firmly with the glass tamping rod. Then 18 ml. of 3 to 1 concentrated sulfuric acid–fuming sulfuric acid is triturated with 30 grams of dry Celite 545 in a mortar

Table III. Spectrophotometric and Organic Chlorine Analyses on Grain Extracts[a]

	Toxaphene, P.P.M.	
	Org. Cl	Spectro
Controls		
Barley 1	2.6, 2.8	0.6, 0.4
Barley 1, whole	0.3	0.4
Barley 2	2.5, 3.1	0.5
Barley 2, whole	0.7	0.4
Wheat 1	0.6	0.2
Rice 1, whole	1.2	0.4
Samples, corrected for control		
Barley 3	1.1	0.7, 0.9
Barley 4	1.7	1.4
Barley 5	6.7	7.1
Barley 6	13.0, 16.0	14.2
Wheat 2	0.3	0.3
Wheat 3	0.5	0.3
Wheat 4	0.3	0.5
Wheat 5	1.8	1.4
Wheat 6	1.4	1.6
Rice 2, whole	0.6	0.5
Rice 3, whole	2.3	1.6
Rice 4, whole	4.4	5.8
Rice 5	1.3	2.1
Rice 5, whole	1.2	1.6, 2.8

[a] Grain mealed in coffee grinder before extraction, except when designated "whole."

and packed above the dry Celite in at least three portions. The column is covered with a circle of coarse filter paper and acid-purified *n*-hexane is added until the column is saturated. An *n*-hexane solution of the fat sample, concentrated to 5 to 10 ml., is forced into the column with the aid of approximately 2 pounds of nitrogen or air pressure. The column is developed with the acid-purified *n*-hexane; the first 50 ml. is discarded and the next 150-ml. cut which contains the toxaphene is collected.

For crop extract samples, a smaller tube is adequate. Using a No. 2 chromatographic tube, the column is prepared as described above; 2 grams of dry Celite 545 are used in the bottom and 10 grams of dry Celite mixed with 6 ml. of the acid mixture on the top. The column is eluted with *n*-hexane; the first 15 ml. is discarded. The toxaphene is eluted in the following 60 ml.

Procedure C. Sulfuric Acid–Methylene Chloride Extraction. For samples containing large amounts of fat, Procedure B is inadequate. A separatory funnel extraction procedure, similar to Hornstein's (*2*), with subsequent column cleanup has proved satisfactory.

An aliquot containing approximately 30 grams of fat is concentrated by evaporation, and the residue transferred to a separatory funnel with 200 ml. of methylene chloride. The fat solution is extracted with three 50-ml. portions of concentrated sulfuric acid. A little sodium sulfate may be added to reduce emulsions. The acid layers are combined and back-washed with 100 ml. of methylene chloride. After 15 minutes have been allowed for the layers to separate, the acid is drained into a large beaker of crushed ice and discarded. The methylene chloride extracts are combined and concentrated to about 100 ml. Traces of acid are removed by passing through a chromatographic tube 28 mm. in inside diameter packed to a height of 15 cm. with 1 to 1 sodium sulfate–Celite 545, tamped firmly. The column is rinsed with 100 ml. of methylene chloride, and the solution is again concentrated to 100 ml.

The toxaphene and waxes must be transferred (without taking to dryness) to *n*-hexane solution, so that the final Florisil column cleanup described in Procedure A may be used to remove the waxes. This is done by adding an equal volume of *n*-hexane, evaporating to about 25 ml., again adding an equal volume of *n*-hexane, and finally concentrating to about 10 ml. All of the methylene chloride is thereby removed, and the final *n*-hexane solution is chromatographed according to Procedure A; a 10-ml. wax cut is discarded.

Isolation and Determination of Toxaphene Residues

Toxaphene-treated crop samples are appropriately fragmented and subsampled. The subsample, usually 0.5 to 1 kg., is extracted by rolling or shaking for at least 1 hour with *n*-hexane, or 2 to 1 *n*-hexane–isopropyl alcohol if the moisture content is high. The extract is water-washed several times with portions at least one third of the organic solvent volume, and then dried over anhydrous granular sodium sulfate.

Any toxaphene in a sample of milk or meat will be concentrated in the fat. Butterfat may be isolated by precipitating the milk proteins and fat at a low pH, drying, and extracting the fat with *n*-hexane, or by freeze-drying the whole milk and extracting the fat from the solids. Meat samples are generally ground with sodium sulfate before extraction. The *n*-hexane extracts from milk or meat are then washed and dried as for crop extracts.

The recovered volumes of the extracts are measured after filtration from granular sodium sulfate, and the proportion of sample represented by the recovered extract is calculated. The extracts are concentrated without loss of insecticide in Kuderna Danish evaporative concentrators equipped with a simple spray trap. The concentrated extract is then transferred to a volumetric flask and diluted quantitatively to a known volume with *n*-hexane. Aliquots containing an estimated 0 to 700 γ of toxaphene are taken for purification by Procedure A, B, or C. The resultant toxaphene cut, freed of all pigments and fats and all but a few milligrams of waxes, is evaporated to a low volume (but never to dryness) and transferred with acetone and *n*-hexane to a test tube for the diphenylamine reaction. The procedure given under Preparation of Standard Curves is followed, and the absorbance of the colored toxaphene complex is measured at 640 mμ. If a small amount of wax is present, it may inhibit the dissolving of the colored complex in acetone or cause a slight cloudiness in this solvent. Up to 40% *n*-hexane may be used in the final dilution to overcome this.

It is advisable to run several standards along with the samples in the spectrophotometric procedure to check the standard curve. By doing this, the likelihood of gross errors caused by unrealized variations in reagents and procedure is greatly reduced. The absorbance of the sample solution is translated to micrograms of toxaphene by reference to the standard curve.

Assay of Toxaphene Formulations

Dusts and wettable powders are weighed in fritted glass crucibles; at least 1-gram portions should be taken to ensure a representative sample. Each sample is extracted with a total of 35 ml. of boiling methanol in several increments; vacuum is used to speed the filtration. The methanol extract is cooled to room temperature and diluted to 50 ml., with filtering before dilution to volume if a sulfur precipitate appears. An aliquot of this solution is further diluted with acetone, so that the final solution contains about 500 to 700 γ of toxaphene per 5 ml. Emulsifiable concentrates are weighed and diluted with acetone to this concentration. A 5-ml. aliquot if sulfur was absent, or a 3-ml. aliquot from sulfur-containing formulations, is then pipetted into a test tube and the procedure given under Preparation of Standard Curves is followed. For the reasons noted in the residue determination section, a few standards should be run with the samples in the fusion reactions. Micrograms of of toxaphene in the aliquot are obtained graphically from the standard curve and the per cent toxaphene in the sample may then be calculated.

Discussion

Recovery from Fortified Extracts. Recovery data for various extracts to which known amounts of toxaphene have been added are shown in Table II. All are in the range of 70 to 100%.

Separation Procedure A yields a blank value equivalent to 10 to 30 γ of toxaphene, and the average control values are a reflection of this blank with

Table IV. Reaction Products of Other Chlorinated Insecticides and Interference in Toxaphene Method

Insecticide	Product Color	Abs. Max., Mμ	% Apparent Toxaphene Recovery from Mixture[a]
Rhothane	Yellow	<420	96
DDT	Orange-red	490	102
Lindane	Colorless	None	102
Endrin, 1-hr. soln.	Lt. green	General	104
Heptachlor	Lt. green	General	107
Methoxychlor	Yellow-green	<420	113
Aldrin	Lt. purple	525, >700	129
Endrin, 72-hr. soln.	...	...	130
Dieldrin	Lt. purple	490, 680	132
Chlordan, 72-hr. soln.	...	...	138
Chlordan, 4-hr. soln.	Blue	650	175

[a] Approximately 300 γ of each insecticide combined with 300 γ of toxaphene just before analysis.

but one exception. Rice straw control extract was contaminated with toxaphene, or contained an impurity which was incompletely removed by Procedure A and reacted with diphenylamine. Recovery of 0 to 100 γ of toxaphene in the absence of plant extractives was 98 ± 10% from Procedure A after correction for the separation procedure blank.

Comparison of Spectrophotometric and Organic Chlorine Analyses. Grain extract analyses by spectrophotometric and organic chlorine (*5*) methods, corrected for the appropriate control, are in good agreement, as shown in Table III. Comparison of data obtained from the whole grain and mealed grain extracts of the same sample shows clearly that all of the toxaphene is at or near the surface. The barley contains about 2 p.p.m. of naturally occurring organic chlorine, which is extracted from the mealed samples only.

The organic chlorine method was not applied to many of the other samples because they had been treated with other chlorinated materials in addition to toxaphene or because they contained high and variable amounts of naturally occurring organic chlorine.

Interference from Solvents and Other Insecticides. Interferences in the spectrophotometric method for toxaphene occur, but can usually be detected and eliminated. A trace impurity in carbon tetrachloride and chloroform (possibly phosgene) gives an azure blue color with an absorbance maximum at 600 mμ. Redistilled methylene chloride is free of such impurities. Nitromethane gives the same type of interference and is not recommended for preliminary separations or cleanup. Sulfur, which may be present in dust formulations, gives an emerald green color with toxaphene in the diphenylamine method, and hydrogen sulfide is evolved during the fusion reaction. Sulfur is difficult to separate from toxaphene at the residue level, and greatly enhanced absorbance results if the amount present is more than 10% of the toxaphene residue. Interference in the assay procedure is eliminated by using a limited volume of methanol, in which sulfur has a very low solubility, as the extracting solvent. Pentachlorophenol, which may be sprayed just prior to harvest to reduce moisture in rice, does not interfere.

A number of other chlorinated insecticides have been tested alone and in 1 to 1 mixtures with toxaphene. All except lindane give some color, but absorbance at 640 mμ is weak for most. Aldrin, dieldrin, and chlordan have absorbance peaks at other wave lengths which overlap the 640-mμ band. Toxaphene recoveries from mixtures (300 γ of each insecticide) are shown in Table IV. A freshly prepared solution of endrin in acetone did not interfere, but upon aging the reactivity approached that of dieldrin and aldrin. Chlordan solutions in acetone showed a decrease in reactivity after aging, but interference in toxaphene determinations was still serious. In practice, no serious interference problem from other chlorinated organic insecticides in this method is anticipated, as 1 to 1 residue mixtures of toxaphene with dieldrin, aldrin, or chlordan are unlikely. They are seldom, if ever, applied together and dieldrin and aldrin are used at much lower dosage levels than toxaphene. There is no problem in distinguishing between chlordan and toxaphene, because methods specific for chlordan are known.

Literature Cited

(1) Geer, H. A., Kingsley, P. L., "Analytical Methods for Determination of DDT," NRC Coordination Center Review 4, Insect Control Committee Rept. 59, U. S. Dept. Commerce, Washington, OTS, PB Rept. **11335** (1946).
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INSECTICIDE RESIDUES

Toxaphene Residues on Pangolagrass

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THERE are approximately one-half million acres of improved pangolagrass in Florida, and toxaphene is one of the leading commercial insecticides recommended for the control of caterpillars on this pasture grass. One of the primary needs of all cattlemen at this time is definite information on a safe interval between pasture grass insecticide treatment and turning in beef cattle for indefinite periods of grazing.

This paper summarizes data involving toxaphene residues on pangolagrass exposed to the varying weather conditions of north, central, and south Florida

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A tolerance of 7.0 p.p.m. of toxaphene residues in the fat of beef cattle has been established by the Food and Drug Administration. Toxaphene residues were determined on pangolagrass sampled 2 hours, and 3, 7, and 14 days after treatment. Range grass treatments included three formulations and two dosage rates at three widely separated locations in the state. Seven days after insecticide application, all toxaphene residues on the pangolagrass were below 20.0 p.p.m., regardless of previous treatment. Two weeks after application, the highest toxaphene residues were 12.0 p.p.m. U. S. Department of Agriculture tests indicate that beef cattle may be fed 25 p.p.m. or less of toxaphene in the diet for 10 consecutive weeks without exceeding the beef fat tolerance. Therefore, a 7-day interval has been recommended to local cattlemen between toxaphene applications on pangolagrass and the time cattle are allowed to graze on the treated range.

in late summer. All three experiments were coordinated as much as possible, including a common source of three formulations of toxaphene, in order to eliminate differences other than climatic. Applications and samplings were supervised by staff entomologists who had previously decided on standardized mode of application and approximate height of grass at application time. Experiments were designed in randomized blocks and the pangolagrass was sampled and extracted in a uniform manner.

Materials and Methods

At all locations a plot size of 1/100 acre (21 × 21 feet) was used. Each treated field and check plot was replicated three times at the three locations (location A, Gainesville; location B, Bradenton; and location C, Belle Glade). Adequate border areas were maintained to avoid excess contamination between treatments. One insecticide application was made using 1 and 2 pounds of active ingredient of the following formulation: 10% dust, 40% wettable powder (W.P.), and 60% emulsifiable concentrate (E.C.). Random sampling of grass was performed in nine individual 1-sq. foot areas in each plot per cutting. The grass from these nine subsamples was cut into 1-inch lengths and mixed and a 100-gram sample was extracted with 600 ml. of *n*-hexane for 1 hour. Extractions were placed in sealed Mason jars and stored at 40° F. until analyzed.

Considerable effort was made to have all coworkers apply the toxaphene to grass of uniform height. However, because of factors beyond control, at locations A and B the toxaphene was applied to grass approximately 18 to 24 inches high, whereas at location C it was applied to grass only 10 to 15 inches high. Variations in height could have considerable effect on the calculated parts per million, particularly in the first samplings.

All samples were analyzed by the colorimetric diphenylamine procedure for toxaphene (*4*), with minor modifications. Chromatographic columns used for cleanup were 19 mm. in inside diameter and were packed to a height of 18 cm. with 60- to 100-mesh Florisil previously conditioned at 120° C. for 7 hours in a forced-draft oven. Elution curves were run on each new batch of Florisil. A typical elution curve is illustrated in Figure 1. For all batches of Florisil treated, 90 to 95% of the toxaphene added to the chromatographic column generally came through in the 80- to 130-ml. elution fraction. Therefore, depending on the Florisil batch, a 50- to 60-ml. wax forecut was discarded before the eluent was collected for analysis. On the basis of elution curve studies, approximately 95% of the toxaphene was collected from the column subsequent to the wax forecut.

Standard curves were run in the 100- to 700-γ toxaphene range. Table I indicates recoveries observed using untreated pangolagrass extract and carried through entire cleanup and subsequent analytical steps.

Results

The toxaphene residue data obtained in these studies are indicated in Table II. Only insignificant differences in relative humidity and mean daily temperatures were recorded at the three locations. However, considerable rainfall occurred during the critical first 3-day period at locations A and B, whereas practically no rain fell at location C during this period. Locations A and C reported rather consistently heavy precipitation between the 3- and 14-day sampling dates, whereas at location B less than 0.5 inch of rainfall was recorded between the 3- and 7-day and the 14-day samplings.

The rather high initial residues at location C can probably be partially explained by the fact that the grass was considerably shorter than at the other two locations. Despite the initial high values, the final toxaphene residues were the lowest at this location. Contrary to the data from the other two locations, the Belle Glade initial toxaphene residues, resulting from the 10% dust treatment, were significantly higher than from the wettable powder or emulsifiable concentrate treatments. However, at the 3-day sampling, the location C samples had the highest residues following the wettable powder treatment. The 7- and 14-day samplings at Belle Glade resulted in the highest toxaphene residues following the emulsifiable concentrate treatments. At Bradenton and Gainesville the emulsifiable concentrate treatment resulted consistently in the highest toxaphene residues, followed closely by the wettable powder applica-

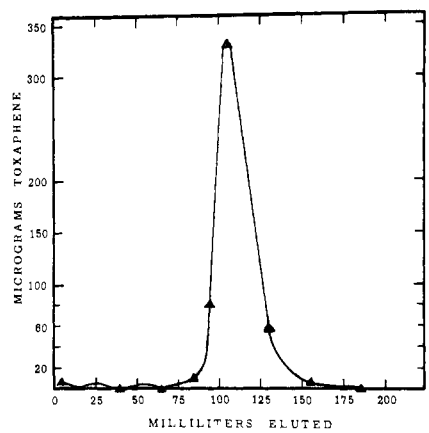

Figure 1. Toxaphene residue in hexane extract of pangolagrass

496 γ of toxaphene added to 100 ml. of extract
18-cm. column packed to 15-cm. height

Table I. Toxaphene Recovery from 100-Ml. Samples of Pangolagrass Hexane Extract

Toxaphene Added, γ	*Recovery, %*
100	100
200	102
300	95
400	97
500	98
600	97
700	103
	Av. 98.9

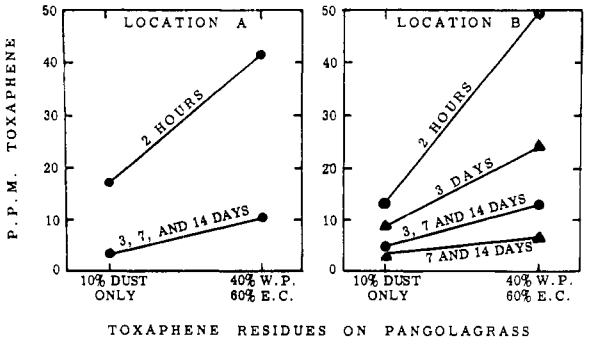

Figure 2. Sampling periods after application of toxaphene

Location A. Significant interaction. Sampling × formulation ($S_3 \times F_2$)
S_3. 2-hour sampling vs. 3-, 7-, and 14-day samplings combined
F_2. 10% dust formulation vs. 40 % W.P. and 60 % E.C.
Location B. Significant interactions. Sampling × formulation ($S_2 \times F_2$), ($S_3 \times F_2$)
S_2. 3-day vs. 7- and 14-day samplings
S_3. 2-hour vs. 3-, 7-, and 14-day samplings
F_2. 10 % dust vs. 40 % W.P. and 60 % E.C.

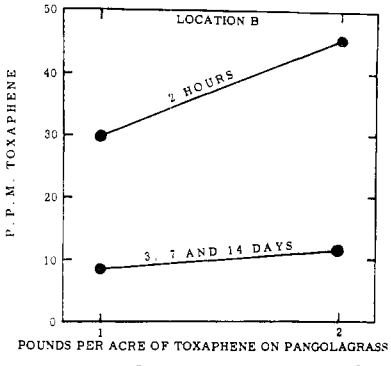

Figure 3. Sampling periods after application

Significant interactions. Sampling × level ($S_3 \times L$)
S_3. 2-hour vs. 3-, 7-, and 14-day samplings combined
L. Pounds per acre of technical insecticide applied

tion. The authors have no explanation for the apparent reversal of residue data for the 1- and 2-pound 10% dust treatment at location A.

Statistical Interpretations

Statistical analysis was performed on toxaphene residue data from identically designed field experiments at three widely separated areas of the state. Table III is an example of the analysis of variance of the Bradenton data, resulting in significant first-order interactions. The pooled error mean square (46 degrees of freedom) was used to test the significance of $S \times F \times L$ interaction. As this second-order interaction was significant at the 5% level, its mean square value was used to test the component first-order interactions for possible significance. The significant first-order interactions were used to test for possible significance of their component main effects. In all three experiments the main effect, S_3(2 hours *vs.* 3, 7, and 14 days), was highly significant. This is to be expected, as during the first few days after the last application of an insecticide, the toxicant usually disappears most rapidly. The only other main effect that showed consistent significance at all locations was F_2, a comparison of the 10% dust treatment *vs.* the 40% wettable powder and 60% emulsifiable concentrate treatments combined.

As it is somewhat difficult to explain a significant first-order interaction, graphical presentation simplifies interpretations. The less the degree of parallelism between the two lines being compared, the greater the significance of the interaction. The significant interaction ($S_3 \times F_2$) for location A is shown graphically in Figure 2. At the initial sampling (2 hours after application) the toxaphene residues due to the 10% dust treatment are significantly lower in comparison with the residues from treatment with the 40% wettable powder or 60% emulsifiable concentrate. However, the toxaphene residues on samples taken 3, 7, and 14 days after application from the 10% dust plots were much more comparable to those found on the grass following the other two formulations. In other words, the great dif-

Table II. Toxaphene Residues on Pangolagrass

(Expressed in parts per million and average of three field replications. Dosages expressed in active ingredient per 100 gallons per acre)

Test Location	R. H., %[a]	Temp., °F.[b]	Rainfall, Inches[c]	Sampling Dates[d]	10% Dust		40% W.P.		60% E.C.	
					1 lb.	2 lb.	1 lb.	2 lb.	1 lb.	2 lb.
Gainesville (Loc. A)				2 hours	19.9	13.9	22.7	52.3	35.7	55.4
	Min. 51 Max. 100	82.2	1.67	3 days	4.5	3.1	7.5	14.9	8.8	22.8
	Min. 56 Max. 100	79.6	1.23	7 days	3.4	2.4	5.4	10.9	7.5	18.2
	Min. 55 Max. 100	80.8	1.80	14 days	3.0	1.9	4.4	9.3	4.9	12.0
Bradenton (Loc. B)				2 hours	10.8	15.0	33.4	60.2	45.0	61.4
	Noon 70 Mid. 98	80.7	1.83	3 days	6.6	8.8	17.3	23.5	24.3	31.7
	Noon 81 Mid. 97	79.9	0.46	7 days	2.8	4.3	6.5	8.5	7.1	9.8
	Noon 74 Mid. 97	81.5	0.47	14 days	2.0	3.2	3.9	6.7	5.2	8.6
Belle Glade (Loc. C)				2 hours	193.1	277.2	86.0	166.2	103.3	138.0
	Min. 47 Max. 100	80.2	0.28	3 days	11.8	30.0	20.8	37.2	17.5	32.2
	Min. 50 Max. 100	80.6	1.51	7 days	3.9	8.9	4.6	11.4	10.6	14.9
	Min. 50 Max. 100	79.6	1.84	14 days	1.3	1.7	1.6	1.9	2.4	6.3

[a] Average daily relative humidity for interval between indicated and previous sampling date.
[b] Average daily temperature for interval between indicated and previous sampling date.
[c] Rainfall for period between indicated and previous sampling date.
[d] Time interval between field application and sampling date.

ference in residues between the 10% dust and the other formulations 2 hours after treatment was much less apparent on subsequent samplings following the insecticide application.

Also in Figure 2, two significant first-order interactions ($S_2 \times F_2$ and $S_3 \times F_2$) selected from location B experimental data are graphically illustrated. Both interactions also indicate that the large differences in toxaphene residues immediately after application resulting from the 10% dust and the other two formulations combined are decreased with each subsequent sampling. After 2 weeks of weathering, very little difference in residues was found among the three formulations. Graphically illustrated in Figure 3 is the significant interaction $S_3 \times L$ for location B. This interaction indicates that at the initial sampling (2 hours after application) the difference in toxaphene residues resulting from 1 pound of active ingredient per acre is significantly lower than from 2 pounds per acre. This difference became less apparent with each subsequent sampling date following the insecticide applications.

Table III. Analysis of Variance of Toxaphene Residues Remaining on Pangolagrass

(Location B, August 1958)

Variance Source	Degrees of Freedom	Sums of Squares	Mean Squares	F Value: Pooled	F Value: S × F × L	F Value: S × F
Blocks, B	2	85				
Samplings, S						
S_1	1	22	22			
S_2	1	2025	2025			N.S.[a]
S_3	1	10238	10238			20.1[b]
Formulations, F						
F_1	1	207	207			N.S.[a]
F_2	1	3807	3807			7.5[c]
$S \times F$	6	3059	510		12.7[b]	
$S_2 \times F_2$	1	421	421		10.5[b]	
$S_3 \times F_2$	1	2535	2535		63.2[b]	
Levels, L	1	743	743		18.5[b]	
$S \times L$	3	565	188		4.7[b]	
$S_3 \times L$	1	539	539		13.4[b]	
$F \times L$	2	165	83		N.S.[a]	
$S \times F \times L$	6	241	40	2.8[b]		
Error						
$S \times B$	6	72	12			
$F \times B$	4	78	19			
$S \times F \times B$	12	145	12			
$L \times B$	2	111	55			
$S \times L \times B$	6	41	7			
$F \times L \times B$	4	50	13			
$S \times F \times L \times B$	12	167	14			
Total	71					
Pooled error	46	663	14			

S_1 7 days *vs.* 14 days.
S_2 3 days *vs.* 7 and 14 days
S_3 2 hours *vs.* 3, 7, and 14 days
F_1 40% W.P. *vs.* 60% E.C.
F_2 10% dust *vs.* other formulations

[a] Not significant.
[b] Significance at 5% level.
[c] Significance at 1% level.

Discussion

A tolerance of 7.0 p.p.m. of toxaphene in beef cattle fat has been established by the Food and Drug Administration. In the three experiments reported here, the toxaphene residues following the highest dosage treatments were all below 20.0 p.p.m., 7 days after the grass had been sprayed or dusted. Two weeks after treatment, the highest toxaphene residues were 12 p.p.m. Allowing for the normal toxaphene breakdown pattern, toxaphene residues on the grass plots treated with the higher dosages should have been somewhat below 10 p.p.m. 3 weeks after treatment.

Normally, in Florida an insecticide is applied to pasture grass only once during a grazing period. A field may be grazed from 3 to 4 weeks to 3 to 4 months, depending on number of head grazed, weather, etc. Consequently, even following the 2 pounds of active toxaphene application per acre, grazing cattle would not be ingesting more than 10 p.p.m. in the grass after the 3-week interval subsequent to treatment. If cattle were allowed in a field treated with 2 pounds of active ingredient per acre 1 week after one toxaphene application, it could be estimated that during a subsequent 1-month grazing period the cattle would ingest in daily grass intake the following quantities of toxaphene:

Grazing Week	P.P.M.	Grazing Week	P.P.M.
1st	12–20	3rd	7–9
2nd	9–12	4th	5–7

Claborn *et al.* (*2, 3*), after extensive U.S. Department of Agriculture tests at Kerrville, Tex., presented data on toxaphene residues in the fatty tissues of steers and heifers. This study indicated that any feed containing less than 25 p.p.m. of toxaphene residue can be fed to beef cattle daily up to 10 consecutive weeks without exceeding the beef fat tolerance at slaughtering. Of the nine chlorinated hydrocarbon insecticides tested, toxaphene tended to be the least stored in the fatty tissues of cattle or sheep. Methoxychlor was the only chlorinated hydrocarbon tested that did not cause some detectable insecticide storage in the fat according to Carter *et al.* (*1*).

The insecticide residues in meat are present only in the fat of the meat. Because the edible meat of a medium fat carcass trimmed for retail trade contains approximately 20% fat, the parts per million of residues in edible meat may be obtained by dividing the results of the fat analysis by a factor of 5.

On the basis of residues remaining on three widely separated pangolagrass experiments in Florida and U. S. Department of Agriculture information on contaminated beef, a 7-day interval is recommended after a toxaphene application before the cattle are allowed to graze on the treated range. This interval should be more than sufficient, as no toxaphene application (up to 2 pounds of active ingredient per acre of three commercial formulations) resulted in residues over 20 p.p.m., 7 days after treatment.

Acknowledgment

The authors thank A. A. Chadwick, Hercules Powder Co., Wilmington, Del., for assistance with this project.

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Metabolism of the Systemic Insecticide *O,O*-Dimethyl 1-Carbomethoxy-1-propen-2-yl Phosphate (Phosdrin) in the Pea Plant

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This study was undertaken to define the degradation mechanism of the Phosdrin isomers in plants, because alkaline hydrolysis yields different degradation products with the two isomers. The two isomers were found to degrade in the same manner but at different rates in pea plants. By administering one metabolite, Phosdrin acid, to plants it was possible to recover a monodealkylated derivative. The enzymatic degradation of Phosdrin in pea plants differs from the alkaline hydrolysis in that at least two pathways are involved, the major one being via dimethyl phosphate directly.

A STUDY of the degradation of substituted vinyl phosphates which are insecticidal is usually complicated by the presence of geometrical isomers. In the case of some, such as *O,O*-dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (Phosdrin) (I), the complexity is increased by the presence of another functional group—an aliphatic ester, attached to the vinyl group. The first problem can be solved by separation of the geometrical isomers on chromatographic columns (*1*) or by liquid-liquid countercurrent extraction (*8*). Where trace amounts of metabolites are produced, labeling of the parent material is essential, while fractionation and identification of the metabolic products may be accomplished by ion exchange (*6*) or paper chromatography with inert material as confirmatory markers as outlined in this paper.

Phosdrin exhibits systemic properties, as it is translocated readily throughout the plant. It has a much shorter life in the plant than most systemic insecticides and yet is moderately stable in vitro. Its half life at pH 8 is 26 days (*7*), while in the pea plant it is less than 24 hours for the cis isomer and 2 days for the trans (*2*). Studies of alkaline hydrolysis indicate that the two isomers do not yield the same end products (*8*). Dimethyl phosphate (III), *O,O*-dimethyl 1-carboxyl-1-propen-2-yl phosphate (Phosdrin acid) (II), and monomethyl phosphate are obtained from the cis isomer in a ratio of approximately 6 to 3 to 1. The trans isomer yields primarily dimethyl phosphate and a small amount of monomethyl phosphate. During the hydrolysis *O*-methyl 1-carbomethoxy-1-propen-2-yl phosphate (desmethyl Phosdrin) (V) was identified as one of the intermediates.

Casida *et al.* (*2*) found two components from Phosdrin metabolism in pea plants—dimethyl phosphate and an unidentified component partitioning equally between water and chloroform. This latter fraction was postulated as being Phosdrin acid (II). They suggested that degradation of Phosdrin in the plant proceeded via this intermediate. In view of the varied pathways of Phosdrin degradation in alkali and the speculative nature of its breakdown in the pea plant, the present study was undertaken in an attempt to help solve the problem.

$(MeO)_2P(O)OC(Me)=CHCOOMe$ I, cis and trans

$(MeO)_2P(O)OC(Me)=CHCOOH$. II

$(MeO)_2P(O)OH$ III

$(MeO)(HO)P(O)OC(Me)=CHCOOH$ IV

$(MeO)(HO)P(O)OC(Me)=CHCOOMe$ V

Methods and Materials

Radioactive Phosdrin. Casida and coworkers have reported briefly the synthesis of P^{32}-labeled Phosdrin which in one case contained 27% of an impurity (*2*, *3*). The method described here, similarly based upon the commercial process of Stiles (*9*), is essentially the same chemically; however, considerable modification in technical detail has provided a significant improvement in yield and purity of the final product as well as of the intermediate, trimethyl phosphite. The difficulty in procuring these labeled materials in good yield would seem to justify a detailed outline of the procedure here.

Trimethyl Phosphite-P^{32}. Pure phosphorus trichloride (approximately 1 gram) was sealed in a 9 × 30 mm. (outside diameter) quartz capsule and irradiated (by Atomic Energy of Canada, Ltd.) for 4 days at 6.6×10^{12} neutrons/sq. cm/sec. to attain a theoretical activity of 20 mc. The radioactive phosphorus trichloride was then transferred in vacuo into a flask containing 3 ml. of nonactive phosphorus trichloride, and the total was again distilled via the vacuum manifold. It was then redistilled at atmospheric pressure through a 15-cm. all-glass column into a weighed dropping funnel. The weight collected was 4.98 grams (36.2 mmoles).

In a 250-ml. three-necked flask, fitted with a wire stirrer, were placed 100 ml. of dry, peroxide-free ether, 4.4 ml. (109 mmoles) of methanol, and 18.3 ml. (114 mmoles) of *N,N*-diethylaniline. The solution was maintained at 0° to 5° C. with ice water and, under anhydrous conditions, phosphorus trichloride was added dropwise with vigorous stirring. On completion, the heavy suspension was kept stirred for an additional hour at room temperature; the flask was then stored in the cold while the precipitate settled.

The diethylaniline hydrochloride was filtered off and washed with three 25-ml. portions of cold ether. Most of the ether was then distilled away from the filtrate, using a 15-cm. column and holding the distillation temperature at 35° C. or less. When the volume was reduced to 8 to 10 ml., the column was replaced by a trap cooled in dry ice–acetone. At a moderate vacuum (12 mm.) at room temperature, the residual ether and all of the trimethyl phosphite soon condensed in the trap, leaving the excess diethylaniline and residual hydrochloride in the flask. The ether–trimethyl phosphite solution (5 to 8 ml.) was fractionated through a 15-cm.

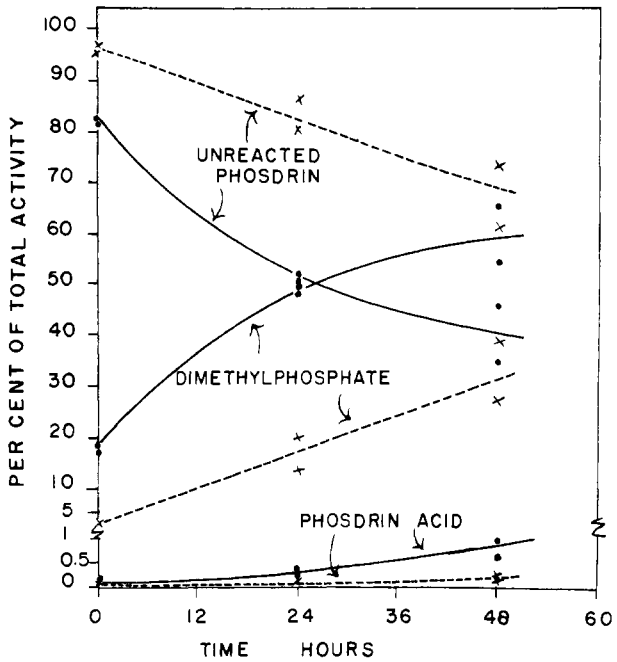

Figure 1. Pea metabolites of *cis*- and *trans*-Phosdrin

● cis isomer and metabolites
× trans isomer and metabolites

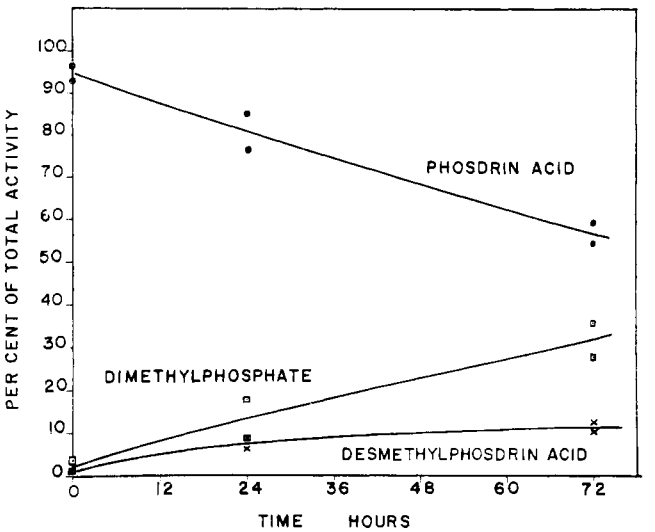

Figure 2. Pea metabolites of *cis*-Phosdrin acid

column at atmospheric pressure, with 1 ml. of bromobenzene as a chaser. The product weighed 3.85 grams (85%) (boiling point 110–112°, n_D^{24} 1.4067). The solvent and all reagents were carefully purified, dried, and distilled immediately prior to use.

Phosdrin-P^{32}. To the trimethyl phosphite, with vigorous stirring, was added 5.5 grams (36.5 mmoles) of methyl 2-chloroacetoacetate. The mixture was stirred and kept at 80° to 90° for 2 hours, while a quantitative yield of methyl chloride distilled off. The residue was fractionated through a 15-cm. column at reduced pressure. The product weighed 4.46 grams (55% based upon phosphorus trichloride) (boiling point 155–58°/10 mm., n_D^{24} 1.4492).

The material was resolved into its cis and trans isomers by countercurrent distribution, with water and ether as solvents (*8*). Each isomer was recovered and washed with 5% aqueous sodium carbonate to remove dimethyl phosphate, then extracted with chloroform and dried over sodium sulfate. On evaporation of the filtered solutions the cis and trans isomers were recovered. Paper chromatograms showed the materials to be radiochemically pure; the activity of the isomers was about 1.5×10^8 counts per minute per millimole, recorded with an end-window counter and corrected for decay and background.

***cis*-Phosdrin Acid-P^{32}.** Radioactive *cis*-Phosdrin acid was prepared by treating the radioactive Phosdrin with sodium carbonate as outlined for nonradioactive material (*8*).

Metabolic Experiments. Alaska variety pea plants about 4 inches high, grown in gravel, were placed in two replicates of five plants each in 0.1% aqueous solution of the labeled material for 15 hours and then transferred to water. At three time intervals two replicates were taken, cut into small pieces, and macerated in an Omni-Mixer with 5 ml. of water. The homogenate was filtered through glass wool and 5 times the volume of acetone was added. After coagulation of the precipitate the mixture was filtered through filter paper and the filtrate concentrated to 5 ml. The residue from the plants and the acetone precipitate were discarded. The unreacted Phosdrin was removed by extraction three times with chloroform. As the R_f of Phosdrin acid is rather close to that of dimethyl phosphate and desmethyl Phosdrin (*8*), Phosdrin acid was effectively removed by acidifying the aqueous residue to less than pH 1, followed by extracting six times with chloroform. Aliquots of the three fractions were then chromatographed on Whatman No. 1 paper using ascending chromatography with isopropyl alcohol–water–ammonium hydroxide (specific gravity 0.90) (75:24:1) as the developing solvent in preference to that used previously (*8*). Because extractable material in some cases affected the R_f values, sufficient nonradioactive material of the possible degradation products of Phosdrin was applied along with the radioactive fractions in quantities that could be located by use of the molybdate spray (*5*). The developed chromatograms were then scanned with an end-window Geiger tube and any concentrated radioactivity was identified from the nonradioactive markers. The radioactive spots were cut out and the activity was counted using a scaler.

The experiment with Phosdrin acid was carried out as in the isomer study, except that the last sampling was done at 72 instead of 48 hours.

Results and Discussion

The rates of degradation of the cis and trans isomers of Phosdrin and the appearance of the significant degradation products over a 48-hour period are shown in Figure 1, where each radioactive component is plotted as a percentage of the total activity of the components isolated. The faster rate of decomposition of the cis isomer is in agreement with previous work (*2*). Although dimethyl phosphate is the chief end product from both isomers, a very small but significant amount of Phosdrin acid was identified. This is in contrast to a much larger amount from alkaline hydrolysis of *cis*-Phosdrin (*8*). No significant amount of desmethyl Phosdrin (V) or desmethyl Phosdrin acid (IV) could be detected from either isomer.

When *cis*-Phosdrin acid was metabolized by pea plants, the degradation was not as rapid as that of *cis*-Phosdrin but was comparable to that of the trans isomer, as shown by a comparison of the rates in Figures 1 and 2. However, in addition to the main hydrolysis product, dimethyl phosphate, desmethyl Phosdrin acid (IV) was also identified. Failure to detect any from the degradation of *cis*- or *trans*-Phosdrin is presumably due to the insignificant amount that would be formed from the very small amount of Phosdrin acid produced.

Casida *et al.* (*2*) suggested that

Phosdrin acid is the first step in the degradation of Phosdrin in plants, followed by subsequent cleavage to dimethyl phosphate. The results shown in Figure 1 might support this hypothesis. However, those in Figure 2 cast doubt on the single pathway. If Phosdrin acid were an essential step in Phosdrin degradation in the plant, desmethyl Phosdrin acid should have been isolated in Phosdrin degradation and been present in the same proportion to dimethyl phosphate as indicated in Figure 2. Since none was detected, there are at least two pathways for Phosdrin degradation, the main one being directly to dimethyl phosphate and the other via Phosdrin acid.

Monodealkylation of organophosphorus insecticides by plants had not been reported until recently. Under conditions of alkaline hydrolysis some occurred with *cis*-Phosdrin (*8*), while in the plant some was shown with Dimethoate (*4*). Although no desmethyl Phosdrin was found in this study, some monodealkylation was shown with the identification of desmethyl Phosdrin acid (IV) from the metabolism of Phosdrin acid as shown in Figure 2.

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HERBICIDE UPTAKE FROM SOILS

Uptake of Radioactive Ethyl-*N*,*N*-di-*n*-propylthio carbamate (EPTC-S^{35}) and Translocation of Sulfur-35 in Various Crops

S. C. FANG and PATRICIA THEISEN

Department of Agricultural Chemistry, Oregon State College, Corvallis, Ore.

A study of the absorption of radioactive EPTC by crops in pre-emergence application indicated an uptake of this chemical from soil. By use of radioautographic technique the differences in accumulation patterns of sulfur-35 from EPTC-S^{35} among crops were demonstrated; above-ground portions of beans, peas, and corn contained slightly more sulfur-35 than the roots, while above-ground portions of radishes, carrots, and other plants contained 70 to 94% of the sulfur-35 from the absorbed EPTC-S^{35}. Total absorption by individual crops at various stages of growth was determined. Generally, an increase in applied EPTC-S^{35} increased absorption, but not in proportion to the increase in rate of application.

AN EARLIER STUDY (*1*) showed that less than 3% of the ethyl-*N*,*N*-di-*n*-propylthiolcarbamate (EPTC) absorbed from soils was left as a residue in a variety of plant tissues. This investigation has been extended to include the total uptake of radioactive EPTC and the distribution of sulfur-35 by various crops, when a pre-emergence application of EPTC-S^{35} is made to soil.

Experimental

The crops used were kidney beans, sweet corn, garden peas, radishes, carrots, cabbage, mustard, swiss chard, table beets, Mung beans, and cucumbers. Beans, peas, and corn were planted in

Table I. Absorption of EPTC-S^{35} from Soil and Translocation of Sulfur-35 by Kidney Bean Plants

Appl. Rate, Lb./Acre	Interval,[a] Days	No. of Plants	Accumulation of S^{35}, %					Total EPTC-S^{35} Absorption, γ/Plant
			Root	Stem	First leaf	Trifoliate	Flower and pod	
1	10	5	48	26	26	..	..	5.0
	17	5	33	32	35	..	..	7.2
	24	5	53	22	16	8	..	11.6
	38	5	48	22	19	8	3	15.7
	52	5	42	14	14	19	11	22.4
4	10	5	35	33	32	..	..	18.8
	17	5	33	40	27	..	..	16.7
	24	5	38	32	30	..	..	33.2
	38	5	39	29	26	4	3	40.7

[a] Interval between treatment and harvest.

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	52	5	42	14	14	19	11	22.4
4	10	5	35	33	32	..	..	18.8
	17	5	33	40	27	..	..	16.7
	24	5	38	32	30	..	..	33.2
	38	5	39	29	26	4	3	40.7

[a] Interval between treatment and harvest.

separate flats. A total of 72 seeds from each crop was sown. The others were planted two crops per flat in three rows each. Newberg sandy loam was used throughout this experiment. One day after the seeds were planted, all flats received a pre-emergence application of EPTC-S^{35} at a rate of either 1 or 4 pounds per acre (*1*).

The plants were harvested at various intervals after treatment, as indicated in the tables. One plant from each crop was used for making a radioautograph in order to study the accumulation pattern of sulfur-35 at various stages of growth. The other plant samples were dried immediately in a vacuum oven at 60° C. and were ground. The radioactivity of a weighed sample was determined and corrected for self-absorption as well as for half life of sulfur-35. The total absorption of EPTC-S^{35} by the plant was then evaluated from the total radioactivity of the plant tissue and the activity of a known amount of EPTC-S^{35}, after impregnation into a sample of weighed ground tissue (*2*).

Results and Discussion

Table I presents the per cent accumulation of sulfur-35 in the various plant parts of the kidney bean, and the total absorption of EPTC in bean plants at various stages of growth. The growth of bean plants which had been treated with 1 pound per acre of EPTC-S^{35} appeared to be normal but growth was slightly inhibited in those treated with 4 pounds per acre. Individual plants, which received the 4-pound-per-acre treatment, absorbed from two to four times more EPTC than those treated with 1 pound per acre.

EPTC-S^{35} was readily absorbed from the soil and translocated throughout the entire plant. This is indicated also in Figure 1, *A*, which is the radioautograph of a bean plant harvested 10 days after application. Absorption of EPTC-S^{35} was continuous throughout the 52-day experimental period. The accumulation of sulfur-35 was highest in the roots at any stage of growth.

Five bean plants treated with 1 pound per acre of EPTC-S^{35} were allowed to grow for 66 days, and the pods were harvested. Determination of radioactivity in both shells and seeds indicated the presence of sulfur-35 in a concentration equivalent to 1.1 and 1.0 γ per 100-mg. dry weight of shells and seeds, respectively. However, these samples were submitted to radiochemical determination (*1*) and were shown to contain no EPTC-S^{35} residue.

The total absorption of EPTC-S^{35} by various crops at various stages of growth is presented in Table II as well as the per cent accumulation of sulfur-35 in the roots and tops. EPTC-S^{35} was translocated readily in all plants. In the pea plant, the sulfur-35 concentration was slightly higher in the tops than in the roots at any stage of growth. The early growth of pea plants was definitely inhibited by treatment with this chemical.

During the first 24 days, pea plants seemed to absorb EPTC-S^{35} continuously from the soil at either rate of treatment. In the last 14-day period, no significant additional amount was taken up from the soil, although a considerable increase in weight of the tops of pea plants was noted. This phenomenon was very different from that observed in bean plants and may reflect the degree of sensitivity of these two crops to EPTC treatment. The detailed accumulation pattern of sulfur-35 from EPTC-S^{35}–treated pea plants is shown in Figure 1, *B*.

The higher rate of EPTC-S^{35} application (4 pounds per acre) did not cause a decrease in the germination or growth rates of corn plants. On the contrary, the

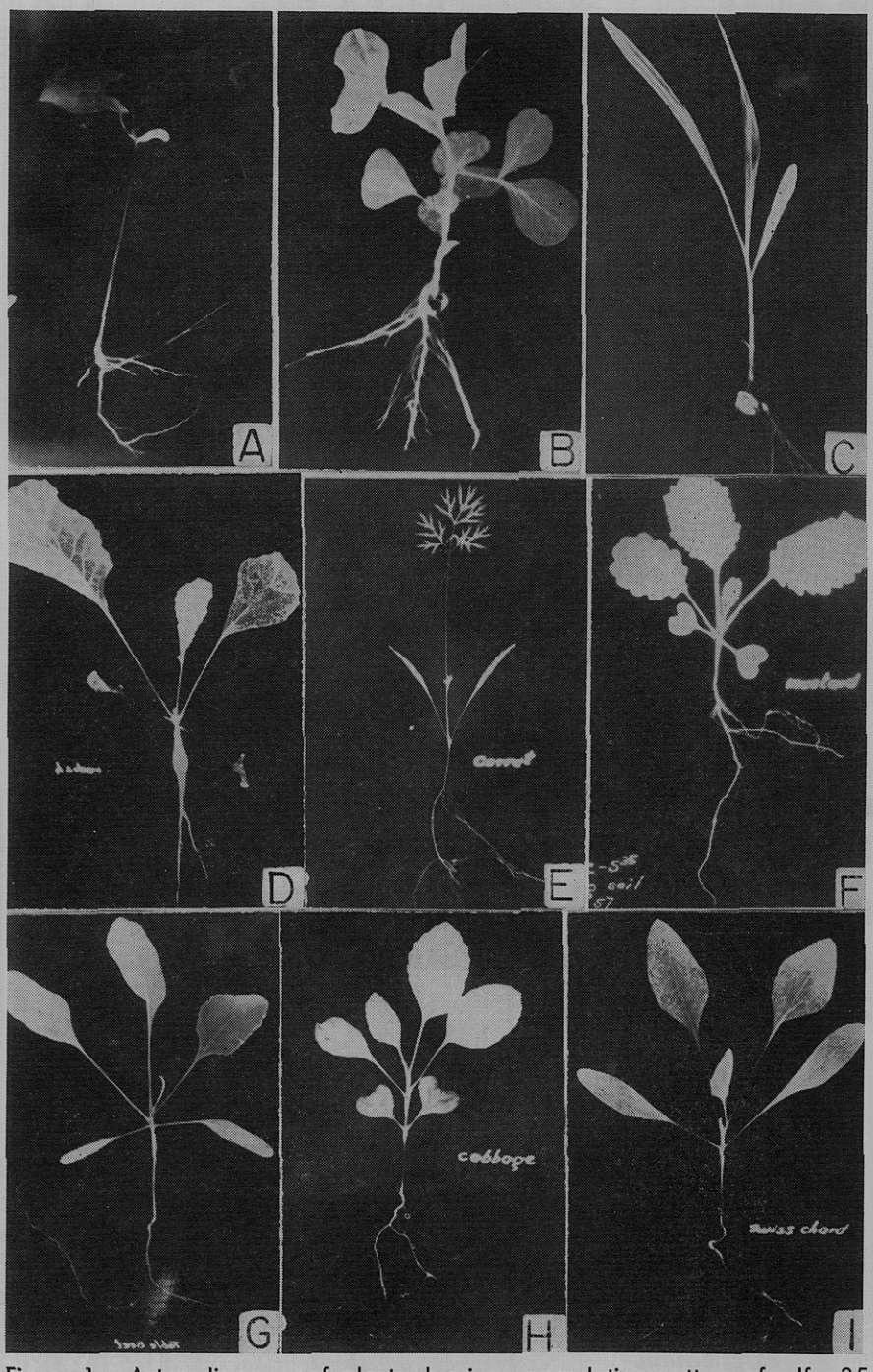

Figure 1. Autoradiograms of plants showing accumulation pattern of sulfur-35 after pre-emergence treatment of EPTC-S^{35} in soil at a rate of 1 pound per acre

A. Kidney bean plant, 10 days old
B. Pea plant, 17 days old
C. Corn plant, 17 days old
D. Radish plant, 24 days old
E. Carrot plant, 24 days old
F. Mustard plant, 24 days old
G. Table beet plant, 38 days old
H. Cabbage plant, 24 days old
I. Swiss chard plant, 24 days old

Table II. Absorption of EPTC-S^{35} from Soil and Translocation of Sulfur-35 by Various Crops

Appl. Rate, Lb./Acre	Interval,[a] Days	No. of Plants	Accumulation of S^{35}, %		Total EPTC-S^{35} Absorption, γ/Plant
			Roots	Tops	
		PEA			
1	10	5	45	55	2.2
	17	5	43	57	4.2
	24	5	46	54	6.5
	38	4	39	61	6.1
4	10	5	37	63	5.4
	17	5	41	59	9.6
	24	5	51	49	15.6
	38	5	27	73	16.1
		CORN			
1	10	5	46	54	1.3
	17	5	45	55	3.3
	24	5	40	60	4.2
	38	4	28	72	11.7
4	10	5	43	57	4.4
	17	5	26	74	5.3
	24	5	37	63	6.8
	38	5	28	72	32.7
		RADISH			
1	10	6	8	92	1.3
	17	6	..	..	1.3
	24	6	6	94	3.3
	38	6	21	79	6.7
	52	4	27	73	8.6
4	10	10	8	92	1.2
	17	12	8	92	1.3
	24	10	12	88	1.7
	38	10	8	92	2.5
		CARROT			
1	17	10	..	..	0.1
	24	10	..	..	0.2
	38	10	..	..	0.4
	52	6	11	89	0.9
4	17	24	19	81	0.2
	24	28	11	89	0.3
	38	40	13	87	0.9
	66	20	18	82	2.5
		CABBAGE			
1	17	4	..	..	1.5
	24	4	11	89	4.4
	38	4	8	92	6.0
	52	4	12	88	5.9
		MUSTARD			
	17	4	..	..	0.5
	24	4	..	..	2.4
	38	4	..	..	2.1
	54	6	8	92	4.9
		SWISS CHARD			
	38	4	20	80	3.0
	54	4	29	71	1.7
		TABLE BEETS			
	54	6	..	..	1.4
4	38	9	28	72	4.0
		CUCUMBER			
	10	5	17	83	1.2
	17	5	14	86	2.2
	24	5	12	88	3.3
	38	5	10	90	5.0
		MUNG BEAN			
	10	10	18	82	5.6
	17	10	20	80	5.1

[a] Interval between treatment and harvest.

growth of plants receiving 4 pounds per acre of EPTC treatment was apparently slightly stimulated. With both rates of treatment, the absorption of EPTC-S^{35} was slow during the first 24-day period but increased immensely during the last two weeks of the experiment. The absorbed EPTC-S^{35} was translocated throughout the entire plants, the tops having a slightly higher percentage of sulfur-35 than the roots. Figure 1, *C*, is the radioautograph of an EPTC-S^{35}–treated corn plant showing the accumulation pattern of sulfur-35.

Two corn plants from the flat which received the 1-pound-per-acre treatment were allowed to grow to maturity. Two corn ears, weighing a total of 69 grams, were harvested after 114 days and tested for radioactivity as well as for EPTC-S^{35} residue. The kernel, the cob, the sheath, and the tassel contained amounts of radioactivity equivalent to 0.45, 0.41, and 0.47 γ of EPTC-S^{35} per 100-mg. dry weight, respectively. This suggested that the sulfur atom from EPTC was evenly distributed throughout the ears. Further determination for EPTC-S^{35} showed the absence of residue.

In radish plants, about 90% of the absorbed EPTC-S^{35} accumulated in the above-ground portion. This distribution differs distinctly from that noted in bean, pea, and corn plants. The absorption of this compound by radish plants which received the 4-pound-per-acre treatment was not greater than the absorption by those receiving a lower rate of treatment. The early growth of radishes in the 4-pound-per-acre flat appeared to be stunted by the EPTC treatment. It is not known at this stage of investigation whether or not the absorption system of radish roots is completely or temporarily impaired by higher concentrations of EPTC in soil. From the increased absorption observed in the 1-pound-per-acre flat, it is possible to assume that at higher concentrations the EPTC-S^{35} would have impaired the absorption system. However, if the plants had been allowed time to recover, further continual absorption might have taken place. Figure 1, *D*, is the radioautograph of a radish plant showing the accumulation pattern of sulfur-35. Two radish plants from the 4-pound-per-acre flat were allowed to grow to maturity. After 55 days, they were harvested and submitted to radiochemical analyses for EPTC residue. The results showed the absence of a detectable amount of EPTC-S^{35} in the roots and the presence of 0.51 γ of EPTC from 21.6 grams of fresh radish leaves.

The accumulation of sulfur-35 in the carrot plant was similar to that in the radish. Eighty to 90% of the absorbed sulfur-35 was found in the foliage rather than in the root system. The data suggest definitely that carrot plants absorb EPTC continuously from the soil, although the amount absorbed by each plant is rather small at the early stage. Figure 1, *E*, is the radioautograph of an EPTC-S^{35}–treated carrot plant, harvested 24 days after treatment, showing the detailed accumulation of radiochemical.

Miscellaneous Crops. Cabbage, mustard, Swiss chard, cucumber, table

beet, and Mung bean analyses also indicated that EPTC-S^{35} was taken up readily from soil and translocated throughout the entire plant, with most of the sulfur-35 accumulating in the tops in all plants. The radioautographs of these vegetables are shown in Figure 1, *F* to *I*.

Literature Cited

(1) Fang, S. C., Theisen, Patricia, J. AGR. FOOD CHEM. **7**, 770 (1959).
(2) Mitchell, J. W., Linder, P. J., *Bot. Gaz.* **112**, 126–9 (1950).

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HERBICIDAL ACTIVITY

Molecular Size *vs.* Herbicidal Activity of Anilides

C. W. HUFFMAN[1] and S.E. ALLEN[2]

Lion Oil Co., A Division of Monsanto Chemical Co., El Dorado, Ark., and Midwest Research Institute, Kansas City, Mo.

The search for better herbicides would be facilitated by some guiding principle. Molecular size was found to be very helpful. A number of the most effective herbicides were shown to have two common dimensions. Anilides were powerful foliage toxicants and the most active anilides possessed the two desired dimensions. Substitution of the phenyl ring also had a very marked effect on phytotoxicity. Maximum toxicity of anilides was associated with 3,4-chlorination of the phenyl ring. A chlorine atom in the 2 position greatly inhibited activity. Thioanilides were as active as anilides.

PREVIOUS INVESTIGATORS have made a number of attempts to correlate molecular size with physiological activity Success has been limited to a few which relate activity to a single dimension. Maximum hormonal activity of diethylstilbestrol and related compounds was associated with a specific long dimension (*7*, *10*, *16*). On a similar basis it has been postulated (*14*) that the high insecticidal activity of the gamma isomer of hexachlorocyclohexane is due to its being the only isomer small enough to enter an insect cell membrane interspace of 8.5 A.

A number of the most active herbicides known at the present time were found to have two common dimensions. This may be the first time that biological activity has been correlated with two dimensions of a number of molecules.

An excellent tabulation of structure *vs.* herbicidal activity was given by Shaw and Swanson (*17*). We measured the dimensions of Fisher-Hirschfelder-Taylor models of the compounds listed. Two dimensions were necessary for most carbamates to have high herbicidal activity: 13 and 10 A. A third dimension could not be found which could be correlated with herbicidal activity.

Measurement of models of several of the most active herbicides being marketed showed them to have about the same 13-A. extended dimension as the highly active carbamates. The collapsed dimension was 10 A. for carbamates and phenoxyacetic acids (*19*) and 11 A. for ureas and phenols. Table I records the structures and dimensions of models of these four herbicides. Each represents a different type of molecule, but all contain a phenyl group. It was assumed that esters of 2,4-D hydrolyze to the free acid; thus measurement of models of the various esters was not warranted.

Therefore the desired dimensions are: Extended 13 A. Collapsed 10 or 11 A.

Some active herbicides do not have the dimensions described. Several smaller compounds (about 10-A. maximum length) have high herbicidal activity. These include the α-chloroacetamides (*8*), 3-amino-1,2,4-triazole (*1*), and pentachlorophenol. Possibly different mechanisms of herbicidal action are operative with molecules which do not have the same dimensions.

The desired size concept was of value in the selection of the compounds to be synthesized. A preliminary seed-germination test had shown that most of the compounds with the desired dimensions

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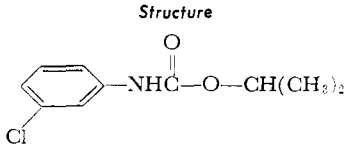
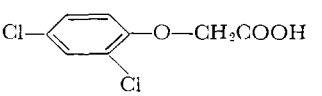
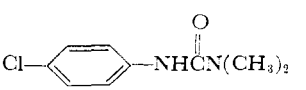
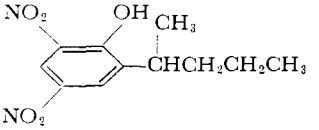
CIPC		13.2	9.8
2,4-D		12.9	10.2
CMU		13.1	10.8
DNAP		13.5	11.0

[1] Present address, Central Research Laboratories, International Minerals and Chemical Corp., Skokie, Ill.
[2] Research Center, U. S. Rubber Co., Wayne, N. J.

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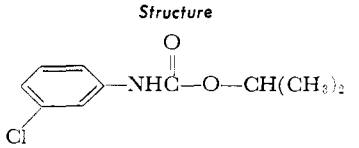
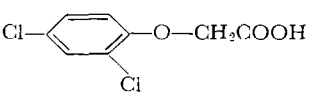
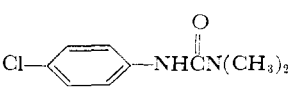
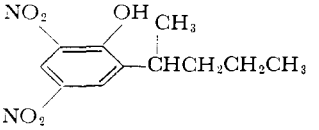
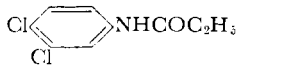
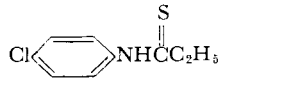
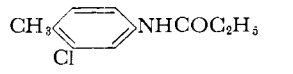
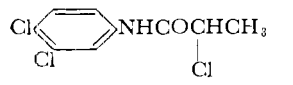
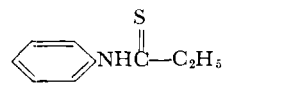
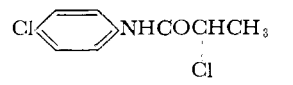
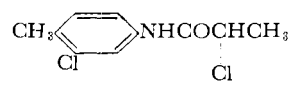
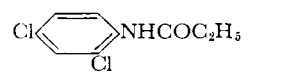
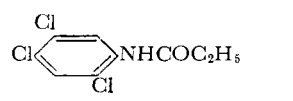
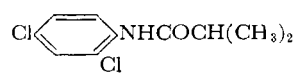
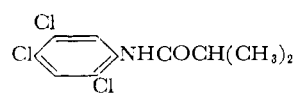
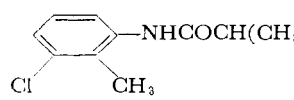
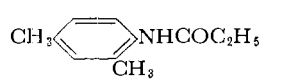
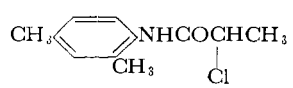
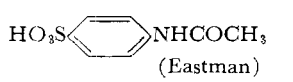
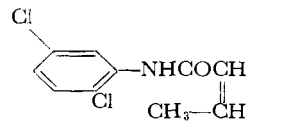
CIPC		13.2	9.8
2,4-D		12.9	10.2
CMU		13.1	10.8
DNAP		13.5	11.0

[1] Present address, Central Research Laboratories, International Minerals and Chemical Corp., Skokie, Ill.
[2] Research Center, U. S. Rubber Co., Wayne, N. J.

Table II. Effect of Substituents in Anilides with the Desired Dimensions

Compound No.	Structure	Size, A. Extended	Size, A. Collapsed	Activity (8 Lb./Acre) Pre-emergence	Activity (8 Lb./Acre) Post-emergence
1		13.1	10.5	1	4
2		13.1	10.3	1	4
3		13.1	10.8	1	3
4		13.0	10.2	0	3
5		13.3	10.4	0	3
6		13.2	10.2	0	3
7		13.4	11.0	1	2
8		12.8	10.0	0	2
9		13.4	11.0	1	2
10		13.2	10.5	0	1
11		13.1	10.3	0	1
12		12.9	10.4	0	1
13		13.1	10.5	0	0
14		13.0	10.5	0	0
15		13.0	9.8	0	0
16		13.2	10.5	0	0
17		13.3	10.4	0	0
18	 (Eastman)	13.2	10.5	0	...
19		12.8	9.8	0	0
20[a]		13.0	9.7	0	0

[a] Both isomers tested.

were active. This test was too sensitive. Further greenhouse tests showed that several types of compounds were active. An illustration of the desired size concept is given in this article, using some of the data obtained from a study of anilides. Little is available (*8, 17*) on the herbicidal activity of anilides. It should be obvious that the desired size concept would reduce the number of compounds to be tested. Nevertheless, many anilides with dimensions outside the desired range were tested to confirm the desired size concept.

Measurement of Atomic Models

Prior to synthesis, a model of each compound was constructed with Fisher-Hirschfelder-Taylor atomic models (scale: 1 cm. = 1 A.). The longest possible extended dimension of the model was measured with 30-cm. calipers (No. 122M, Starrett Co., Athol, Mass.), then the model was compressed to its smallest size and the maximum collapsed dimension was measured. The first step in the application of the desired size concept was the preparation of anilides with the desired dimensions.

Greenhouse Experiments

Formulation. Compounds were prepared for testing, by solution in an appropriate solvent (dimethylformamide or methyl isobutyl ketone), followed by dispersion in water with aid of an emulsifier (Agrimul GM or C). By preparing the emulsions in suitable volume and concentration, all treatments could be applied at a rate of 8 pounds per 100 gallons per acre. Testing of blank formulations showed no toxicity in any treatment.

Seeds Used

Corn, *Zea mays*, variety U-S-13
Cotton, *Gossypium sp.*
Beans, *Phaseolus vulgaris*, variety top crop
Rye grass, *Lolium perenne*
Bindweed, *Convolvulus arvensis*
Morning glory, *Ipomea purpurea*
Mustard, *Brassica kaber*
Pigweed, *Amaranthus retroflexus*

Pre-emergence Test. The seeds were planted in soil in 1 × 2 foot flats. Within 48 hours after planting, the soil surface was sprayed with the test compounds. All treatments were duplicated. Results were evaluated by observation, emergence counts, photography, and estimates of stand and yield at termination of the tests (usually 6 to 8 weeks after planting). Based on these results, a toxicity rating was assigned.

Postemergence Test. This test was conducted as above, but the plants were not treated until 3 to 4 weeks after emergence, at which time they were in

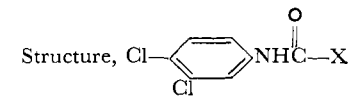
approximately the following stage:

Corn	8 to 12 inches tall, 4 to 5 leaves
Cotton	2 to 4 inches tall, 3 leaves
Beans	6 to 10 inches tall, 2 to 3 trifoliate leaves
Rye grass	4 to 6 inches tall
Bindweed	2 to 3 inches tall, 3 leaves
Pigweed	3 to 6 inches tall

Toxicity Rating System. To present the results with minimum confusion and facilitate a rapid comparison of compounds, the following rating system was established:

0. Inactive, no effect on any crop plant.
1. Low activity, injury to sensitive crops, with little or no effect on resistant plants.
2. Moderate activity, lethal to sensitive crops, with moderate injury or no effect on resistant plants.
3. High activity, lethal to sensitive crops, moderate to severe injury to resistant plants.
4. Maximum activity, lethal to all plants.

Table III. Molecular Size *vs.* Herbicidal Activity of 3,4-Dichloroanilides

Structure, 

Compound No.	X	Size, A. Extended	Size, A. Collapsed	Activity (8 Lb./Acre) Pre-emergence	Activity (8 Lb./Acre) Post-emergence
21	—H (*9*)	11.0	10.4	0	3
22	—CH_3	11.5	10.3	1	3
2	—C_2H_5	13.1	10.3	1	4
1	—$CH(CH_3)_2$	13.1	10.5	1	4
7	—$CHCH_3$ | Cl	13.4	11.0	1	2
23	—$CH(C_2H_5)_2$	14.0	10.4	0	1
24	—$CH_2CH(CH_3)_2$	14.1	10.3	0	3
25	—$(CH_2)_3CH_3$	14.5	10.2	1	3
26	—$(CH_2)_4CH_3$	15.2	10.3	0	3
27	—$(CH_2)_5CH_3$	18.0	10.3	0	1
28	—$(CH_2)_{10}CH_3$	22.4	14.5	0	0

Results. The foliage effects produced by anilides usually begin to develop within 24 hours after treatment and gradually increase in severity for about 7 days. If plants are not killed within a week, slow recovery starts with a minimum of physiological effects, as indicated by the lack of appreciable malformation of newly formed tissue. As might be expected of poisons primarily contact in nature, meristematic regions are most sensitive. Plants treated with sublethal doses are characterized by rapid development of marginal leaf burning, followed within 48 hours by the appearance of chlorotic areas in less severely damaged tissues. Young corn plants sprayed from above often deteriorate near the ground level because of toxic material entering the opening whorl of leaves and touching the meristem below.

Table II lists anilides with the desired dimensions and in the order of their decreasing herbicidal activity.

Table II shows that maximum activity was obtained with 3,4-dichloroanilides (compounds 1 and 2). Replacement of the acyl oxygen by sulfur did not significantly alter the activity (No. 4 *vs.* No. 5). A sharp decrease in activity resulted from a 2-chloro (Nos. 11 and 12) substituent. Many compounds with 2-chloro atoms were inactive (Nos. 13, 14, and 20). A 2-methyl group also caused inactivation (Nos. 15, 16, and 17). A chlorine in the acyl group decreased postemergence activity (No. 7 *vs.* No. 2, No. 9 *vs.* No. 4, No. 10 *vs.* No. 6), but not pre-emergence activity.

Evidence that the desired size concept was valid for 3,4-dichloroanilides is given in Table III. These anilides are listed in accordance with their increasing extended size. It was gratifying to predict that the two 3,4-dichloroanilides with the desired dimensions (Nos. 1 and 2) would show the greatest herbicidal activity. Other variously substituted anilides (not shown) which did not have the desired dimensions had little or no activity.

Table IV records the foliage data obtained with specific plants and a dosage of 8 pounds per acre. These results were the basis for the herbicidal ratings given in Tables II and III. The selectivity of the various compounds as well as the degree of herbicidal activity is apparent from the data. The maximum figure, 1200, signified no activity. A low total shows high activity. Consequently, the value of 15 for compound 2 (3,4-dichloropropionanilide) represents a very high activity, or 4 on the toxicity rating system. CIPC is normally used as a pre-emergence herbicide. Its activity in postemergence treatments was not high.

Preparation of Compounds

Melting points (uncorrected) were taken with Anschütz thermometers.

The compounds listed in Table V were prepared by the procedure described for the preparation of 2,3′,4′-trichloropropionanilide. In a few cases the product was obtained from the reaction mixture by precipitation with petroleum ether; in others it was necessary to remove the solvent by distillation. Details are given for the preparation of *p*-chlorothiopropionanilide.

2,3′,4′-Trichloropropionanilide (No. 7). Benzene (50 ml.) and triethylamine (15.2 grams, 0.15 mole) were heated to boiling in a flask equipped with a stirrer, to dissolve technical 3,4-dichloroaniline (24.3 grams, 0.15 mole). A condenser, dropping funnel, and drying tube were installed. The heating mantle was removed, and α-chloropropionyl chloride (19.0 grams, 0.15 mole) was added dropwise to the black solution, with cooling, over a 15-minute period. The mixture was refluxed 1 hour and then cooled to room temperature. Water (50 ml.) was added to dissolve the crystalline triethylamine hydrochloride. About 1 hour later, the light tan product was removed by filtration and washed with water (20 ml.). The dry crude material weighed 36.2 grams (yield 96%) and melted at 124–9° C. with sintering at 110° C. A sample was recrystallized from benzene (charcoal). The white crystals had a melting point of 132.6–3.8° C.

***p*-Chlorothiopropionanilide** (No. 5). Beilenson and Hamer's (*2*) procedure for thioanilides was employed. Xylene (125 ml.) and *p*-chloropropionanilide (18.4 grams, 0.1 mole, m.p. 141° C.) were stirred on a steam bath. Phosphorus pentasulfide (11 grams, 0.5 mole) was added to the colorless solution. A lump formed in the reddish suspension, which was crushed after the reaction had proceeded about 15 minutes. Stirring was resumed for 1 hour. A hot filtration removed orange solid, which was washed with 3 × 50 ml. of hot xylene. The combined filtrate and washes were stored overnight in the icebox. Additional sticky yellow solid was removed by filtration. Petroleum ether (200 ml.) was added to the yellow xylene filtrate, and the mixture was allowed to cool overnight. The first crop of crude product was collected by filtration and weighed 6.1 grams (30% yield). Concentration of the filtrate under vacuum gave 10.4 grams (52%) of crude second crop material as a dark oil. The first crop material (6.1 grams) was heated with cyclohexane (300 ml.) and the cyclohexane solution was decanted from an insoluble orange oil and treated with Nuchar C. The filtrate was cooled, and the product (4.7 grams) was collected by filtration (m.p. 78–80° C.). A second recrystallization of a sample (1 gram) from cyclo-

Table IV. Postemergence Stand and Yield Results at 8 Pounds per Acre

Compound No.		Broadleaf[a]		Pigweed		Rye Grass		Snap Beans		Corn		Cotton		Total
		% S[b]	% Y[b]	% S	% Y	% S	% Y	% S	% Y	% S	% Y	% S	% Y	
Effect of Substituents in Anilides with Desired Dimensions														
CIPC	G	50	15	0	0	100	35	20	5	100	75	75	25	500
1	B	0	0	0	0	0	0	0	0	25	5	0	0	30
2	B	0	0	0	0	10	5	0	0	0	0	0	0	15
3	G	0	0	0	0	100	50	0	0	100	50	0	0	300
4	B	50	25	0	0	100	100	0	0	100	75	20	5	475
5	B	0	0	0	0	100	75	0	0	65	25	0	0	265
6	B	0	0	0	0	100	85	0	0	50	10	50	25	310
	M	0	0											
7	B	100	75	0	0	100	100	100	20	100	50	100	100	845
	M	0	0											(670)
8	B	100	75	0	0	100	100	100	25	100	85	100	75	860
9	B	100	75	0	0	100	100	100	20	100	85	100	75	855
	M	0	0											(680)
10	B	100	100	10	5	100	100	100	100	100	100	100	100	1015
11		...	...	100	75	100	100	100	75	100	100	100	50	[900]
12	B	100	100	100	100	100	100	100	50	100	100	100	100	1150
13 through 20			Little or no effect											1200
Molecular Size *vs.* Herbicidal Activity of 3,4-Dichloroanilides														
21	B	0	0	0	0	100	100	100	25	100	50	50	25	550
	M	0	0											
22	B	0	0	0	0	100	75	50	5	60	30	100	75	495
	M	0	0											
2	B	0	0	0	0	10	5	0	0	0	0	0	0	15
1	B	0	0	0	0	0	0	0	0	25	5	0	0	30
7	B	100	75	0	0	100	100	100	20	100	50	100	100	845
	M	0	0											(670)
23	B	100	100	30	10	100	100	100	100	100	100	100	100	1040
	M	75	35											(950)
24	B	0	0	0	0	100	100	50	5	75	35	100	75	540
	M	0	0											
25	B	25	10	0	0	100	75	20	5	75	35	100	75	520
	M	0	0											(485)
26	B	30	10	0	0	100	100	50	5	60	30	100	75	560
	M	0	0											(520)
27	B	100	100	0	0	100	100	100	85	70	50	100	100	905
	M	100	50											(855)
28		No effect												1200

[a] B. Bindweed. G. Wild morning glory. M. Mustard. [b] S. Stand. Y. Yield. ( ) Test with alternate broadleaf weed. [ ] Broadleaf weed omitted from test.

Table V. Anilides Prepared

Compound No.[a]	Ref.	Solvent[b]	Crude, % Yield	Recrystallization Solvent[b]	Pure, M.P., °C.	Formula	Analyses % Calcd. C	H	Cl	N	% Found C	H	Cl	N
1		B	96	Dioxane-water	134.6–5.2	$C_{10}H_{11}Cl_2NO$	51.8	4.74	30.6	6.03	52.0	4.60	30.3	5.94
2		B	79	Cyclohexane	90.6–1.6	$C_9C_9Cl_2NO$	49.6	4.13	32.5	6.42	49.6	3.90	32.4	6.34
3	(*20*)	B	95	B	152.6–3.0	$C_{10}H_{12}ClNO$	60.8	6.08	17.9	7.09	61.0	6.26	18.1	7.03
4	(*6*)	C	98	C–pet. ether	141	$C_9H_{10}ClNO$								
5		..	82	Cyclohexane	87–8	$C_9H_{10}ClNS$	54.2	5.01			54.6	4.86		
6		B	65	Cyclohexane	95.5–6.2	$C_{10}H_{12}ClNO$	60.8	6.08			61.1	5.97		
7		B	88	B	132.6–3.8	$C_9H_8Cl_2NO$	42.8	3.17			43.5	3.12		
9		B	88	Cyclohexene	113.8–14.6	$C_9H_9Cl_2NO$	49.6	4.13			50.1	4.50		
10		B	88	Cyclohexane	116.2–16.4	$C_{10}H_{11}Cl_2NO$	51.8	4.74	30.6		52.1	4.68	30.6	
11	(*6*)	B	79	B–pet. ether	121	$C_9H_9Cl_2NO$								
12		B	75	Ethanol	150–1	$C_9H_8Cl_2NO$	42.8	3.17			42.2	3.51		
13		B	87	Ethanol	123.5–4.5	$C_{10}H_{11}Cl_2NO$	51.8	4.74			52.1	4.78		
14		B	80	B	148.0–8.4	$C_{10}H_{10}Cl_2NO$	45.1	3.75	39.9		45.3	3.79	39.8	
15		B	99	Cyclohexene	142–3	$C_{11}H_{14}ClNO$	62.4	6.62	16.8		62.8	6.54	16.8	
16	(*4*)	B	100	2-Propanol	139.8–40.2	$C_{11}H_{14}NO$								
17		B	100	B	143.8–44.2	$C_{11}H_{14}ClNO$	62.4	6.62	16.8	6.62	62.9	6.54	16.6	6.69
19	(*13*)	B	98	Dioxane-water	127	$C_9H_{10}ClNO$								
20		B	Total	Methylcyclohexane	70–6	$C_{10}H_9Cl_2NO$	52.2	3.91		6.09	51.0	3.94		6.20
	(*18*)	B	94	Cyclohexene	150–1						52.8	4.05		
22	(*3*)	B	67	B	120.5	$C_8H_7Cl_2NO$								
23		B	98	Cyclohexene	124.2–25.8	$C_{12}H_{13}Cl_2NO$	55.4	5.77			55.8	5.72		
24		B	60	2-Propanol	81.8–7.4	$C_{11}H_{13}Cl_2NO$	53.9	5.31			54.4	5.47		
25		B	65	B	76.6–7.9	$C_{10}H_{11}Cl_2NO$	51.8	4.74			51.7	4.98		
26		B	58	B	71.4–3.2	$C_{11}H_{13}Cl_2NO$	53.9	5.31			53.7	5.34		
27		B	90	Cyclohexane	59.8–61.4	$C_{13}H_{17}Cl_2NO$	57.0	6.20			57.0	6.31		
28		B	91	A	81.1–2.5	$C_{18}H_{27}Cl_2NO$	62.8	7.85			62.5	7.84		

[a] Structures given in Tables II-III.
[b] A. Acetonitrile. B. Benzene. C. Chloroform.

hexane gave 0.8 grams of fine white needles (m.p. 87–8° C.).

P. J. Stoffel, Monsanto, St. Louis Laboratories, has suggested pyridine as a useful solvent for thiations (*11*).

Discussion

Although anilides are structurally similar to carbamates and ureas, their mode of action and structural requirements for maximum herbicidal activity differ to a marked degree. For example, the anilides are primarily foliage toxicants, while carbamates and ureas are much more effective in pre-emergence applications. Carbamates (*17*) show the greatest activity with a 3-chlorophenyl group, while phenyl, 2,4-dichloro-, and 4-chloro- analogs are lower in activity. Ureas with these substituents are all toxic (*5*). In contrast, the 3,4-dichloroanilides are by far the most active anilides; 4-chloro- and phenyl derivatives are less active.

The clear-cut inhibition of activity produced by a 2-chloro- substituent was unique, as it did not occur in ureas, carbamates, or phenoxyacetic acids (*19*). Maximum activity was obtained with 2,4-dichlorophenoxyacetic acid, while 3,4-chlorination imparted a decreased activity (*12*). This was reversed in the anilide series. The cinnamic acids (*15*) follow a structure *vs.* activity pattern very similar to phenoxyacetic acids.

How should the data on the desired size concept be interpreted? Perhaps two enzyme interspaces were involved—one required a molecule with a maximum length of 13 A., the second a 10- or 11-A. size. Apparently the second interspace was not satisfied by a molecule which could be compressed to a size less than 10 A.

Molecular size was an important factor in the herbicidal activity of varied compounds. Properly placed substituents were also necessary for maximum activity. Consequently, the desired size concept enables the prediction of the most active compound in a series with similar substituents. Such a prediction was of value in reducing the number of compounds to be tested. Acceptance of the premise that the desired size concept would apply to anilides would have eliminated many compounds from the test program. A study is being made of lower rates of application of several of the active anilides. These studies, along with directed spraying, may enable their use as selective herbicides on various crops. Selectivity might also be achieved by choosing the less toxic compounds, or variants of these compounds.

Several years elapsed between the discovery of 2,4-D and the discovery of other active herbicides such as CMU and CIPC. This time would have been shortened considerably if the extended and collapsed dimensions of 2,4-D had been accepted as the desired dimensions. This reasonable conclusion establishes the validity of the desired size concept. Unfortunately, many compounds with these dimensions had little or no pre- or postemergence activity. Consequently, the desired size concept remains as only one facet of herbicidal activity.

Possibly the desired size concept will prove applicable to other biological problems. Molecular size measurements of models of fungicides, insecticides, nematocides, etc., may prove fruitful.

Acknowledgment

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Measurement of atomic models and preparation of compounds were done at the Lion Oil Co. and greenhouse experiments at the Midwest Research Institute.

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Preparation and Fungitoxicity of Some Trialkyl Phosphorotetrathioates

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Homologous series of both symmetrical and unsymmetrical trialkyl phosphorotetrathioates were prepared and tested in agar plates as fungitoxicants for eight species of phytopathogenic fungi. Certain of the shorter homologs, such as trimethyl phosphorotetrathioate, proved to be more fungitoxic than standard commercial fungicides. These studies showed that the fungitoxicity of trialkyl phosphorotetrathioates generally decreased as alkyl groups were lengthened.

During a recent screening program on the biological properties of several types of compounds, it was found that trialkyl phosphorotetrathioates containing methyl groups had significant fungitoxicities. To obtain more information on the toxicity-structure relationships of these little known materials, 16 compounds were synthesized and evaluated. This paper is a brief account of that work, and the unique toxicity found for the methyl substituent.

Preparation of Compounds

Two methods of preparing some of the trialkyl phosphorotetrathioates were described recently (*2*). As the third method (Reaction 5) is somewhat similar, only the pertinent data are recorded (Table I). The reactions involved are:

$$6RSH + P_2S_5 \rightarrow 2(RS)_3PS + 3H_2S \quad (1)$$

$$2CH_3SH + P_2S_5 \rightarrow 2CH_3SPS_2 + H_2S \quad (2)$$

$$CH_3SPS_2 + RSH \rightarrow CH_3S(RS)P(=S)-SH \quad (3)$$

$$CH_3S(RS)P(=S)-SH + \text{olefin} \rightarrow CH_3S(RS)P(=S)-SR' \quad (4)$$

$$CH_3S(RS)P(=S)-SH + R'SH \rightarrow CH_3S(RS)P(=S)-SR' + H_2S \quad (5)$$

The symmetrical triakyl phosphorotetrathioates were made easily by allowing phosphorus pentasulfide to react with an excess of mercaptan, as shown in Reaction 1. The unsymmetrical trialkyl phosphorotetrathioates were less easily prepared and, in this work, depended upon the great reactivity of methyl phosphenotrithioate, the preparation of which is shown in Reaction 2. When methyl phosphenotrithioate reacts with one mole of a mercaptan, the product is a methyl alkyl hydrogen phosphorotetrathioate (Reaction 3). With two or more moles of a mercaptan, methyl phosphenotrithioate gives methyl dialkyl phosphorotetrathioate (Reaction 5). When a methyl alkyl hydrogen phosphorotetrathioate reacts with an olefin, the product is a methyl dialkyl phosphorotetrathioate (Reaction 4). The addition here is according to Markownikoff's rule—i.e., 1-olefins add in the 2- position.

Most of these reactions are nearly quantitative. Many of the poor yields in Table I were caused by handling losses. Because these compounds have moderately poor thermal stabilities, considerable decomposition occurred during some of the distillations. Residual solvent was removed from many of the compounds by vacuum stripping rather than by distillation. Although some of the products were contaminated with small amounts of solvent, the structures were confirmed by infrared analysis (*1*).

Fungitoxicity Evaluation

The fungitoxicity of these compounds was tested in agar at 1000 and 100 p.p.m. To prepare the 1000-p.p.m. dosage, 2.5 grams of the test compound was weighed into a 200-ml. Waring Blendor, with 2 drops of a nonionic dispersing agent (Triton X-171, Rohm and Haas Co.) and sufficient distilled water to bring the total weight to 50 grams. The mixture was homogenized at high speed and then, with the blender still operating, 1 gram of the homogenate was transferred to 50 grams of sterile liquid potato dextrose agar at 45° C. The treated medium, containing 1000 p.p.m. of test chemical, was then poured into three Petri plates and allowed to solidify. To obtain 100 p.p.m. of test chemical in the agar medium, 10.0 grams of the homogenate was weighed into another blender and the weight brought up to 100 grams with distilled water. This mixture was homogenized and 1 gram transferred to 50 grams of sterile agar medium, which was then poured into three Petri plates and allowed to solidify. In all cases, the homogenate was readily dispersed in the liquid agar medium by swirling.

The solid medium in each plate was inoculated with the mycelia of eight species of fungi: *Rhizoctonia solani*, *Sclerotium rolfsii*, *Phytophthora cinnamomi*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *Monolinia fructicola*, *Botrytis cinerea*, and *Alternaria solani*. Disks of agar with mycelia on the surface were cut from Petri-plate cultures with a sterile, No. 2 cork borer and were transferred to treated agar with the mycelia against the treated surface.

The disks were arranged in a circle on each plate about 1 inch apart and 0.7 inch in from the plate rim. The cultures of fast-growing species were 2 days old and the more slowly growing species 4 or 5 days old at the time of transfer. All cultures were originally isolated from plant hosts.

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[2] Present address, Pentavate Corp., P. O. Box 786, Lindsay, Calif.

Table I. Synthesis of Trialkyl Phosphorotetrathioates

Phosphorotetrathioate	Mercaptan, Moles	CH_3SPS_2, Mole	P_2S_5, Moles	Olefin, Moles	Reaction Pressure, P.S.I.G.	Reaction Temp., ° C.	Reaction Time, Hours	Boiling Point of Product, ° C./Mm. Hg	Yield, Mole %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Phosphorus, % Calcd.	Phosphorus, % Found	Sulfur, % Calcd.	Sulfur, % Found
$(CH_3S)_3PS$	42	...	3.5	...	195	100	6	126–130/0.2	86	17.7	17.1	4.4	4.3	15.2	15.1	62.7	62.8
$(C_2H_5S)_3PS$	15	...	3	...	0	100	14	110/0.15	46	29.3	28.8	6.1	6.3	12.6	12.6	52.0	51.8
$(CH_3)_2CH-S)_3PS$	3	...	0.5	...	0	70	40	123–125/0.3	79	37.5	36.6	7.3	7.4	10.8	10.7	44.4	44.7
$(CH_3CH_2CH_2S)_3PS$	8.2	...	2	...	0	100	5	136–137/0.15	38	37.5	37.1	7.3	7.4	10.8	10.9	44.4	44.6
$(CH_3S)_2P(S)SCH(CH_3)_2$	CH_3SH 0.15	0.5	...	$CH_2=CHCH_3$ 1.0	[a]	40[b] 80–100	21.5[c] 2.5	125–130/0.8	22	25.8	26.0	5.6	5.7	13.4	13.7	55.2	55.2
$(CH_3S)_2P(S)S-CH_2CH_2CH_3$	n-C_3H_7SH, CH_3SH 0.5 1.0	0.5	...	...	65	50 80	0.5 3.0	140–150/0.5–0.7	57	25.8	24.6	5.6	5.6	13.4	14.1	55.2	56.9
$(CH_3S)_2P(S)S$-n-C_4H_9	CH_3SH, n-C_4H_9SH 0.5 1.0	0.5	...	...	33	40 100	2 17	[d]	28	...	...	...	...	12.6	13.2	51.9	49.2
$(CH_3S)_2P(S)S-CH(CH_3)-C_4H_9$	CH_3SH 0.5	0.5	...	$CH_2=CHC_4H_9$ 1.0	50	5 100	2 16	[d]	80	...	...	...	...	11.3	12.1	46.8	47.8
$(CH_3S)_2P(S)S$-n-C_6H_{13}	CH_3SH, n-$C_6H_{13}SH$ 1.0 0.4	0.4	...	...	0 65	40 100	0.5 4	[d]	75	...	...	...	...	11.3	12.2	46.6	46.3
$(CH_3S)_2P(S)S$-n-C_8H_{17}	CH_3SH, n-$C_8H_{17}SH$ 0.5 0.3	0.3	...	...	0 60	40 100	0.5 1.0	[d] [d]	75	39.8	41.3	7.6	7.7	10.3	10.3	42.3	39.6
$(CH_3S)_2P(S)S$-n-$C_{10}H_{21}$	CH_3SH, n-$C_{10}H_{21}SH$ 1.0 0.2	0.2	...	...	0 60	30 100	1.2 16	[d]	77	43.6	46.3	8.2	8.8	9.4	8.8	38.8	35.7
$(CH_3S)_2P(S)S$-n-$C_{11}H_{23}$	CH_3SH, n-$C_{11}H_{23}SH$ 1.0 0.2	0.2	...	...	0 40	40 65	0.5 5.5	[d]	76	45.3	45.3	8.4	8.1	9.1	9.8	37.2	35.1
$(CH_3S)_2P(S)S$-n-$C_{12}H_{25}$	CH_3SH, n-$C_{12}H_{25}SH$ 0.2 0.2	0.2	...	...	5 0	40 50	2 17	[d]	74	...	...	...	...	...	...	...	
$CH_3S-P(S)(SC_2H_5)_2$	C_2H_5SH 0.4	0.4	...	$CH_2=CH_2$ (excess)[f]	[f]	25 100	66 18	109–129/0.5	34	25.8	24.4	5.6	5.7	13.4	14.2	55.2	56.3
$CH_3S-P(S)(SC_2H_5)(S-CH(CH_3)_2)$	C_2H_5SH 0.4	0.4	...	$CH_2=CHCH_3$ 1.0	[a]	25 100	66 16	105–115/0.2	15	...	...	...	...	12.6	12.8	52.1	53.2
$CH_3S-P(S)[S-CH(CH_3)_2]_2$	i-C_3H_7SH 0.7 0.7	0.7	...	$CH_2=CHCH_3$ 1.0	[a]	34 100	2 1.5	130–135/0.6	45	32.3	31.6	6.5	6.8	11.9	11.7	49.3	49.0

[a] Autogenous pressure.
[b] First number is temperature of reaction of CH_3SPS_2 and RSH. Second number is temperature of addition of olefin.
[c] First number is time of reaction of CH_3SPS_2 and RSH. Second number is time and addition of olefin.
[d] Product vacuum stripped rather than distilled because of instability at high temperatures.
[e] Structure confirmed by infrared analysis.
[f] 300-ml. bomb at 500 p.s.i.g./25° C., 3000 p.s.i.g./95° C.

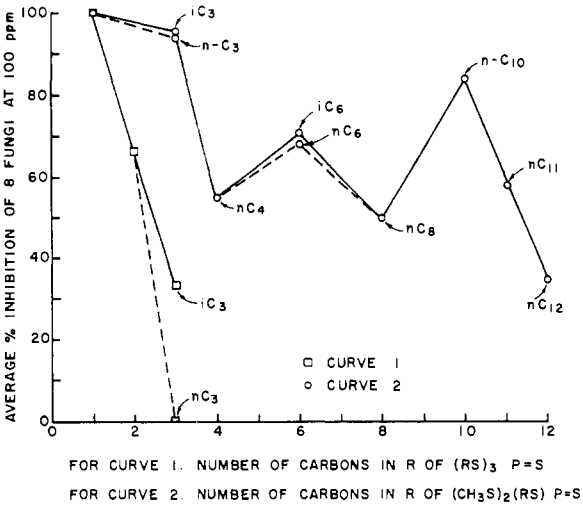

Figure 1. Effect of side chain length on agar-plate fungitoxicities of dimethyl alkyl and trialkyl phosphorotetrathioates

Inoculated plates were incubated for 2 days at 25° C. and the millimeters of mycelial growth from the edge of the inoculum disk to the margin of radial development recorded. The per cent inhibition of mycelial growth was calculated from the average of three replicates as follows:

$$100 - \left(\frac{\text{average growth on chemical-treated agar}}{\text{average growth on untreated agar}}\right) \times 100 = \% \text{ inhibition}$$

Discussion

The compounds in Tables I and II are separated into three groups: sym-

Table II. Agar-Plate Fungitoxicities of Trialkyl Phosphorotetrathioates

(Per cent inhibition of radial growth)

Phosphorotetrathioates	*Rhizoctonia Solani*		*Sclerotium Rolfsii*		*Phytophthora Cinnamomi*		*Pythium Ultimum*		*Sclerotinia Sclerotiorum*		*Sclerotinia Fructicola*		*Botrytis Cinerea*		*Alternaria Solani*		*Average*	
	100	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*	*100*	*1000*
	p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
$(CH_3S)_3PS$	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
$(C_2H_5S)_3PS$	79	89	95	100	24	100	43	90	90	94	89	89	70	91	40	70	66	90
$(CH_3)_2CHS)_3PS$	63	68	61	89	10	40	16	70	48	77	58	77	0	52	14	36	34	64
$(CH_3CH_2CH_2S)_3PS$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$(CH_3S)_2P(S)-SCH(CH_3)_2$	100	100	100	100	100	100	94	100	98	100	100	100	95	95	70	100	95	99
$(CH_3S)_2P(S)-S-CH_2CH_2CH_3$	100	100	100	100	100	100	82	100	97	100	97	100	95	100	83	83	94	98
$(CH_3S)_2P(S)-S-n-C_4H_9$	60	68	76	93	89	100	48	100	39	90	60	87	55	90	12	35	55	83
$(CH_3S)_2P(S)-S-CH(CH_3)-C_4H_9$	62	82	91	99	100	100	38	100	63	100	83	100	91	96	29	59	70	92
$(CH_3S)_2P(S)-S-n-C_6H_{13}$	42	90	83	100	91	100	80	100	70	98	62	94	67	100	50	89	68	96
$(CH_3S)_2P(S)-S-n-C_8H_{17}$	40	72	48	96	100	100	62	100	41	100	60	90	49	90	0	47	50	82
$(CH_3S)_2P(S)-S-n-C_{10}H_{21}$	86	100	95	100	100	100	90	100	77	100	87	100	91	100	44	67	84	96
$(CH_3S)_2P(S)-S-n-C_{11}H_{23}$	32	87	73	94	91	100	81	100	44	98	66	100	75	94	0	77	58	94
$(CH_3S)_2P(S)-S-n-C_{12}H_{25}$	19	76	29	96	68	100	72	100	11	95	31	91	30	91	28	61	35	89
$CH_3S-P(S)(SC_2H_5)(SC_2H_5)$	92	95	100	100	81	90	76	92	87	97	100	100	93	93	54	62	86	91
$CH_3S-P(S)(SC_2H_5)(S-CH(CH_3)_2)$	80	100	100	80	80	80	80	100	100	100	...	...	80	100	...	...	87	93
$CH_3S-P(S)(SCH(CH_3)_2)_2$	58	76	73	96	12	82	21	83	72	87	53	75	42	78	0	38	41	77
Phygon[a]	72	83	15	60	90	100	65	87	56	88	74	89	52	80	50	71	59	82
Captan[b]	75	85	58	85	90	90	74	88	72	95	83	91	91	92	100	100	80	91
Terrachlor[c]	70	76	94	100	43	100	66	100	70	87	91	100	91	92	57	79	73	92
Ferbam[d]	68	92	53	100	90	97	31	100	59	95	26	91	56	95	79	100	58	96

[a] 2,3-Dichloro-1,4-naphthoquinone from U. S. Rubber Co.
[b] *N*-Trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide from Stauffer Chemical Co.
[c] Pentachloronitrobenzene from Olin Mathieson Chemical Corp.
[d] Ferric dimethyldithiocarbamate from E. I. du Pont de Nemours and Co., Inc.

metrical triakyl phosphorotetrathioates, $(RS)_3PS$; unsymmetrical dimethyl alkyl phosphorotetrathioates, $(CH_3S)_2P(=S)-SR$; and unsymmetrical methyl dialkyl phosphorotetrathioates, $CH_3S-P(=S)(SR)(SR')$. To compare the fungitoxicities of these compounds, the average inhibitions of eight fungi at 100 p.p.m. were used. Although there were variations between fungi, examination of the data in Table II shows that the variations generally were small and that the structure-toxicity relationships are consistent.

Figure 1 shows these structure-toxicity relationships graphically for the first two groups of compounds in Tables I and II. Curve I represents the symmetrical compounds and shows the linear relationship among CH_3, C_2H_5, and i-C_3H_7. The same superiority of branched chains over straight chains is shown again in curve 2 for the dimethyl propyl phosphorotetrathioates and the dimethyl hexyl phosphorotetrathioates. The differences here are not so exaggerated, however, because of the overwhelming effect of the two methyl groups. The increase in toxicity with the *n*-decyl side chain is not surprising, as this phenomenon occurs in many other toxicity-structure relationships. It is interesting to note, however, that there is a linear relationship with the *n*-decyl, *n*-undecyl, and *n*-dodecyl groups.

In view of the many reactions and modes of action in which these compounds could be involved it would be unwise to infer too much from these preliminary screening results. It is clear, however, that these compounds have significant biological activity, particularly those with small alkyl groups, and are worthy of further investigation.

Acknowledgment

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Literature Cited

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FUNGICIDE RESIDUES

Rapid Determination of Mercury in Apples by Modified Schöniger Combustion

WALTER H. GUTENMANN and DONALD J. LISK

Pesticide Residue Laboratory, Department of Entomology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

A modified Schöniger combustion of dried apple tissue replaces wet ashing prior to the determination of mercury. Loss of mercury by volatilization is eliminated in the closed combustion flask. Apple tissue is dried on cellophane overnight under vacuum, then burned in an oxygen-filled flask with a balloon attached for pressure control. Mercury is determined spectrophotometrically after extraction of the absorbing solution with dithizone. About 12 samples can be burned and analyzed in one day. Recovery of mercury from apples in the 0.3- to 0.6-p.p.m. range averaged 83.6%. Up to 0.18 p.p.m. of mercury was found in apples treated with mercurial fungicides for scab control.

MERCURIAL FUNGICIDES have been used in New York during the past three seasons for control of scab in apples. During analysis of these apples for mercury residues this laboratory was unable to recover small amounts of mercury consistently by wet-ashing procedures. Although oxidizing conditions were always maintained, mercury was lost by volatilization during acid digestion of apple tissues. Attempts to reduce this loss by the use of a low temperature initial digestion, hydrogen peroxide, selenium, and various condensing systems proved unsuccessful.

Southworth, Hodecker, and Fleischer (*4*) recently determined mercury in organic compounds by combustion in a Schöniger flask (*2, 3*). In the work reported a modified Schöniger flask is used to determine mercury in apples. Apple tissue containing organic mercury fungicides is dried on cellophane and burned in an oxygen-filled flask. The combustion products are absorbed in 0.1*N* hydrochloric acid and mercury is determined spectrophotometrically after extraction of the absorbing solution with dithizone (*1*). Loss of mercury by volatilization cannot occur in the closed system and recovery of mercury is consistently good.

Combustion Flask

The combustion flask and platinum holder are shown in Figure 1.

The flask is round-bottomed, of 5-liter capacity, and made of borosilicate glass. A 40/35 standard-taper, female ground joint is sealed on the neck. A side arm (1 cm. in inside diameter, 4 cm. long) is

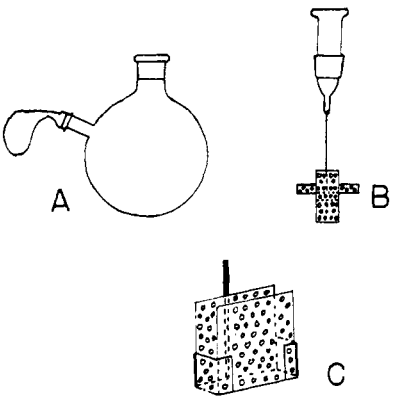

Figure 1. Combustion equipment

A. Flask
B. Platinum holder open
C. Platinum holder closed

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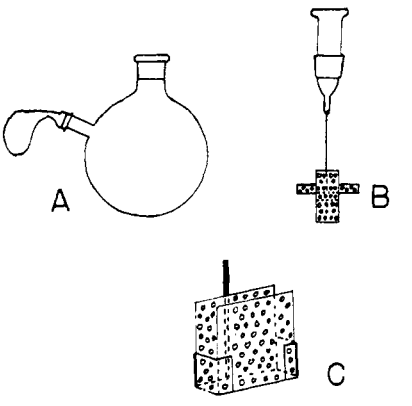

Figure 1. Combustion equipment

A. Flask
B. Platinum holder open
C. Platinum holder closed

Table I. Per Cent Recovery of Mercurial Fungicides[a] from Apples

Apple Variety	Phenylmercuric Acetate	Phenylmercuric Oxyquinolate (Quinex)	Di(phenylmercuric) Ammonium Oxyquinolate (Metasol DPO)
MacIntosh	81.9		
	71.5		
	84.9		
	80.0		
	81.7		
	79.7		
	74.7		
	78.5		
	82.9		
	82.2		
Cortland	89.3	85.0	
	84.1	83.2	
	79.2	75.0	
		80.6	
		80.6	
Red Delicious	82.9	76.8	80.7
	84.1	83.8	80.5
	86.9	63.5	103.9
		89.6	106.3
		70.1	
Rome Beauty	83.9	77.2	94.7
	85.6	89.8	100.8
	82.5		93.8
			109.9
Rhode Island Greening	77.8	76.7	98.8
	91.9	69.3	63.5
	85.4		

[a] 0.60 p.p.m. (calculated as mercury) of fungicide added to each sample except first two samples of MacIntosh apples treated with phenylmercuric acetate, to which 0.30 p.p.m. was added.

Table II. Residues of Mercury in Apples Treated with Organic Mercurial Oxyquinolate

Apple Variety	Phenylmercuric Oxyquinolate (Quinex)	Di(phenylmercuric) Ammonium Oxyquinolate (Metasol DPO)
	(P.p.m.)	
Cortland[a]	0.03	
	0.00	
	0.07	
	0.05	
Red Delicious[a]	0.06	0.00
	0.05	
Rome Beauty[a]	0.04	0.00
	0.05	
	0.02	
Rhode Island Greening[a]	0.04	0.00
	0.04	
	0.02	
Cortland[b]	0.13	
	0.06	
	0.15	
	0.14	
Red Delicious[b]	0.15	
	0.18	
Cortland[c]	0.00	
	0.00	
Red Delicious[c]	0.02	0.00
	0.00	0.00
Rome Beauty[c]		0.05
		0.02
Cortland[d]	0.02	
	0.04	
Red Delicious[d]	0.03	
	0.03	

[a] 7 applications through May 26; apples sampled July 16.
[b] 7 applications through May 26 with bimonthly thereafter; apples sampled July 16.
[c] 7 applications through May 26; apples sampled September 16.
[d] 7 applications through May 26 with bimonthly applications until August 16; apples sampled September 16.

sealed to the flask as illustrated. A rubber balloon, about 8 cm. long, is secured to the side arm with a rubber band.

The platinum holder is constructed by sealing a 12-cm. length of No. 16 B. and S. gage platinum wire onto a 40/35 standard-taper, male ground joint drawn out 8 cm. below the ground portion. A piece of Style 4, perforated platinum sheet (J. Bishop and Co., Platinum Works, Malvern, Pa.), 2.5 cm. wide and 7.5 cm. long, and bent as shown is electrically welded to the last 3 cm. of this wire. Two platinum tabs (1.25 × 2.5 cm.) are welded on to form the two remaining sides of the holder and to keep the sample compressed. The bottom of the resulting holder is 1 × 2.5 cm. and the holder is 3 cm. deep.

Procedure

Preparation of Sample for Combustion. Blend apples in their own liquid until a homogeneous mixture is obtained. Spread a 10-gram portion of the mixture on a 3 × 3 inch square of cellophane (Du Pont No. 300, MSAT No. 87 is satisfactory) supported on a watch glass. Place the watch glass and sample in a desiccator containing sulfuric acid. Several samples may be dried at once. Evacuate the desiccator and allow the samples to remain under vacuum overnight.

Combustion and Determination of Residues. Remove the sample from the desiccator and wrap the cellophane around it by folding the opposite sides up. Place the package in the partially open platinum holder and compress by bending the tabs around it. Insert a fuse cut from filter paper (0.5 × 10 cm.) into the sample. Place 200 ml. of 0.1*N* hydrochloric acid and an egg-shaped, magnetized stirring bar ($2^1/_2$ inches long, Teflon-sealed) in the combustion flask. Place the flask on a magnetic stirrer and support it from a ring stand. Thoroughly purge the flask with oxygen. Light the fuse and place the holder in the flask as soon as about 1 inch of the fuse has burned. Hold the top on until combustion is complete and the balloon has collapsed. Start the magnetic stirrer and operate it fast enough to wash the inside surface of the flask with the splashing solution. Stirring from 7 to 10 minutes is usually sufficient. Remove the flask from the stirrer and tilt it to rinse the upper portion of the flask. Do not allow the acid to enter the balloon, as rubber absorbs mercury. Remove the platinum holder. The magnetized stirring bar is best removed by using a large horseshoe magnet externally to draw the bar out of the flask. Pour the absorbing solution into a 500-ml. separatory funnel while rinsing the platinum holder. Rinse the flask, holder, and balloon with 50 ml. of 0.1*N* hydrochloric acid and add the rinse solution to that in the funnel. Repeat with a second 50-ml. portion of the acid. Carry each sample separately through the procedure from this point.

To the combined acid solution in the funnel add 10 ml. of 20% hydroxylamine hydrochloride and 5 ml. of 30% acetic acid and mix. Add exactly 25 ml. of dithizone in chloroform (4 mg. per liter) and shake exactly 1 minute. Drain the lower layer through a cotton pledget placed in the stem of the funnel into a 5-cm. cuvette. Measure the absorbance of the solution at 490 mμ with dithizone in the reference cell. Use fresh dithizone with each sample.

Prepare the calibration curve (0 to 10 γ of mercury) as follows:

Pipet 0, 1, 2, 3, 4, and 5 ml. of a standard mercuric chloride solution in 0.1*N* hydrochloric acid (2 γ of mercury per ml.) into a series of 500-ml. separatory funnels, and add 0.1*N* hydrochloric acid to make a total volume of 300 ml. Proceed as in the analysis of samples, beginning with addition of the hydroxylamine solution.

Results and Discussion

The method was used to recover phenylmercuric acetate, phenylmercuric oxyquinolate (Quinex), and di(phenylmercuric) ammonium oxyquinolate (Metasol DPO) from apples. These fungicides have been used here for the control of apple scab.

Solutions of these fungicides in glacial acetic acid were added to the 10 grams of apple tissue on the cellophane before drying. The recoveries obtained after drying and combustion of the tissue are shown in Table I.

The check value for 29 analyses averaged 0.037 p.p.m. of mercury. The method will detect about 0.01 p.p.m. of mercury in a 10-gram apple sample.

During 1959, several varieties of apple trees and fruit were treated with Quinex and Metasol DPO for scab control. Quinex was formulated with $^1/_4$ pint of a 20% active liquid formulation contained in 100 gallons of water. Metasol DPO was formulated with $^1/_2$ pint of a 10% active liquid formulation contained in 100 gallons of water. About 12 gallons of this dilute spray was applied to each tree (about a gallon per bushel of fruit). Seven applications were made between April 11 and May 26, 1959. In one experiment spray applications were not continued after May 26 and fruit was

sampled on July 16 and September 16. In a second experiment spray applications were continued bimonthly until August 16, samples being taken on July 16 and September 16. Table II shows the residues of mercury found in replicate samples following analysis.

Some varieties in Table II were not available for the September sampling.

The weight of dried apple burned was about 2 grams, which represented about 10 grams of fresh apple tissue. If a larger weight of tissue was used, combustion was incomplete. Expansion of gases into the balloon allowed the combustions to be carried on safely. The size of the sample and the rapid rate of burning of cellophane necessitated the use of a balloon, which was replaced after about five combustions. Nearly 200 combustions have been conducted in the flask. About 12 samples can be burned and analyzed in a day.

Acknowledgment

This work was done in cooperation with John Van Geluwe, Grange League Federation, Ithaca, N. Y.

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(1) Assoc. Offic. Agr. Chemists, Washington, D. C., "Official Methods of Analysis," 8th ed., pp. 441–2, 1955.
(2) Schöniger, W., *Mikrochim. Acta* **1955,** 123–9.
(3) *Ibid.*, **1956,** 869–76.
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FUNGICIDE RESIDUES

Effects of Treatment Conditions on *o*-Phenylphenol Residues in Oranges

F. W. HAYWARD and WILLIAM GRIERSON

Citrus Experiment Station, University of Florida, Lake Alfred, Fla.

Sodium o-phenylphenate is effective as a postharvest fungicide in controlling decay of citrus fruits, but it sometimes causes peel injury. A study was made of the effects of treatment conditions on residues of o-phenylphenol in oranges. Residues increased with concentration, temperature, and length of treatment, but decreased as pH was raised. By control of treatment conditions residues can be maintained at any desired level. Peel injury was not a problem when residues were below the legal tolerance of 10 p.p.m.

ORTHO-PHENYLPHENOL and its sodium salt are effective as postharvest fungicides for citrus fruits when applied from aqueous solutions (*8*). A major problem has been injury to the peel by these materials (*7*). Efforts to prevent this injury have been directed toward adjustment of formulation (*9*) and use of additives such as hexamethylenetetramine (*5*). Recently it has been found (*6*) that both peel injury and fungicidal effectiveness are related to the pH of sodium *o*-phenylphenate solutions used for treatment of oranges.

The authors found (*4*) that addition of sodium *o*-phenylphenate to the water used for hydrocooling oranges was effective in reducing subsequent decay. The search for optimum conditions has been extended to study the effects of concentration, temperature, pH, and length of treatment on residues of *o*-phenylphenol and extent of peel injury of oranges.

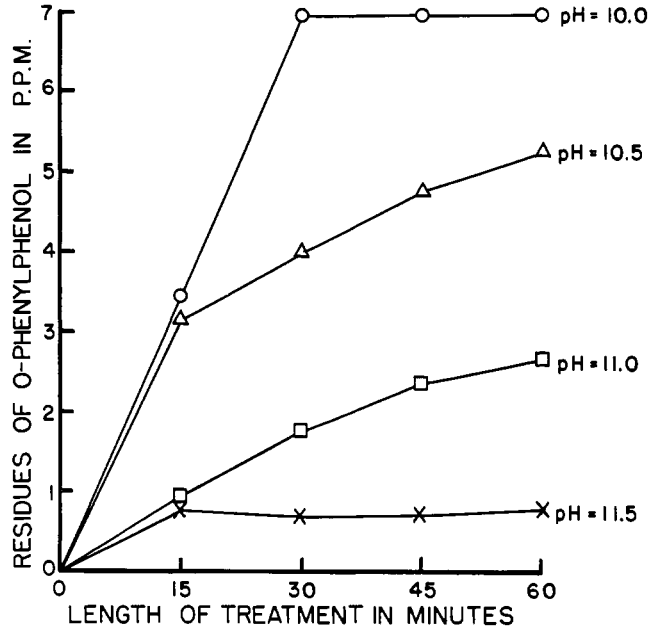

Figure 1. Effect of length of treatment on o-phenylphenol residues in oranges treated with 0.1% Dowicide A at 40° F. at various pH levels

Fruit Handling

Oranges used in these experiments came from groves of the Citrus Experiment Station. The entire series of experiments was run using Pineapple oranges, a midseason variety, and repeated with Valencias, which mature much later. Fruit was washed, dried, graded, and automatically packed in 4/5-bushel fiberboard cartons using small, commercial-type machinery in the experimental packinghouse. Storage before and after fungicidal treatments was at 70° F.

Low temperature treatments were

sampled on July 16 and September 16. In a second experiment spray applications were continued bimonthly until August 16, samples being taken on July 16 and September 16. Table II shows the residues of mercury found in replicate samples following analysis.

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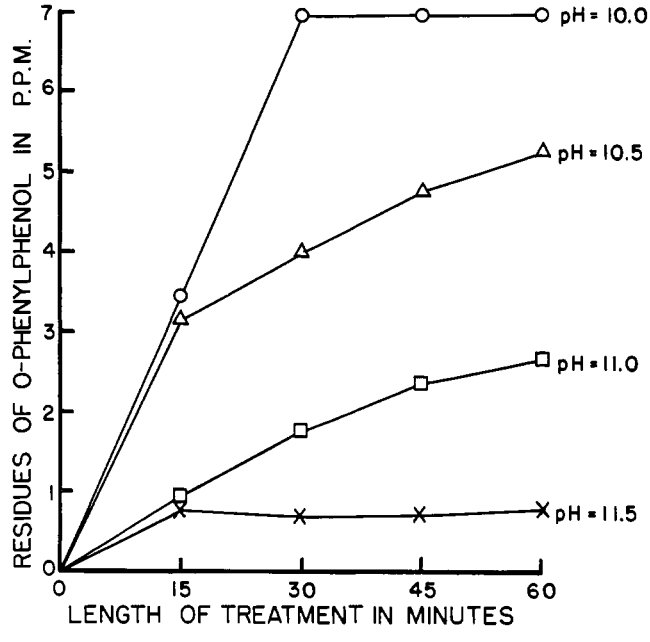

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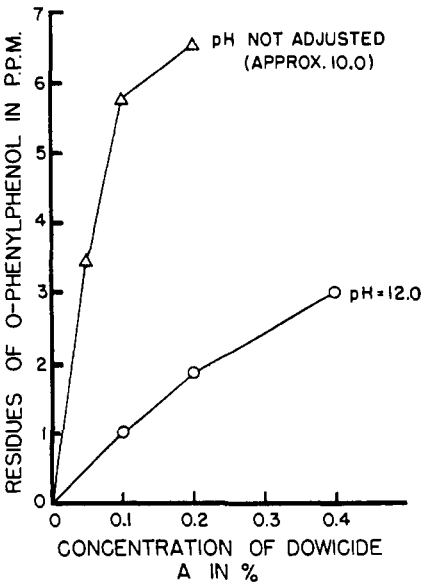

Figure 2. Effect of concentration of Dowicide A on o-phenylphenol residues in oranges treated for 30 minutes at 40° F. at two pH levels

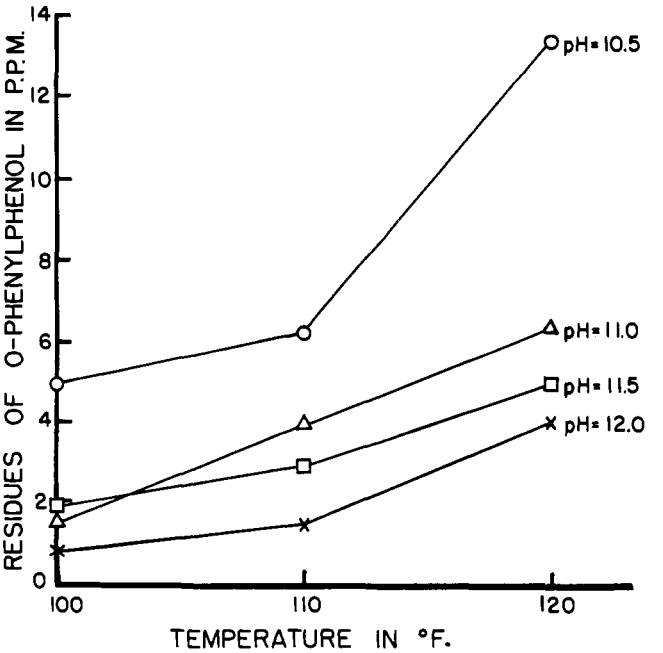

Figure 3. Effect of temperature on o-phenylphenol residues in oranges treated for 3 minutes with 0.1% Dowicide A at various pH levels

carried out in a bin-type hydrocooler (*3*) having cloth baffles for the fruit and a pump for flooding with fungicidal solutions maintained at 40° F. by mechanical refrigeration. Oranges were coated with a commercial, solvent-type wax and run directly into the hydrocooler for treatment. Samples were removed at scheduled times, packed wet into cartons, and stored at 70° F. for analysis and examination.

At higher temperatures, a flood-type, color-add tank with a thermostatically controlled steam coil for heating the solution was used. Fruit was placed on a roller conveyor running at a speed adjusted to give an exposure time of 3 minutes under the flooding solution, rinsed in a water spray, dried, waxed, packed in cartons, and stored at 70° F.

Fungicidal Solutions

A calculated amount of Dowicide A, a technical grade (97%) of the tetrahydrate of sodium *o*-phenylphenate, was added to a known volume of water in the tank to give a solution of the lowest concentration required for a given series of treatments. More Dowicide A was added when higher concentrations were desired in the same series.

The pH was adjusted by slowly adding a 25% solution of sodium hydroxide using the color change of pHydrion test papers as a measure of pH. Samples at the lowest pH were run first and the level was increased as necessary by addition of more alkali.

Residue Analysis

Two oranges were taken at random from each treatment sample, cut into pieces, and ground in a Waring Blendor. A 20-gram portion was transferred, using 90 ml. of water, to a 250-ml. flask containing 2 grams of citric acid and a drop of Dow Corning Antifoam B. The flask was placed on an electric heater and connected by a distilling head to a vertical condenser dipping into 25 ml. of 0.025% sodium carbonate solution in a 150-ml. beaker. Boiling was continued until 50 ml. had distilled, making a total volume in the beaker of 75 ml. This solution containing the fungicide was filtered, to remove peel oil, into a 100-ml. volumetric flask, 15 to 20 ml. of sodium carbonate solution being used for washing. This simplified steam distillation method gave an 85 to 90% recovery of *o*-phenylphenol from ground oranges at levels up to 8 p.p.m.

Color was developed by the method of Gottlieb and Marsh (*2*) using 4-aminoantipyrine and potassium ferricyanide. Absorption of standard and unknown solutions was measured at 500 mμ with a Bausch & Lomb Spectronic 20. A standard sample containing 160 γ of *o*-phenylphenol had an absorbance of 0.128 when read in a half-inch square cell. Results were calculated as parts per million of *o*-phenylphenol and corrected for the apparent 1.2 p.p.m. shown consistently by untreated oranges. Averages of the residues found in the two orange varieties were used for plotting graphs showing results of experiments. No varietal differences were observed.

Experimental and Results

Experiments were run in the hydrocooler at 40° F. using a Dowicide A concentration of 0.1% with samples of oranges removed at 15-minute intervals over a period of 1 hour. This series was repeated at four pH levels varying by increments of 0.5 pH unit from 10.0 to 11.5. Results in Figure 1 show that *o*-phenylphenol residues increase with length of treatment but approach or achieve a limit inversely related to the pH of the solution.

In a second low temperature series the concentration of Dowicide A was varied from 0.05 to 0.4% for a 30-minute treatment at 40° F. This series was run once without pH adjustment (approximately 10.0) and again at pH 12.0. Figure 2 shows that *o*-phenylphenol residues increase with concentration of Dowicide A, but are much lower at the higher pH level.

Three series of experiments were run at higher temperatures in the color-add applicator, with treatment time fixed at 3 minutes by the conveyor speed.

Oranges were treated with 0.1% Dowicide A solution at 100°, 110°, and 120° F. for pH levels of 10.5, 11.0, 11.5, and 12.0. The increase in residues of *o*-phenylphenol with temperature is shown in Figure 3, and the inverse relationship of residues to pH is again evident.

In a second series fruit was treated with 2.0% Dowicide A solution for 3 minutes at 90° F. at various pH levels between 11.0 and 11.8. The results shown in Figure 4 indicate the importance of pH control in determining the level of residues in oranges.

The final series was run at 110° F. with a pH of 11.5 for concentrations of Dowicide A ranging from 0.1 to 1.0%.

The straight-line curve which resulted shows that under the conditions of the experiment the *o*-phenylphenol residues vary directly with concentration of the treatment solution.

All samples were examined for chemical peel injury after storage for 1 week at 70° F. Only the few samples having residues of *o*-phenylphenol greater than 15 p.p.m. had appreciable peel injury.

Discussion

Residues of *o*-phenylphenol in oranges increase with concentration, temperature, and length of treatment but decrease as the pH of the Dowicide A solution is raised. By proper control of treatment conditions almost any desired level of residues can be maintained. The time-tested Dowicide A–hexamine method (*5*) gives good control of decay at residue levels of about 2 p.p.m. and the legal tolerance for *o*-phenylphenol in citrus fruits (*1*) is 10 p.p.m. As no peel injury was found at levels below the legal limit, residues in this range should give good decay control without injury. For hydrocooling of oranges, a 30-minute treatment at 40° F. with 0.1% Dowicide A at pH 10.5, resulting in a residue level of about 4 p.p.m., has been found satisfactory.

These experiments have shown the importance of pH control in the use of Dowicide A as a fungicide for oranges. This factor is especially critical, as the pH of the solution tends to decrease because of absorption of carbon dioxide from the air and reaction with citric acid from damaged fruit. Unless checked, this could lead to residues in excess of the legal tolerance, serious peel injury, and ultimate precipitation of the fungicide.

The pronounced effect of pH on residues is probably due to absorption by the fruit of fungicide in the form of free *o*-phenylphenol which is formed by hydrolysis of the sodium *o*-phenylphenate in solution. Lower pH would tend to favor this hydrolysis and higher pH to suppress it. The absorption of fungicide by waxed fruit in the hydrocooling experiment supports this view.

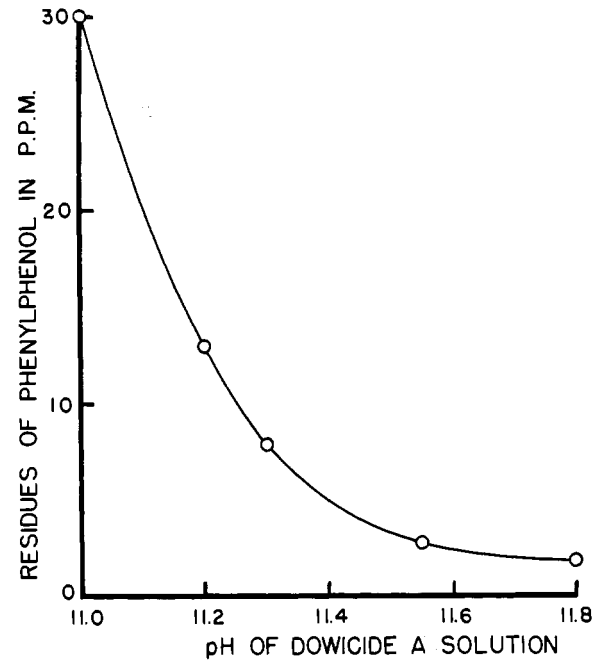

Figure 4. Effect of pH on o-phenylphenol residues in oranges treated for 3 minutes with 2.0% Dowicide A at 90° F.

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FERTILIZER TECHNOLOGY

High-Analysis Superphosphate by the Reaction of Phosphate Rock with Superphosphoric Acid

A. B. PHILLIPS, R. D. YOUNG, F. G. HEIL, and M. M. NORTON

Tennessee Valley Authority, Wilson Dam, Ala.

A NEW SUPERPHOSPHATE that contains about 54% available P_2O_5 has been introduced to the fertilizer industry by TVA. Because of its higher grade, this material is referred to as "high-analysis superphosphate." It is produced by acidulating phosphate rock with superphosphoric acid. Superphosphoric acid, also a TVA development, is an electric furnace acid that contains about 76% P_2O_5, which is equivalent to about 105% orthophosphoric acid (H_3PO_4). This compares with 54 to 56% P_2O_5 for the usual furnace and wet-process phosphoric acids used in the fertilizer industry. Chemically, the superphosphoric acid contains about 49% of its phosphate as orthophosphate, 42% as pyrophosphate, and the remainder as higher polyphosphates. TVA has done considerable work in studying the production, properties, and use of superphosphoric acid (*3, 5, 6*); first application was in liquid fertilizer processes.

In considering the use of superphosphoric acid in the production of super-

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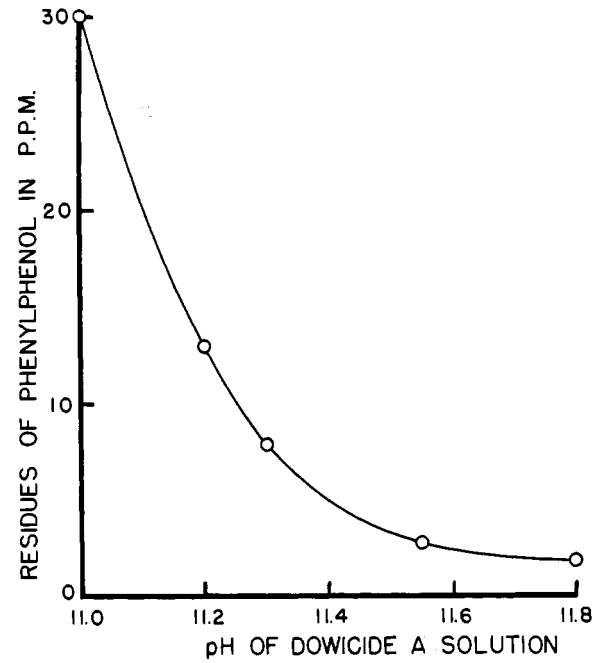

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In considering the use of superphosphoric acid in the production of super-

Production of high-analysis superphosphate (54 to 56% P_2O_5) by reaction of phosphate rock with superphosphoric acid was studied in bench scale, pilot plant, and plant scale equipment. Results were best when the acid concentration was about 74% P_2O_5 and the acid was preheated at 180° to 225° F. When the superphosphate was held in a den or storage pile, its temperature reached a maximum of 300° to 350° F. in about 40 minutes. This high temperature promoted fluorine volatilization and rapid conversion of the P_2O_5 to an available form. The superphosphate contained 1% or less of free moisture, and the P_2O_5 was chiefly in the form of anhydrous monocalcium phosphate. About 60% of the fluorine was evolved, compared with about 15% in the production of conventional triple superphosphate. The comparatively anhydrous nature of the product and evolution of fluorine compounds account for the substantially higher P_2O_5 content. Good ammoniating and processing characteristics have been demonstrated in pilot plant scale production of granular fertilizers. Economies should be realized in handling and shipping the more concentrated superphosphate and in the higher grades of fertilizers that can be prepared.

phosphate, some doubt of obtaining good results existed because conversion of the P_2O_5 in phosphate rock had been poor in earlier studies with stronger than usual phosphoric acid in the range of 80 to 90% H_3PO_4 (7). Also nothing was known about the acidulation properties of the nonortho acids in the superphosphoric acid. However, in exploratory small scale tests, conversion of rock P_2O_5 was high when the acid was diluted slightly to about 102% H_3PO_4 and heated to 170° F. prior to mixing with the rock. The superphosphate thus produced contained 54 to 55% available P_2O_5 when the proportions of acid and rock were about the same as those normally used for the production of triple superphosphate. This represents a substantial increase in grade, compared with 46 to 48% for conventional triple superphosphate. This paper describes the development of the process for production of high-analysis superphosphate.

Small Scale Studies

In the small scale tests, superphosphoric acid and pulverized phosphate rock in amounts sufficient to produce 1 to 2 pounds of superphosphate were mixed for 15 to 30 seconds in a kitchen mixer. The mixture was transferred to a Dewar flask, where it was held for an hour or longer to avoid loss of heat and to simulate denning. It then was removed from the flask and placed in a closed jar in an oven for curing at 250° to 300° F. for 3 days and 150° F. thereafter for a total of 2 to 4 weeks. In tests made to determine the setting time, or the time required for the superphosphate to become firm, the mixture was allowed to remain in the mixer bowl until it solidified. Samples were taken after simulated denning and after curing for various periods.

The superphosphoric acid was obtained from TVA plant operations at the usual strength of about 75.5% P_2O_5 (104.5% H_3PO_4) and used as received or diluted slightly with water to the desired concentration. The phosphate rock used in most tests was Florida land pebble that contained about 32% P_2O_5 and had been ground to the size commonly used in the prodution of superphosphate (75% −200 mesh). Chemical analysis typical of the rock used in the tests showed:

	%		%
P_2O_5	32.2	Al_2O_3	1.7
CaO	46.0	F	3.6
SiO_2	7.1	CO_2	2.8
Fe_2O_3	2.1	H_2O	0.8
Ignition loss	6.6		

Effects of Acid Concentration, Temperature, and Acidulation Ratio (Table I). At 0.98 acidulation ratio and 175° F. highest conversion was obtained with 102% H_3PO_4 concentration. Setting time decreased with decrease in acid concentration.

In tests with acid of 102% H_3PO_4 concentration and an acidulation mole ratio of 0.96, at 200° F. and above, the conversion was 90%. Varying the acid temperature proved an effective means of controlling the setting time.

At acid strength of 102% H_3PO_4 and acid temperature of 150° F., conversion increased from 75 to 99% as the acidulation ratio was increased to 1.10. The setting time, 135 to 180 seconds, was not greatly affected by changes in acidulation. The grade of the product increased from 48 to 56.5% as the acidulation mole ratio was increased from 0.85 to 1.10. Superphosphate made using an acidulation ratio higher than 1.05 was excessively sticky and was considered unsatisfactory.

Particle Size, Grade, and Type of Phosphate Rock. In a few tests with more finely pulverized Florida rock, the most pronounced difference was that the set time decreased with decrease in size of rock. Conversion increased slightly. In these tests, the P_2O_5:CaO mole ratio was 0.96 and the 102% acid was heated to 200° F.

Fineness of Phosphate Rock, % through 200 Mesh	Set Time, Sec.	Conversion, %
75	90	90
85	70	91
93	60	91
96	45	94

In tests with higher grades of Florida phosphate rock (34 to 35% P_2O_5), fluidity during mixing was increased and the set time of the acidulate was prolonged. Conversion was about the same as with 32% rock; however, as expected, higher grades of superphosphate were produced. The acid concentration was 102% H_3PO_4 and the P_2O_5:CaO mole ratio was 1.0.

P_2O_5 Content of Phosphate Rock, %	Grade of High-Analysis Superphosphate Produced, % Available P_2O_5
32	54
34	56
35	57

In a few tests with Wyoming and Idaho rocks of about the same grade and particle size as the ground Florida pebble (75% −200 mesh), results were similar except that, when the western rocks were used, the set time was about twice as long as with Florida rock under the same test conditions. Conversion was slightly lower with the western phosphates. Calcination of Idaho phosphate at about 1200° F. resulted in very hard and very dense superphosphate. It appeared to be too hard to cut from a den with the equipment generally used.

Temperature of Reaction. With acids of some concentrations, exceptionally high temperatures developed in the acidulates during the first hour after mixing, and copious evolution of fluorine was observed. Small scale tests, therefore, were made to study reaction temperature in more detail. The procedure used was the same as described. During

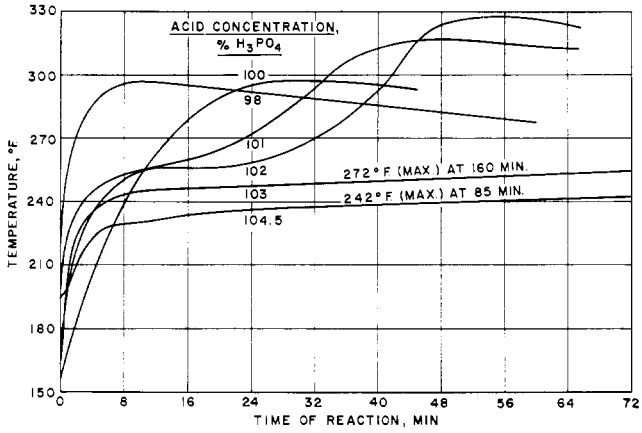

Figure 1. Temperature curves for acidulates of high-analysis superphosphates from acids of different concentrations

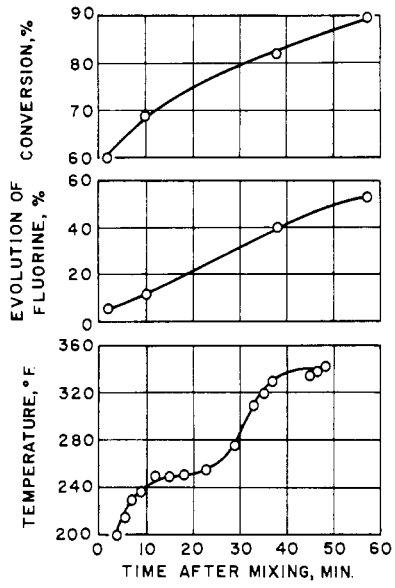

Figure 2. Effect of denning time on temperature, evolution of fluorine, and conversion of rock P_2O_5

Table I. Effect of Acid Concentration, Acid Temperature, and Acidulation Ratio on P_2O_5 Conversion and Setting Time

Acid % H_3PO_4	Acid Temp., ° F.	P_2O_5:CaO Mole Ratio	Setting Time, Sec.	P_2O_5 Conversion, %	Grade of Superphosphate, % Available P_2O_5
		Effect of Acid Concentration			
104.5	175	0.98	390	65	...
103	175	0.98	210	86	...
102	175	0.98	180	92	...
101	175	0.98	...	90	...
100	175	0.98	180	90	...
98	175	0.98	120	90	...
		Effect of Acid Temperature			
102	125	0.96	240	83	...
	150	0.96	180	87	...
	175	0.96	120	89	...
	200	0.96	90	90	...
	225	0.96	75	90	...
	250	0.96	75	90	...
	275	0.96	45	90	...
	300	0.96	45	90	...
		Effect of Acidulation Ratio			
102	150	0.85	135	75	48
		0.90	135	78	50
		0.95	180	85	52
		1.00	150	93	54
		1.05	180	97	55
		1.10	150	99	56 5

the simulated denning period, the temperature of the material in the Dewar flask was measured at 5-minute intervals for 45 to 160 minutes until the maximum temperature was reached. Acidulates were prepared with acids containing the equivalent of 98, 100, 101, 102, 103, and 104.5% H_3PO_4. The acid was heated to 225° F. and an acidulation mole ratio of 0.98 was used in each test. Curves showing the time-temperature relationship for the various acid concentrations are given in Figure 1.

Acid concentration had a pronounced effect on the maximum temperature and the time required to reach the peak temperature after mixing.

In subsequent work, the autogenous rise in temperature of the acidulate was found to be very important in obtaining good conversion and high grade of product and to have a pronounced effect on the evolution of fluorine during processing and storage. Pilot plant tests were made to study further the temperature rise phenomena and their effects on the physical and chemical properties of the superphosphate.

Pilot Plant Tests

The pilot plant, which has been described in detail (*8*), consisted of a cone mixer and a continuous den of the Broadfield type. Capacity was about 1 ton per hour. Tests were made under conditions that had given best results in small scale tests; superphosphoric acid diluted to 102% H_3PO_4 was used to acidulate pulverized phosphate rock (32% P_2O_5, 75% −200 mesh) at a P_2O_5:CaO mole ratio of 0.98 to 1.05. The acid was heated to 170° F. prior to mixing with the rock.

The unit was operated at a production rate to 1 ton per hour, with the speed of the den regulated to allow a retention time of about 1 hour. The block of superphosphate formed in the den was about 18 inches deep and 24 inches wide. The material was very porous and was friable after a few minutes in the den. Temperature of the block was measured periodically, and samples were taken 2, 10, 38, and 57 minutes after mixing. The samples were crushed to simulate cutting and were spread out to cool to interrupt the high-temperature reaction. These samples were submitted for chemical analyses to determine conversion and fluorine content. Data showing the time-temperature curve obtained, the extent of the fluorine evolution, and conversion at the various times are plotted in Figure 2.

The temperature curve was similar to the one obtained in small-scale tests with 102% acid. Conversion was 90% immediately after denning and increased to 92% after 24 hours in a storage pile.

The pilot-plant product was much more porous and, consequently, had a considerably lower bulk density than conventional triple superphosphate as it solidified in the den. It was very friable and could be cut from the den by the usual method. The bulk density was about 25 pounds per cubic foot while in the den and 35 pounds after cutting from the den. After milling in a cage mill and screening at 6 mesh, the bulk density was about 55 pounds per cubic foot, which is not much lower than that of other superphosphates. Somewhat larger denning and storage capacity than is needed for conventional triple superphosphate would be required for proc-

essing high-analysis superphosphate because of its low bulk density before milling.

Recovery of fluorine from the den has been studied extensively in the pilot plant (*2*).

Plant Scale Experience

High-analysis superphosphate was produced on a demonstration scale in the plant used previously to make conventional triple superphosphate from electric furnace phosphoric acid. The facilities, which were used with little modification, included a TVA cone mixer, a "wet belt" for retention of the material until it solidified, and conveying equipment to pile-storage curing. Although the product was satisfactory, the plant was inadequate in some respects. The cone mixer proved satisfactory. However, the lower bulk density and longer set time of the acidulate limited the capacity of the wet belt and it was necessary to operate at a considerably reduced rate of production. A large part of the fluorine was evolved after the material reached the storage piles; the maximum temperature occurred in the storage piles, because the material reached storage only about 10 minutes after mixing. Disposal or recovery of fluorine was impractical under these conditions. The product contained about 54% available P_2O_5 and conversion of the P_2O_5 in the phosphate rock averaged 92 to 94%.

Modified demonstration scale plant facilities that will overcome these deficiencies and provide part of the production as granular material are being constructed. A semigranular material will be produced by acidulating phosphate rock in a rotary drum. The plant will have facilities for denning the superphosphate to allow best conditions for reaction and to facilitate collection and recovery of fluorine. The granular and nongranular products will be obtained by screening the semigranular material after pile curing.

Production of High-Analysis Granular Fertilizers

High-analysis superphosphate was used in a pilot plant continuous ammoniator-granulator as a source of phosphate in formulating granular fertilizers. Several grades including 13–13–13, 6–24–24, 12–24–12, and 13–39–0, which are higher in analysis than can be produced with conventional triple superphosphate, were produced satisfactorily. Substantial tonnages of high-analysis superphosphate have been used in several commercial scale ammoniation-granulation plants, and good results have been reported in formulating a variety of grades. The degree of ammoniation has been about 3.5 pounds of ammonia per unit of available phosphate.

In addition to the advantage of using high-analysis superphosphate in formulating higher analysis mixed goods, this superphosphate is of economic advantage in formulations for conventional grades, because larger proportions of ordinary (20%) superphosphate can be used. It also is advantageous in granulating some grades because of its low moisture content and the high heat of reaction obtained during ammoniation. High-analysis superphosphate has been reported to be much less dusty than the usual superphosphates, and this has been a particular advantage in bulk handling in fertilizer plants.

Chemical Composition and Characteristics

Chemical Analysis of High-Analysis of Superphosphate from Demonstration Plant

	Wt. %		Wt. %
P_2O_5		CaO	21.2
Total	55.4	F	0.7
Available	54.3	Free moisture	1.2
Water-soluble	53.0		
Free acid	3.2		

High-analysis superphosphate usually contains only about 1% of free moisture and is mainly in the form of anhydrous monocalcium phosphate. Only small quantities of nonorthophosphates have been found in the product. The low proportion of free moisture and water of hydration accounts largely for the higher grade. The greater evolution of fluorine, mainly as SiF_4, adds further to the increased grade. Only 10 to 15% of the fluorine in the rock is released in making conventional triple superphosphate, while about 60% is released in making high-analysis superphosphate. The water solubility of the phosphate usually is 92 to 95%, somewhat higher than for conventional triple superphosphate.

High-analysis superphosphate is more hygroscopic than conventional triple superphosphate and will absorb moisture from the air, particularly if small samples are exposed for extended periods during humid weather. The absorption of moisture will decrease the grade of the product but not impair its physical condition. Care is exercised to avoid undue exposure of samples of the material during preparation and handling for chemical analysis, to minimize the effect of absorption of moisture on the analytical results.

Storage and Handling Properties

High-analysis superphosphate stored very well in bulk. It caked less than conventional triple superphosphate. The lumps that formed were easy to crush, and the fine material was not as dusty as the usual superphosphates. Although it absorbed moisture more readily than conventional superphosphate, the absorption was confined to the outer layer of the storage pile. The absorption of moisture during storage did not result in the formation of wet, sticky material, as a large part of the moisture combined chemically with the anhydrous monocalcium phosphate as water of hydration.

Bag-storage tests were made of nongranular high-analysis superphosphate that had been cured in storage piles for 2 to 3 months. The bags were stored in stacks 12 bags high in a well-ventilated building. At the time of inspection, the bags were dropped four times from a height of 3 feet to simulate handling in regular plant and shipping operations. Any failure of the bags on dropping was noted and the amount of +2-mesh lumps remaining after dropping was determined as a measure of caking.

Storage in plain paper bags (no moisture barriers) was unsatisfactory. Because of the hygroscopicity of the superphosphate, it absorbed water through the bag walls and this resulted in lowering of the grade and in caking. The paper became dry and brittle and failed in a short time. This deterioration of the bag was apparently due to dehydration, the presence of free acid, and the continued slow release of fluorine. Fertilizer bags with one or two asphalt-laminated plies gave considerably better results, but failed in about 2 months. Bags with a polyethylene-coated inner ply failed along the bag folds; the rest of the bag was not attacked. It was found that folding of the bags had caused minute breaks in the polyethylene film and that this permitted passage of fluorine-bearing gas to the outer plies with resultant weakening and failure. Untreated cotton thread used in sewing bags containing high analysis superphosphate usually deteriorated during storage. Waxed cotton thread and Dacron thread were satisfactory. Little or no caking of the superphosphate occurred in any of the bags with asphalt-laminated or polyethylene moisture barriers.

Deterioration of bags could be decreased by mixing the cured superphosphate with 3% by weight of pulverized calcined dolomite or 5% by weight of pulverized limestone; however, dilution as a result of these treatments reduced P_2O_5 concentration of the product by 1.5 to 2.5 units. More promising results were obtained when the cured superphosphate was conditioned with 0.5 to 1.0% by weight of ammonia. Bag deterioration was decreased markedly and the available P_2O_5 content of the product was not decreased more than 1 unit by dilution and loss of availability. Treatment of cured product with 0.5%

of ammonia reduced free-acid P_2O_5 content from about 3% to about 1%. Use of 1% of ammonia reduced free-acid P_2O_5 to less than 0.5%. It is believed that conditioning with ammonia will satisfactorily solve the bag-storage problem.

Production of Granular High-Analysis Superphosphate

High-analysis superphosphate also has been produced in a granular form in pilot plant tests. Acidulation and granulation were accomplished simultaneously in a rotary drum by a technique similar to the one-step process developed by TVA for production of granular triple superphosphate (*4*). Granulation was controlled more easily in production of the high-analysis superphosphate. No recycle was required for control, because of the very low moisture content and rapid set time of the acidulate. The primary control of granulation was through regulation of the temperature of the acid. The acid was heated to a range of 225° to 300° F. and fed at a concentration equivalent to about 102% H_3PO_4. The superphosphoric acid was diluted to about 102% H_3PO_4 either in the feed tank or by continuous addition of water in a mixing wye on the acid distributor. Operation was satisfactory with proportioning of acid and rock to give P_2O_5:CaO mole ratios of 0.98 to 1.05.

Tests were made in the pilot plant with the distributor for acid located above the bed of material in the drum and located beneath the bed. Although results were reasonably good with the distributor above the bed, areas of excessive stickiness sometimes resulted and impaired granulation efficiency. The use of a submerged distributor that contained 20 holes 3/32 inch in diameter, which distributed acid over about the first half of the length of the drum, gave best results. The addition of about 40 pounds of steam per ton of product through a parallel distributor beneath the bed also assisted in control of granulation. Use of distributors beneath the bed allowed satisfactory operation with acid temperature as low as 225° F.; a temperature of 275° to 300° F. was required when the acid was distributed above the bed. With distributors beneath the bed, best results were obtained with a rotational speed of the drum of about 35% of critical speed. Critical speed in revolutions per minute is determined by the relationship, $76.5/\sqrt{D}$, where D is the diameter of the drum in feet (*1*). A higher rotational speed of 45 to 50% of critical speed was best when the acid was distributed above the bed.

With the distributors beneath the bed, it was possible to control granulation to produce a high percentage of granular product in the −6- +20-mesh range or to produce a semigranular product containing about half granular material and half fines. The fine (−20-mesh) material produced in this manner ammoniated satisfactorily in the production of granular high-analysis fertilizers. The semigranular product could be screened to provide both granular superphosphate for direct application and fine material for ammoniation and use in complete fertilizer formulations.

Data for a typical test of the production of granular high-analysis superphosphate in the pilot plant are given in Table II.

As in the production of nongranular material, it was necessary to den the granular product at least 1 hour to obtain good conversion and a high product grade. The temperature of the granular product increased to a range of 300° to 350° F. during denning. The amount of fluorine evolved increased as the denning time was increased. The percentage of fluorine evolved, conversion, and grade of product at various denning times and after curing for 3 days are shown in the following tabulation. The acid concentration was 102% H_3PO_4 and the acid P_2O_5:CaO mole ratio was 0.98.

Time of Denning, Hr.	Evolution of Fluorine, %	Conversion, %	Grade, % Available P_2O_5
1	52	87	52.5
8	54	89	53.5
12	56	89	54
20	60	90	54
Cured 3 days at 250° F.	66	92	55

In producing the granular material, screening should be deferred until denning is completed or until after pile storage for a few days. This allows maximum evolution of fluorine and high denning and curing temperatures, which are favorable to conversion.

Bulk density of the granular product is about 65 pounds per cubic foot and the angle of repose is about 38°.

Materials of Construction

Type 316 stainless steel was satisfactory for the cone mixer for high-analysis superphosphate and for pumps and piping for handling the superphosphoric acid up to 200° F. Mild steel was satisfactory for the granulation drum in the pilot plant. Corrosion test data indicate that red brass should be satisfactory for handling heated superphosphoric acid at 250° to 300° F.; the corrosion rate was about 15 mils per year. Hastelloy B had a corrosion rate of 1 mil per year or less in this service. Stainless steels showed excessive corrosion in contact with superphosphoric acid at 250° F. and higher.

Conclusions

Superphosphoric acid can be used to acidulate phosphate rock to produce superphosphate of unusually high analysis. Both granular and nongranular products can be produced in equipment commonly used in the fertilizer industry. Heating of the acid and provisions for denning the material for at least 1 hour are required to obtain satisfactory reaction and to facilitate fluorine recovery, which is expected to become of more and more importance in the future. The high proportion of fluorine evolved, about 20 pounds per ton of high-analysis superphosphate, should make recovery particularly attractive.

Savings should result in handling and shipping the more concentrated superphosphate and the higher analysis grades of complete fertilizers that can be formulated with it. Additional economy in formulating the usual grades should result because more ordinary (20%) superphosphate can be used. The low moisture content, higher heat of reaction during ammoniation, and the fact that high-analysis superphosphate is less dusty in handling also are of importance in its use in fertilizer formulations. Satisfactory bulk handling has been demonstrated, and promising results have been obtained in treating the material to prevent excessive attack on bags.

Table II. Production of Semigranular High-Analysis Superphosphate

Production rate, tons/hr.	1.7
Formulation, lb./ton product	
Phosphate rock (32.3% P_2O_5, 46.4% CaO, 0.5% H_2O)	921
Phosphoric acid, 102% H_3PO_4 (73.9% P_2O_5)	1047
Steam	30–60
Temperature, ° F.	
Acidulator product	326
Acid preheat	276
Screen analysis (Tyler), %	
+6 mesh	15.4
−6 +10 mesh	12.5
−10 +16 mesh	38.8
−16 +20 mesh	6.3
−20 +28 mesh	10.4
−28 mesh	16.6
Chemical analysis of product after curing 4 days at 250° F., %	
Total P_2O_5	55.8
Available P_2O_5	54.9
W.S. P_2O_5	52.1
Free acid P_2O_5	3.8
F	0.5
CaO	21.1
H_2O	2.0
Net conversion of P_2O_5 from rock, %	
Denned 20 hr.	89
Cured 4 days at 250° F.	94
Further cured 10 days at 150° F.	94
Fluorine evolved, %	
Denned 20 hr.	52
Cured 4 days at 250° F.	60
Further cured 10 days at 150° F.	60

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plant tests, and George Hoffmeister, Jr., who supervised conditioning and storage tests. They are indebted also to T. P. Hignett, chief of the Applied Research Branch, whose advice helped direct the course of the work. A large part of the analytical work was done by R. D. Duncan and L. J. Sheffield.

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FERTILIZER TECHNOLOGY

Calcium Superphosphate Manufacture by a Low Acidulation-Quick Conversion Process

SHAW-CHEN LEE and CHIU-CHIN LIN

Factory No. 2, Taiwan Fertilizer Co., Ltd., Keelung, Taiwan, China

A low acidulation and quick conversion process for the manufacture of calcium superphosphate was developed on a pilot plant scale, starting with acidulation of phosphate rock with a comparatively low acid-rock ratio. The acidulated mass was then autoclaved, concentrated, and converted under controlled conditions. Good conversion was obtained in various experiments, and the product contained 3 to 7% of citrate-soluble phosphorus pentoxide and 11 to 15% of water-soluble phosphorus pentoxide with excellent physical properties.

THE CONVENTIONAL PROCESS for calcium superphosphate requires acidulation of phosphate rock for the formation of monocalcium phosphate and subsequent curing of the green product for 4 to 6 weeks. To save curing space and time, a quick-curing process (*2, 4*) was developed. To save the mineral acid required in acidulation, experimental work on dicalcium phosphate has been done. Based on previous work on dicalcium phosphate (*3*) and superphosphate (*7*), a low acidulation–quick conversion process for the manufacture of calcium superphosphate is presented.

Materials, Equipment, and Methods

Three phosphate rock samples and crude chamber sulfuric acid were used (Tables I and II).

The equipment (Figure 1) consisted essentially of an autoclave reactor and an electrically heated paddle-type continuous converter. The function of the reactor was to perform the acidulation and hydrolysis reactions and that of the converter was to evaporate and to convert the partially hydrolyzed mixture.

Total water was determined by heating 5 grams of the sample at 99 to 101° C. for 5 hours. For free acid determinations a 2-gram sample was extracted with 50 ml. of 95% alcohol instead of acetone. The total, citrate-insoluble, and water-soluble phosphorus pentoxides were determined by the AOAC volumetric titration method (*1*), calcium oxide and combined oxides by the usual method, and fluorine by the Willard-Winter method (*6*).

Table I. Analysis of Phosphate Rock

	Phosphate Rock		
	P_1, Florida	P_2, Florida	P_3, Morocco
Chemical Analysis (Air-Dry Basis), %			
Total P_2O_5	34.23	33.95	34.07
Citrate-insol. P_2O_5	31.09	31.29	...
CaO	49.30	...	50.90
R_2O_3	2.52	...	0.65
CO_2	3.21	...	3.89
F	3.87	...	3.94
Screen Analysis (Tyler Standard), Weight %			
Mesh			
+ 60	...	6.00	3.32
− 60 + 80	6.0	6.60	5.73
− 80 +100	1.5	8.40	5.03
−100 +150	12.0	18.85	29.11
−150 +200	13.5	11.15	22.35
−200	67.0	49.00	34.46

Table II. Analysis of Crude Chamber Sulfuric Acid

(Product from Factory No. 2)

	%
H_2SO_4	62.53
Total solids	0.15 –0.35
Suspension solids	0.04 –0.05
Total Fe	0.0053–0.00248
Soluble Fe	0.0047–0.00246
N_2O_3	0.023 –0.03
As	0.0259–0.0929

Procedure

Phosphate rock and 50° Be'. sulfuric acid were mixed, and water was added in the reactor which was made vapor-tight. The reactor was then kept at 100° to 105° C. by the embedded electric heater for the hydrolysis of the mixture. Speed of the stirrer in the reactor was 1300 to 1500 r.p.m. After heating, the material was discharged through the bottom outlet of the reactor into the paddle converter, the temperature of which had been regulated in the range of 110° to 150° C. by rheostat. The material was then continuously dried and

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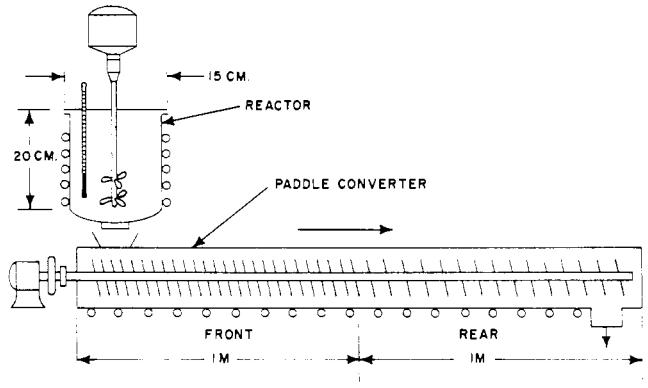

Figure 1. Arrangement of pilot plant

Reactor. 304 stainless steel with embedded Nichrome wire heater
Paddle converter. Casing and paddle of mild and 304 stainless steel, respectively. Nichrome wire heater, 1 kw. for front section and 0.8 kw. for rear section. Pitches of paddles, 3.5 and 7 cm. for front and rear sections, respectively. Rotating speed of paddle shaft, 73 seconds per revolution. Driving motor, 1 hp.

Table III. Effect of Conversion Temperature,[a] Conversion Time,[b] and Number of Cycles[c]

Expt.	No. of Runs	Converter	Conversion Temp., ° C.	Moist., %	Acidity, %	P_2O_5 in Product, %					Availability, %
						Total	Citrate-insol.	Available	Water-sol.	As DCP[d]	
					SERIES A[e]						
T-147	...	Oven	90–5, 4 hr.	16.78	...	19.51	3.55	15.96	12.96	3.00(15.4)	81.8
T-140	...	Pad.	127–37	1.82	1.64	23.28	4.81	18.47	12.53	4.93(21.2)	79.3
		Oven	150, 30 min.	1.75	1.94	23.75	5.76	17.99	11.88	6.11(25.7)	75.8
T-134	...	Pad.	156–9	2.83	0.95	22.48	4.71	17.77	13.09	4.68(20.8)	79.0
		Oven	130, 30 min.	2.31	0.92	22.70	4.21	18.49	14.62	3.87(16.7)	81.5
T-133	...	Pad.	150–8	3.59	1.11	22.32	4.57	17.75	13.40	4.35(19.5)	79.7
					SERIES B[f]						
T-148	...	Oven	90–5, 4 hr.	17.22	...	18.53	3.01	15.52	11.84	3.68(19.9)	83.8
T-139	...	Pad.	125–35	1.63	1.56	22.32	3.60	18.72	14.36	4.36(19.6)	84.0
		Oven	150, 30 min.	1.32	1.45	23.01	3.80	19.18	14.14	5.04(21.9)	83.0
T-138	...	Pad.	122–38	2.41	1.26	21.95	3.21	18.74	15.00	3.74(17.1)	85.00
		Oven	150, 30 min.	0.74	2.15	22.61	3.61	19.00	13.91	5.09(22.5)	84.1
T-132	...	Pad.	143–57	1.41	2.40	22.75	3.72	19.03	11.13	7.90(34.8)	83.1
		Oven	130, 30 min.	3.74	1.32	22.28	2.47	19.81	15.95	3.86(17.3)	88.8
T-131	...	Pad.	160–55	1.97	2.29	21.63	3.73	17.90	10.92	6.98(32.4)	82.8
					SERIES C[g]						
	No. of Passages										
T-142	1	...	110–38	2.70	1.34	22.96	4.42	18.54	14.43	4.11(17.9)	80.8
	2	...	112–39	1.00	1.66	23.49	5.39	18.10	11.63	6.53(27.8)	77.1
T-143	1	...	112–35	2.63	1.19	22.67	4.93	17.73	13.29	4.45(19.6)	78.2
	2	...	111–34	1.06	1.56	23.40	5.82	17.58	10.78	6.80(29.1)	75.2
					SERIES D[h]						
T-144	1	...	105–30	4.57	1.45	21.27	3.24	18.03	14.70	3.33(15.6)	84.7
	2	...	109–34	2.72	2.01	22.45	3.90	18.55	12.98	5.57(24.8)	82.5
T-145	1	...	115–39	3.24	1.94	22.12	3.65	18.47	14.09	4.38(19.8)	83.7
	2	...	116–40	2.48	2.69	22.18	4.11	18.70	12.18	6.52(29.4)	82.1
					SERIES E[i]						
	No. of Cycles										
T-146	1	...	120–10	2.64	2.07	21.23	3.21	18.02	15.08	2.94(13.9)	84.8
T-147[j]	2	...	119–14	2.31	1.55	21.31	2.91	18.40	14.58	3.82(17.9)	86.3
T-148	2	...	121–15	2.42	1.41	21.49	2.89	18.51	14.67	3.84(17.9)	86.1
T-149	1	...	118–08	2.98	2.18	21.12	3.35	17.77	14.65	3.12(14.8)	84.2
	2	...	120–11	3.23	1.80	20.90	2.81	18.09	14.45	3.64(17.4)	86.7
	3	...	128–35	2.56	1.96	21.02	2.48	18.54	13.94	4.60(21.9)	88.3
T-151	1	...	120–18	3.42	2.53	21.00	3.25	17.75	14.94	2.81(13.4)	84.6
	2	...	118–31	2.91	2.08	21.13	2.81	18.32	14.99	3.33(15.8)	86.8
	3	...	126–29	2.36	1.53	21.30	2.53	18.77	15.00	3.77(17.7)	88.2

[a] Experimental conditions for A and B. One cycle. One passage through converter. Hydrolysis time, 5 hours. Hydrolysis temp., 100–20° C.
[b] Experimental conditions for C and D. Hydrolysis time for 1st cycle, 5 hr. Hydrolysis temp., 100–03° C.
[c] Experimental conditions for E. Hydrolysis time. For 1st cycle 5 hr., for 2nd and 3rd cycles 1 hr. Hydrolysis temp. 100–03° C.
[d] Dicalcium phosphate. Figures in parentheses indicate percentage on total P_2O_5 basis.
[e] Acidulation mole ratio 1.9, phosphate rock (P_1) 1430 g., H_2SO_4 50° Be.' 1040 g., water 500 cc. (free moist. 30%).
[f] Acidulation mole ratio 2.1, phosphate rock (P_1) 1430 g., H_2SO_4 50° Be.' 1140 g., water 500 cc. (free moist. 30%).
[g] Acidulation mole ratio 1.9, phosphate rock (P_2) 1430 g., H_2SO_4 50° Be.' 1020 g., water 500 cc. (free moist. 30%).
[h] Acidulation mole ratio 2.1, phosphate rock (P_2) 1430 g., H_2SO_4 50° Be.' 1120 g., water 500 cc. (free moist. 30%).
[i] Acidulation mole ratio 2.1, phosphate rock (P_3) 1072.5 g. H_2SO_4 50° Be.' 847.5 g.
[j] Grinding of 1st cycle product, all passing 80 mesh.

Table IV. Average Results of Conversion

Acidulation Ratio, Moles H_2SO_4/ Mole P_2O_5	No. of Cycle	Product Moisture, %	Product Acidity, %	% P_2O_5 in Product					P_2O_5 Availability %
				Total	Citrate-insol.	Available	Water-sol.	DCP	
1.9	1	2.15	1.24	22.74	4.09	18.05	13.55	4.50	79.4
2.1	1	2.98	2.18	21.12	3.35	17.77	14.65	3.12	84.2
	2	3.23	1.80	20.90	2.81	18.09	14.45	3.65	86.7
	3	2.56	1.96	21.02	2.48	18.54	13.94	4.60	88.3

Table V. Comparison of Results of Proposed Process with Conventional Process

Phosphate Rock, P_3			Acidulation Ratio			P_2O_5 Conversion, %	
	Composition, %	Gram Moles/Kg. Rock	Gram Moles/Kg. Rock				
			Theoretical	Actually used	% theoretical	Result obtained	Conventional
P_2O_5	34.07	2.4	4.8	2.1 mole ratio series			
CaO	50.90	9.08	1.53				
F	3.94	0.35[a]					
			6.33	5.04	79.6	88.3	110.6

[a] As SiF_6.

granulated in the converter. In normal operation the material took 60 minutes to pass through the paddle converter.

Results

Experiments were made with an acidulation mole ratio equivalent to 1.9 and 2.1 moles of sulfuric acid per mole of phosphorus pentoxide, and a heating period of 5 hours for the first cycle and 1 hour for subsequent cycles in the autoclave for each series of experiments. The conditions for the acidulation step were kept almost constant as specified in each series of experiments. Other conditions of acidulation and heating time have been tried with poor results. Impurities in acidulation gave obscure experimental results. Results in Table III are arranged according to the order of the influence of varying conditions.

Effect of Conversion Temperature

In this series of experiments, conversion temperature for the partially hydrolyzed mixture varied from 95° to 150° C. (Table III, A and B). For comparison of performance, the conversion step for each hydrolyzed mass was run in parallel with both the continuous paddle converter and the laboratory electric drying oven with automatic temperature-controlling device. These results show that a suitable conversion temperature is 110° to 130° C., while the formation of dicalcium phosphate increases with increasing temperature and time of conversion. Apparently reversion of available phosphate occurred above 130° C. in the continuous paddle converter.

Effect of Conversion Time

Conversion time for the hydrolyzed mass was varied from 1 hour for single passage through the continuous paddle converter to 2 hours for double passage, by which the product discharged from the first passage was subjected to second passage through the paddle converter without intermediate grinding and moistening (Table III, C and D). Although time intervals for conversion shorter than 1 hour have not been tried, time required for conversion between 110° and 130° C. in the paddle converter should be from 30 minutes to 1 hour. Citrate-soluble phosphorus pentoxide mainly as dicalcium phosphate increased from 3% to around 7% at the expense of total phosphorus pentoxide when the first passage product was put through the double passage.

Effect of Number of Cycles

Experiments were made with an acidulation mole ratio of 2.1, and recycling of hydrolysis and conversion steps were carried out without intermediate grinding of the first cycle product in most instances (Table III, E). Up to 3 cycles of processing an increment of more than 2% in phosphorus pentoxide availability and 3% in citrate-soluble phosphorus pentoxide can be effected through each cycle.

Conversion temperature seems to be the most important factor influencing phosphorus pentoxide availability. The resultant data are averaged in Table IV.

The product from the continuous converter had good physical properties with a granular size yield of around 70%. The screen analyses of the product from experiments 143 and 145 are:

Size, Tyler Mesh		Wt., % T-143	Wt., % T-145
	+ 3	14.1	16.8
− 3	+10	62.5	63.7
−10	+20	12.1	11.3
−20	+35	4.4	4.4
−35		6.9	3.8

Discussion

When the proposed process is considered as underacidulation in which calcium fluorosilicate in rock does not consume sulfuric acid, the degree of acidulation and resultant conversion of the proposed process are compared with those of the conventional process in Table V.

As compared with the conventional process for calcium superphosphate, the proposed process requires an acidulation mole ratio of around 2.1 instead of 2.6 and a reaction time of about 10 hours instead of 4 to 6 weeks for curing. Although the loss in insoluble phosphorus pentoxide and the processing and equipment cost of the proposed process may seem higher than those of the conventional process, the net savings of about 15% in acidulation and in the costs of storage, curing, handling, and granulation should be appreciable.

As compared with the process for dicalcium phosphate, the proposed process seems to be more convenient, direct, and feasible, with comparable results.

The proposed process for calcium superphosphate includes direct acidulation, hydrolysis, and thermal conversion performed in a simple and continuous operation. Because good conversion can be obtained with less sulfuric acid, the process seems to be adaptable for superphosphate manufacture.

As the process converts only 15 to 30% of the phosphorus pentoxide in phosphate rock to dicalcium phosphate and the remainder to monocalcium phosphate, the product should be less liable to water loss than ordinary superphosphate and more effective than dicalcium phosphate fertilizer. However, this experiment is only a preliminary new approach to calcium superphosphate. Process conditions and plant equipment need to be further explored and developed.

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TRACE ELEMENTS ANALYSIS

Preparation of Plant Tissues for Micronutrient Analysis. Removal of Dust and Spray Contaminants

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Procedures for removal of contamination from dust and spray residues from plant tissues prior to analysis for micronutrients are described. Three herbaceous plants—rye, tomato, and sugar beet—were dusted with soil or sprayed with a simulated Bordeaux spray containing copper, iron, manganese, molybdenum, and zinc. Effectiveness of removal of contamination by dilute hydrochloric acid or dilute hydrochloric acid plus detergent was measured. Dust contamination was removed satisfactorily by both wash solutions. Spray contamination was difficult to remove by washing. The wash solutions and procedures used did not result in loss of elements from within the tissues.

REPORTS on the need for removal of surface contamination from plant tissue prior to determination of micronutrients have largely been confined to work with iron in tissues such as leaves of citrus (*7*) and pears (*2*). Jacobson (*2*) considered it necessary to wash corn and tobacco leaves with dilute acid prior to iron determination, and Jacobson and Oertli (*3*) washed sunflower leaves with 2% Dreft detergent solution for the same purpose. Nicholas, Lloyd-Jones, and Fisher (*6*) studied methods of removing surface contamination from tomato tops and showed that immersion of fresh tissue in a detergent (0.37% Teepol) or in detergent and then 0.25*N* hydrochloric acid, followed by distilled water rinsing, removed iron contaminant from leaf surfaces without causing loss of potassium, phosphorus, manganese, or internal iron (tagged with iron -59).

This paper describes studies designed to obtain additional information, for three herbaceous species, on the efficiency of procedures for removing contamination with manganese, copper, zinc, and molybdenum as well as iron. This information is of consequence because of increasing recognition of the need for reliable chemical analyses in order to assess potential micronutrient deficiencies in annual crops where the analytical hazard from soil and spray residue contamination is large.

Because of the nature of the foliar sample—usually small and easily bruised leaves—it did not appear that procedures recommended for cleaning surfaces of tree leaves would be generally applicable. Smith, Reuther, and Specht (*7*) and others have recommended either scrubbing citrus leaves with a fiber-bristled hand brush or wiping with moistened clean cloth, and Jacobsen (*2*) rubbed pear leaves with the fingers in 0.3*N* hydrochloric acid. These procedures would be time-consuming when applied to tissues such as grasses or tomato leaves and also subject to the risk of damaging the tissue and permitting leakage of ions from the interior. Therefore, the procedure of Nicholas *et al.* (*6*), of simply immersing and agitating the samples in the washing and rinsing solutions, was studied to determine whether surface contamination could be removed from the species under consideration.

The question of whether elements are leached from within plant materials is usually raised when decontamination by washing is suggested. In a recent review of foliar leaching, Tukey, Tukey, and Wittwer (*8*) presented data which show that the continuous leaching of plant leaves with distilled water for periods up to 24 hours results in the loss of appreciable quantities of some absorbed nutrients. However, their data show that the leaching losses are a direct function of time, and that losses to be expected even in 2 hours' leaching from detached leaves appear to be 5% or less, depending upon the element and plant species studied. Their results therefore support the conclusions of Nicholas *et al.* (*6*) and of the authors (as will be shown) that there is negligible hazard of leaching losses during washing procedures of short duration (2 minutes in this study).

Materials and Methods

The three species used—sugar beet, rye, and tomato—represent a variety of leaf types. The plants were cultured on nutrient solutions according to the procedure of Johnson *et al.* (*4*) in a relatively dust-free greenhouse (filtered ventilating air and concrete floor), and were about 2 months old when contaminated with dust or spray. Six 18-liter containers of nutrient solutions were provided for each species. For tomato and sugar beet, six plants were grown in each container; for rye there were 18 plants per container.

Within each species, plants were randomized among containers just before application of contaminants. This precaution to minimize the effects of possible variation between culture solutions was perhaps unnecessary, as the media were carefully made up and growth for a given species was visibly uniform.

Contamination Treatments

The plants of two containers of each species were retained uncontaminated as controls. Those of two other containers were sprayed with a Bordeaux-type spray containing 1 gram per liter

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The plants of two containers of each species were retained uncontaminated as controls. Those of two other containers were sprayed with a Bordeaux-type spray containing 1 gram per liter

of copper, zinc, manganese, iron, and molybdenum, prepared by dissolving ammonium molybdate in water together with ferrous, manganous, and zinc sulfates, adding an amount of commercial Bordeaux dry mixture (Bordox) equivalent to 1 gram of copper, and finally neutralizing to pH 7 with calcium carbonate.

The spray used was about one third as concentrated (0.1% w./v. of each element) as commercial Bordeaux sprays (approximately 0.3% copper, w./v.). The application was made to the runoff point. Therefore the degree of contamination is approximately that which might be encountered in agricultural practice. The composition of this spray material is unusual; the purpose of preparing such a contaminant was to provide a severe test of the washing procedures.

Plants in the remaining two containers were lightly sprayed with a fine mist of water and then dusted with fine peat soil while moist. The soil contained 16,300 p.p.m. of iron, 260 p.p.m. of manganese, 53 p.p.m. of copper, 45 p.p.m. of zinc, and 3.5 p.p.m. of molybdenum. The apparent contamination from iron indicated that the amount of the soil adhering to the plant tissue was in the order of 4 to 10 mg. per gram of dry tissue.

Harvesting and Washing

Each container was harvested separately, thus providing duplication of each contamination treatment. All plants were cut off 1 inch above the root crown. Blades of the tomato plants were separated from petioles and used as sample material, the remainder of the plant being discarded. With sugar beet, both blades and petiole were sampled and processed separately. All the blade material of the rye was used. Fresh weights of samples were from 60 to 120 grams, depending on species. Of the plants in each container for each species, one third were harvested and dried for analysis without washing (treatment 1 of Figure 1); one third were harvested, washed in 0.1N hydrochloric acid, rinsed twice with distilled water and finally with borosilicate glass–redistilled water (treatment 2), then dried for analysis. The remaining plants in each pot were harvested, washed in a solution of 10 ml. of liquid detergent in 3 liters of 0.1N hydrochloric acid, and rinsed twice with distilled water and finally with borosilicate glass–redistilled water, (treatment 3), before being dried for analysis. The samples were agitated in 3 liters of wash solution for 30 seconds. Rinses with distilled water were also of 30-second duration. Washing times of 20 to 90 seconds have been shown by Nicholas *et al.* (*6*) to be adequate, with no loss of nutrients at the longer time.

Samples were drained on aluminum screen trays and dried at 75° C. for 16 hours.

Blades of rye were cut into small pieces with new, clean, stainless steel scissors prior to ashing. Blades of sugar beets and tomato were hand crushed in kraft bags prior to sampling or ashing.

Analytical Methods

Iron was determined by the *o*-phenanthroline method, manganese by periodate oxidation, zinc by the Zincon method, copper by the diethyldithiocarbamate method, and molybdenum by the thiocyanate method (*5*).

Five-gram (dry weight) samples were taken for wet ashing, with nitric and perchloric acid, the ash solution was made to 250 ml., and suitable aliquots were taken for determination of the elements.

Results and Discussion

Mean values for analytical results are presented graphically in Figure 1. The analytical values were subjected to statistical analysis after logarithmic transformation. In the following discussion statements of differences in apparent elemental concentration resulting from contamination and washing treatments are based on evidence of statistical significance at the 5% probability level.

Dust Contamination Removal

Contamination of plant samples by soil dust, at least in the case of the peat dust used here, resulted in large increases in apparent concentrations of iron in unwashed samples in all cases. Washing with either 0.1N hydrochloric acid or detergent in 0.1N acid reduced the apparent iron content of the samples to that of the uncontaminated samples. Washing of samples of foliage from these species with either acid alone or acidified detergent solution did not remove iron initially present within the tissues as shown by comparison of treatments 1, 2, and 3 for the initial (uncontaminated) samples.

Copper contamination was significant only with dusted and unwashed sugar beet blades. Both washing treatments were effective in reducing the copper concentration to their initial values—compare treatments 2 and 3 of the dusted group with treatments 1, 2, and 3 of the initial group.

Within the limits of biological and analytical variation the dust did not lead to a significant contamination of any of the tissues with manganese, molybdenum, or zinc. Analytical values for any of these elements after either washing technique were not significantly different from those of the initial (uncontaminated) samples.

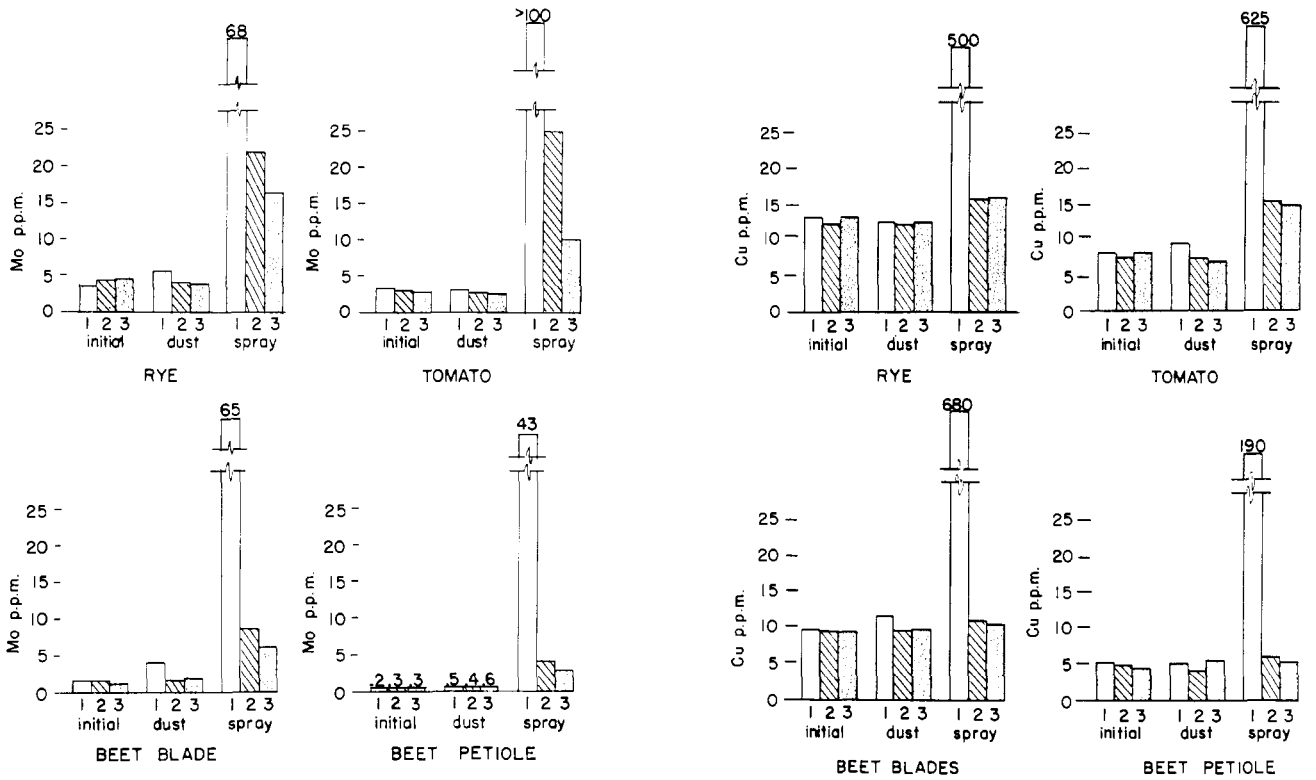
Removal of Spray Contamination

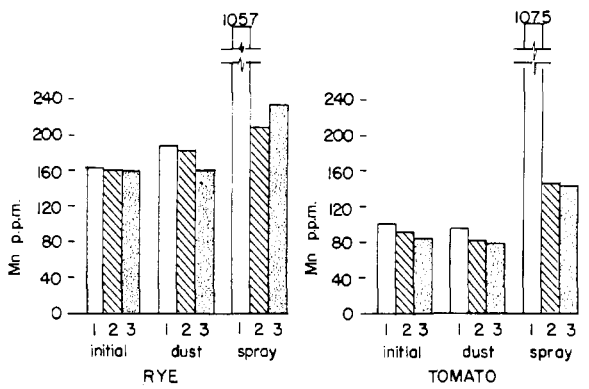
In normal practice samples would be avoided which were obviously contaminated by spray material. However, in some instances local spraying history might be unknown, or it might be necessary to attempt investigation of the effectiveness of spray application of increasing the micronutrient content in plants. Thus it was of interest to investigate the usefulness of the washing procedures in removing spray residues. The concentrations of the elements remaining on the sprayed and unwashed foliage were large, ranging from 43 p.p.m. for molybdenum to over 1000 p.p.m. for manganese. Evidently the various elements were distributed differently between solid and liquid phases, and hence between spray and sediment in the sprayer, and between runoff and residue on the plant.

Estimation of the extent of removal of surface contamination from spray residues is complicated by the probability that some of the applied compounds are actually absorbed by the tissues and thus not amenable to removal by washing. However, in many instances the washing procedures were remarkably effective in reducing the apparent micronutrient concentrations to levels near that of the initial (uncontaminated) samples as is shown in Figure 1. The extent of the removal depends on the particular element and the nature of the foliar sample.

Iron, copper, and zinc contaminants from spray applications were removed effectively by the washing procedures from sugar beet blade and sugar beet petiole. Tomato blades and rye, probably because of their hairy or rough surfaces, appear to retain all elements from the spray in rather large amounts despite washing. There is the possibility that a portion of the applied spray is actually absorbed by the plant. It is difficult to assess the distribution between surface and interior content of the elements from the data of this experiment.

The apparent concentrations of molybdenum and manganese in the sprayed samples were reduced by both washing procedures as compared with the concentrations in the unwashed samples. However, except in sugar beet blades, the apparent concentration of manganese remaining after washing was considerably higher than that in the uncontaminated foliage samples. Much of the molybdenum from the spray application was removed by each of the washing treatments, but the final apparent molybdenum concentration in all tissues after washing remained severalfold higher than that in the uncontaminated material. Effectiveness of foliar application of molybdenum in supplying molybdenum-deficient plants has been





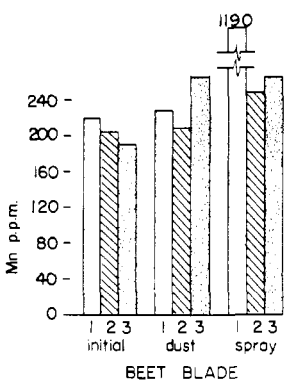


Figure 1. Efficiency of washing procedures in removing copper, iron, manganese, molybdenum, and zinc from contaminated foliage samples

Initial. Foliage samples taken from relatively dust-free greenhouse
Dust. Similar samples contaminated by dusting moistened foliage samples with peat soil
Spray. Similar samples sprayed with a simulated Bordeaux spray containing copper, iron, manganese, molybdenum, and zinc
Washing treatment numbers
1. No washing
2. Agitated 30 seconds with 0.1*N* HCl, washed twice with distilled water, and once with borosilicate glass–redistilled water
3. Agitated 30 seconds with 0.1*N* HCl and detergent, rinsed twice with distilled water, and once with borosilicate glass–redistilled water

observed for many years. Much of the increase in molybdenum concentration in the sprayed and washed tissues probably can be explained on the basis of rapid absorption of the element.

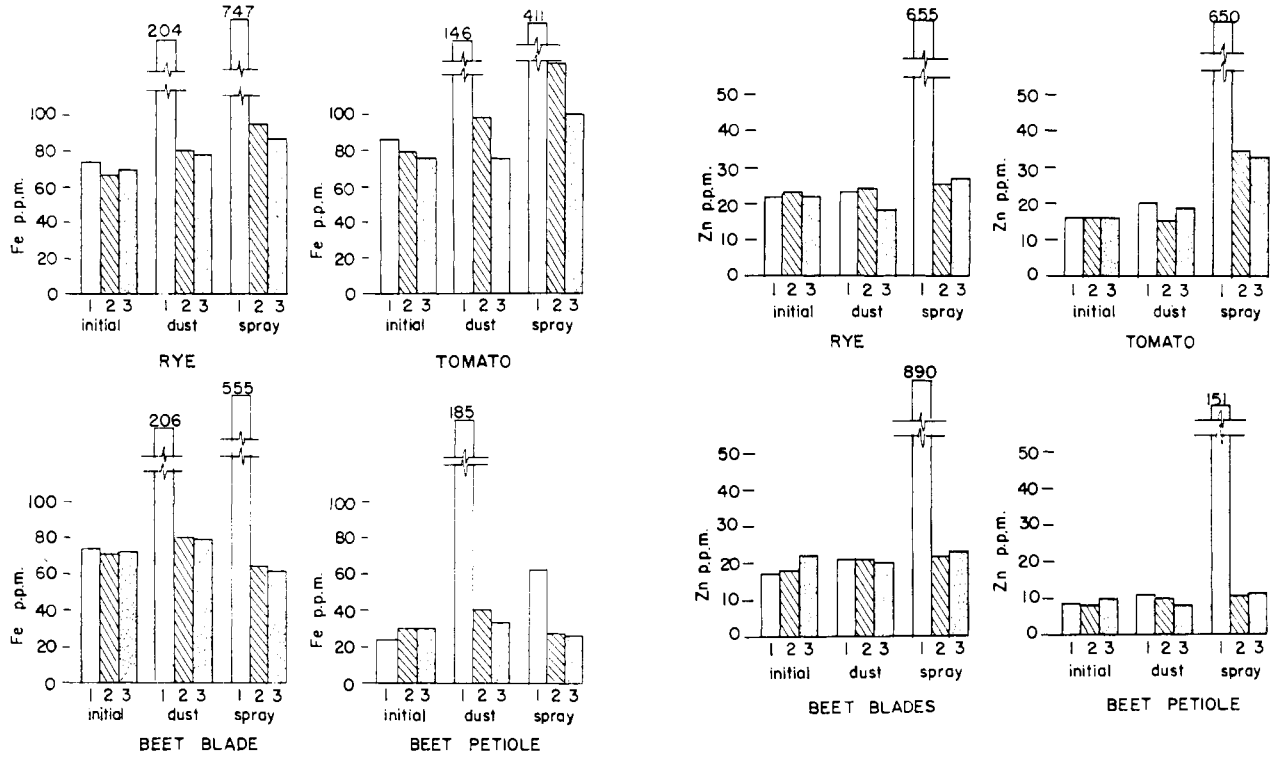
Summary

Contamination from aqueous sprays was less effectively removed by either of the wash solutions than was dust contamination. Therefore analyses of tissue known to have been contaminated by sprays should be suspect even after washing.

With the soil used in this experiment, iron contamination was the only one of statistical significance and this was removed by either washing technique. However, soils may vary widely in their micronutrient content (*1*) and errors in the apparent concentration of any or all of the elements arising from dust contamination must be recognized as a possibility. These experiments show that internal concentration of none of the five elements is reduced by the washing procedures when applied to diverse tissues. This conclusion is supported by the work of Nicholas *et al.* (*6*), and is not discordant with the data of Tukey *et al.* (*8*), since the period of immersion of the plant material in the washing and rinsing solutions was short in contrast to the long leaching studies of Tukey *et al.* (*8*). Therefore, it is recommended that all tissues be washed in preparation for all micronutrient analyses.

These experiments indicated that dilute acid alone was as effective as acid plus detergent in removing surface contamination. However, as noted by Jacobson (*2*), the dilute acid would not be expected to dissolve iron oxide or iron silicate. Thus the efficiency of the washing is largely a matter of dispersing dust particles in the washing solution. With field samples where there has been considerable build-up of layers of dust and perhaps spray materials, the use of the combined acid and detergent wash solution should prove superior. Because no leaching of ions occurs with this wash solution, it is recommended for routine use.

Although emphasis has been placed on procedures for minimizing spurious results from surface contamination in developing correlations between analytical values for micronutrient concentrations and foliar symptoms of deficiency, it is not implied that proper washing procedures alone will ensure good correlations. Proper removal of surface contamination is only one re-



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PLANT COMPOSITION AND VIGOR

Hemicelluloses and Winter Hardiness in Raspberry Canes

EMMETT BENNETT and WALTER D. WEEKS

Massachusetts Agricultural Experiment Station, Amherst, Mass.

In a general study of winter hardiness in plants, hemicelluloses from winter-hardy and nonwinter-hardy varieties of raspberry canes were isolated and studied to determine a possible relationship. The hemicelluloses were found to be similar in quantitative and qualitative aspects. Xylose was present in by far the greatest amounts. The data on the present basis do not seem to indicate a positive relationship.

VARIETIES OF RASPBERRIES vary in their ability to survive when exposed to extremely low temperatures. The resistance of a given variety is not necessarily constant for the winter season. It may be lost and regained during the same season. The phenomenon of winter hardiness is not generally understood. Excellent reviews on the hardiness of plants have been published by Dexter (*2*) and Levitt (*4*). Hardiness has been related to many plant substances, including hemicelluloses. Although these substances are sufficiently hydrophilic to play an important role in the water relationships of the plant, published reports vary as to the importance of their role in winter hardiness (*1, 5, 8*). To investigate further the possibilities of an indirect relationship, a chemical investigation has been conducted on the hemicelluloses of hardy (Latham) and tender (Milton) varieties of raspberry canes.

Material and Methods

Representative samples of both Latham and Milton canes were collected approximately every two weeks from November through February, dried in a forced draft oven at 75° C., and composited later. Approximately 500 grams of each were extracted successively with a

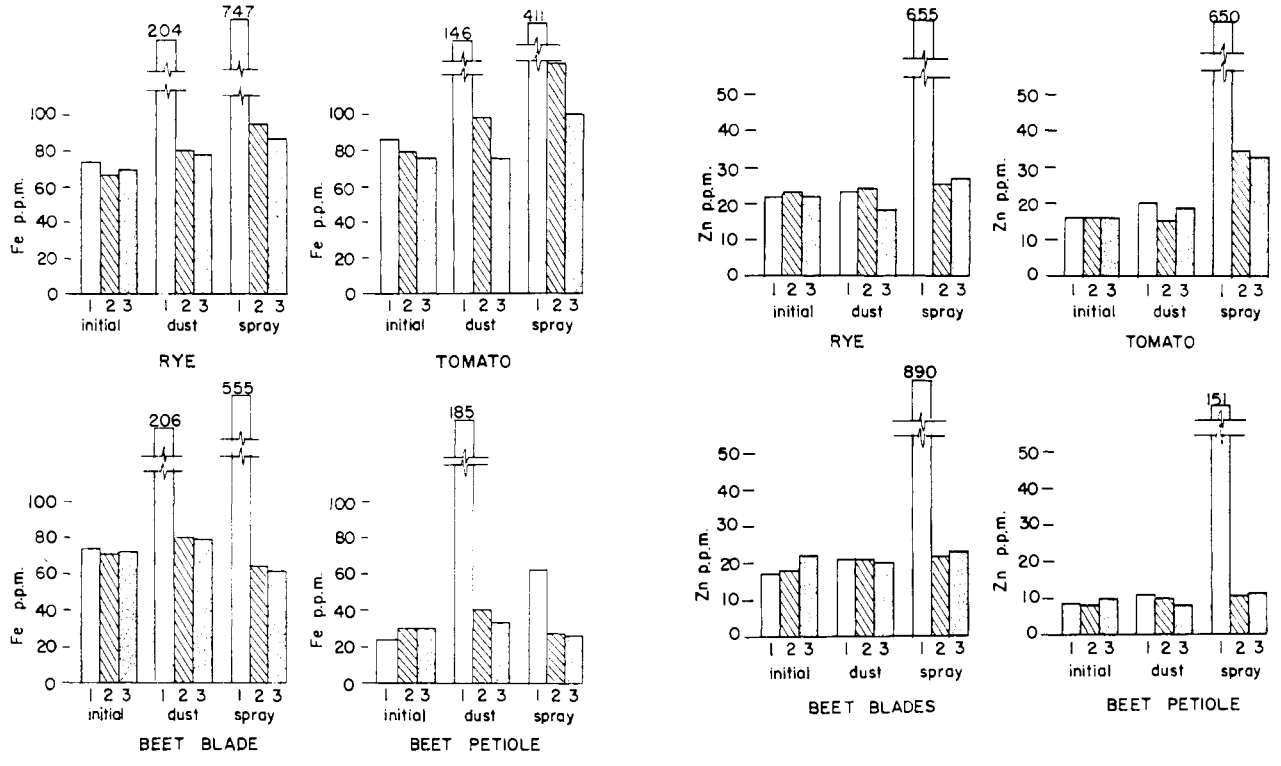

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Table I. Hemicelluloses of Latham and Milton Raspberry Canes on an Ash-, Moisture-, and Lignin-Free Basis

	Latham, %	Milton, %
Hemicellulose	15.12	15.13
Xylose	78.33	66.84
Arabinose	5.70	2.98
Glucose	6.39	6.63
Galactose	2.32	2.32
Uronic acids	11.13	14.23
Specific rotation, $[\alpha]_D^{25}$	−100.6	−64.90

solution of alcohol and benzene in the volume ratio of 1 to 2 and of 0.5% ammonium citrate. Holocellulose was prepared from the extractive-free material by the use of sodium chlorite essentially according to Whistler's (*9*) modification. Hemicelluloses were extracted from the holocellulose with 12% potassium hydroxide at about 25° C. for 20 hours in the presence of nitrogen. The entire fraction was precipitated by adjusting the pH to 3.5 and adding 3 volumes of ethanol. The analytical data are shown in Table I.

Solutions for specific rotations were made by dissolving 0.3 gram in 50 ml. of 4% sodium hydroxide. The readings were made on a Schmidt and Haensch saccharimeter using sodium light at 25° C. The factor 0.34620 was used for conversion to angular degrees. Uronic acids were determined by the method of Phillips, Goss, and Browne (*7*). Hydrolysis of the samples was effected by approximately 1*N* sulfuric acid in a boiling water bath for 15 hours in an atmosphere of nitrogen. The hydrolyzate was recovered in the usual manner after treatment with barium hydroxide and barium carbonate. The sugars were resolved on Whatman filter paper No. 1, using as solvent a mixture of equal parts of ethyl acetate, pyridine, butanol, and water. Under our conditions this system produced a satisfactory resolution of glucose, galactose, xylose, arabinose, and uronic acids in 24 hours. The position of the sugars was determined by spraying the paper with aniline hydrogen phthalate (*6*). The individual sugars were quantitatively estimated as follows:

Bits of paper approximately 50 mm. square known to bear a single sugar were folded in a serrated form about 5 mm. on an edge and placed in a 10 × 75 mm. test tube. The papers were just covered with distilled water, after which the tubes were placed in a water bath at 60° C. for 2 to 3 minutes. The extracts were drained into prepared glass-stoppered 20 × 130 mm. test tubes. The process was repeated five times. The sugars were then determined directly by the Hagedorn-Jensen method (*3*). The content of sugar was calculated by reference to a curve constructed from data obtained under identical conditions from solutions of known concentration. A volume of 10 μl. containing from 20 to 80 γ provides a satisfactory concentration.

Results

From the data shown in Table I it is immediately evident that both varieties have about the same amount of hemicellulose, that they are both chiefly xylans, and that the β form of pentose predominates. No other single sugar was noted except those recorded, although there was evidence to indicate the presence of a trace of a pentose polymer of extremely low R_f value in both samples. The sugars recorded in Table I were identified chromatographically. Although the effect of impurities has not been satisfactorily assessed, preliminary data on the sorption of water under controlled conditions indicate that the greater amount of water is sorbed by the Latham hemicellulose.

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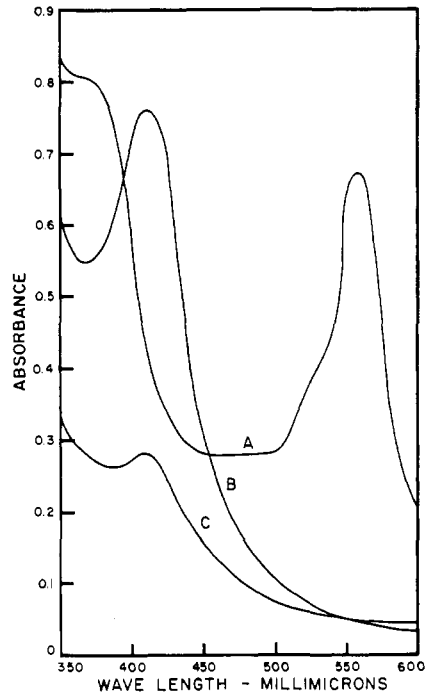

Figure 3. Absorption curves of color complexes (each with 20 γ of reactant)

A. Heptachlor (Polen-Silverman reagent)
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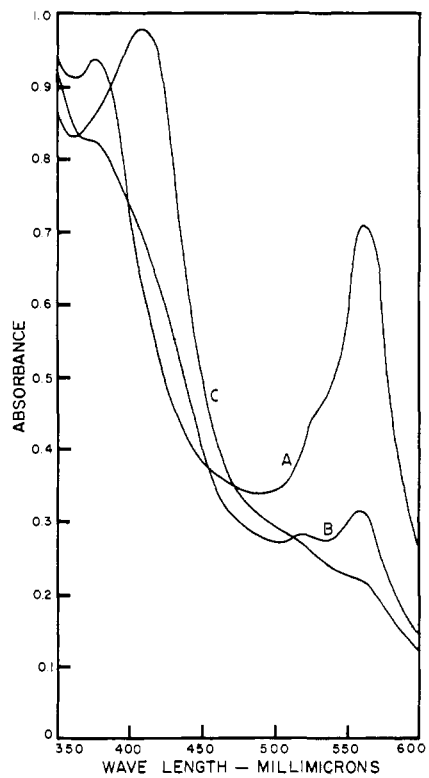

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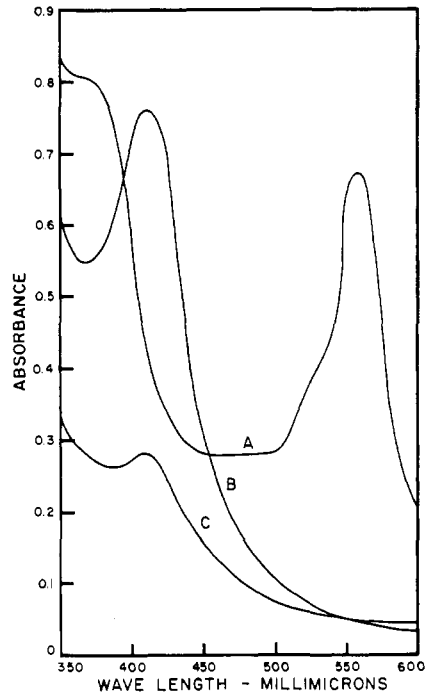

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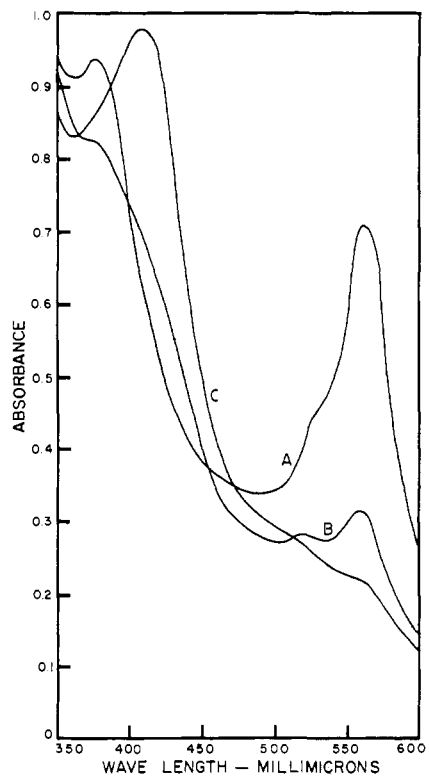

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Evaluation of Protein in Foods for Regulatory Purposes

J. A. CAMPBELL

Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada

The determination of the protein efficiency ratio of individual foods fed at a 10% protein level in a standardized 4-week rat growth assay has been found to be the most generally applicable procedure for evaluating protein quality. Based on the determination of three amino acids, lysine, methionine, and cystine, a simplified chemical score was found to correlate well with PER data on a series of 43 foods. The method was rapid and reproducible. To evaluate the protein contribution of different foods for regulatory purposes, a protein rating was developed based on both the quantity and quality of protein in a reasonable daily intake of food. This procedure was applied to a variety of foods and appeared to classify them in a logical manner.

RECENT RESEARCH on the role of protein in nutrition has prompted commercial emphasis on this constituent. This emphasis on protein has been applied to a wide variety of foods, some of which are recognized as important sources of protein and others which are not. The Food and Drug Laboratories in Canada have been concerned about claims made for the protein content of some foods and have tried to determine which are justified and which are misleading or likely to create an erroneous impression regarding the value of the food.

Allison (*2*) has stated, "The prime purpose of a dietary protein is to provide a pattern of amino acids appropriate for the synthesis of tissue proteins and for other metabolic functions." The chief problem, therefore, was to evaluate the pattern of amino acids in a food. Methods for protein evaluation have been detailed by Allison (*1, 3*), Grau and Carroll (*13*), Bender (*6*), and others and will not be reviewed here. Although there are many methods available, no definite recommendation has been made as to which is the most suitable or satisfactory for determining the value of proteins. Obviously, if any progress were to be made in the regulatory aspects of the problem, certain more or less arbitrary decisions regarding a method had to be made. The majority of workers in the field seemed to favor the determination of the protein efficiency ratio (PER) for routine purposes.

General Considerations

Some of the implications of different approaches to the evaluation of proteins were considered in this study. Mitchell (*23*), Hart (*16*), and others have pointed out that, because of the supplementary action of amino acids, the classification of proteins individually is of relatively little significance in assessing the protein value of diets. It could be argued, therefore, that the protein contribution of a food should be evaluated only in terms of the complete diet with which it is fed. As Canadians (and Americans) consume, on the average, about 100 grams of protein daily, more than half of which is animal protein, it would be difficult to distinguish between the effect of a good or a poor quality supplement to such a diet. For example, there would be little difference between a supplement of 20 grams of protein as meat or as white bread. Under these conditions, quality of protein would be relatively unimportant and the only factor to be considered would be the amount of protein. Because most individuals, however, consume almost 50% more protein than they require, additional amounts would have little or no value. It might be argued, therefore, that it would be misleading in advertising material to stress the importance of the protein contribution of a food. If one follows this argument to its conclusion it would appear that, for the average individual or the great majority of people, all claims for the importance of and need for protein would be misleading and any statement regarding protein quality would have little if any meaning.

This position is a rather extreme one and, although tenable from one aspect, lacks complete justification. It ignores the vast amount of study on the protein quality of individual foods and the view that protein quality is important. Furthermore, foods are sold individually and advertisements are based on the individual food. The application of average consumption figures to individual cases is undoubtedly open to question. If the diet of some people is not fully adequate in protein, it is useful to add significant amounts of good quality protein to their diet. In these circumstances, it would not be misleading to encourage the use of protein and it would seem desirable to improve existing sources of protein. This stand would imply that, at least for regulatory purposes, it is important to know the value of individual protein foods but to realize, also, that this value may change, and usually increases, when the food is consumed as part of a mixed diet. This is the approach taken in this laboratory. This paper describes experiments with a rat growth method (*10, 24*), the development of a simplified chemical score for use as a rapid screening procedure (*21*), and a protein rating for the classification of foods into categories according to their protein contribution (*9*) for regulatory purposes.

Determination of Protein Efficiency Ratios

Table I presents the details of the method which has been recommended (*10*) for the evaluation of protein quality. The protein to be tested is added at the expense of cornstarch to a level of 10% and the fat content is maintained also at 10%. Possibly the chief innovation is the use of casein as a reference standard and the correction of all PER values to 2.5 for casein. With this exception, the method is very similar to that suggested recently by Derse (*11*) and now being tested collaboratively by the A.O.A.C.

Results obtained by this procedure are affected by several factors. The importance of the age of the animal at the time it is put on test is shown (Figure 1) and marked differences occurred

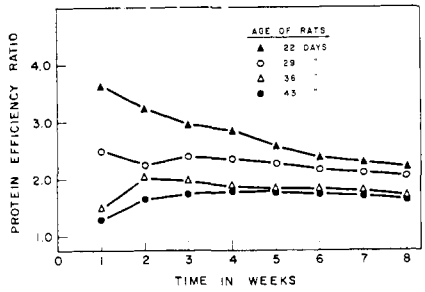

Figure 1. Effect of age of rat when put on test on PER values of casein

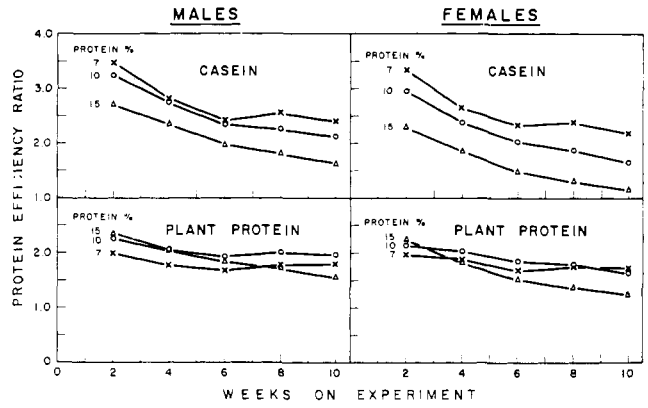

Figure 2. Effect of sex of rat, level of protein, and time on test on PER values of two proteins

Table I. Recommended Method for P.E.R. Assay

Animals, weanling males, 20–23 days old

Diet	%
Cornstarch	80
Corn oil	10 (maximum)
Cellulose	5
Salt U.S.P. XIV	4
Vitamins	1
Protein (N × 6.25)	10 (at expense of starch)

Reference standard, A.N.R.C. casein

Assay period, 4 weeks

Calculation, PER × $\frac{2.5}{\text{PER for casein}}$

Table II. Effect of Strain of Rat on PER Obtained with Different Foods

Food	PER Values[a] Found with Strain A	Strain B Uncorrected	Strain B Corrected[b]	Strain C Uncorrected	Strain C Corrected[b]
Casein	2.59 (10)	3.35 (1)	...	3.18 (1)	...
Dried whole egg	3.44 (9)	4.00 (1)	2.99	4.00 (1)	3.14
Wheat flour–soybean flour mixture	2.09 (1)	2.64 (1)	2.00	2.56 (1)	2.01
Whole wheat flour	1.17 (1)	1.44 (1)	1.07	1.50 (1)	1.18

[a] Figures in parentheses indicate number of experiments in which 10 male weanling rats received each food at the 10% protein level for 4 weeks.

[b] Obtained by multiplying the determined PER value by the fraction 2.5/determined PER of reference standard casein.

as a result of variations in this factor. PER values for casein tended to decrease with age and with time on test. On the basis of these data, and other considerations, rats 20 to 23 days old were specified for use in a 4-week test.

The level of protein to be used in a rat assay is controversial. Barnes *et al.* (*5*) and Barnes and Bosshardt (*4*), confirming the earlier work of Osborne *et al.* (*25*), showed that the effect of the level of protein on PER values varied with the type of protein and they emphasized the importance of this factor. Most workers favor about 10% but Harris and Burress (*15*) recently suggested that a level of 15% is more realistic, as it is closer to the proportion of protein consumed on the average by Americans. Figure 2 illustrates data from an experiment in which the level of protein, sex, and length of the experimental period were studied with casein and a plant-protein mixture. Female rats tended to have lower PER values than males, but not consistently so. They tended to give maximum values at lower protein levels than did males. PER values found with both sexes dropped as the experiment progressed but the decline was greater with animals fed casein than with those fed the plant-protein mixture. Differences between casein and plant protein were greatest during the early stages of the experiment. At the 10% level, both proteins exhibited near maximum values while casein gave low values at 15% protein and the plant protein gave low values at 7%. Because sex, time on test, and protein level influenced PER values, these factors must be controlled if results are to be reproducible in different laboratories. Furthermore, if one level of protein is to be used, 10% seems to be the most generally satisfactory.

Some laboratories have reported markedly higher PER values for casein than others. This laboratory has exchanged casein with other laboratories, found essentially the same low values with the acquired sample of casein as with its own, and concluded that strain of rat may be the most important factor. Data on the response of three different strains are presented in Table II. Strain A was the original strain used in our laboratories. While differences were marked between this strain and rats from two other sources, the use of the correction for the value obtained with the casein standard largely eliminated the variation and placed the results in the same perspective.

Development of Simplified Chemical Score

While there appears to be general agreement at the present time that animal assay procedures are necessary for evaluating proteins, the use of simpler and more rapid techniques was also considered. These would be particularly valuable in development work and where facilities for animal assay are not available. They would also be useful as preliminary screening procedures.

Several microbiological methods had been suggested (*14*, *19*, *29*) for this purpose. Results obtained with modifications of these procedures using *S. faecalis* and *L. mesenteroides* were compared with PER values determined on the same samples of a variety of foods and were unreliable (*27*) as an indication of protein quality.

From these studies, however, it became evident that it might be possible to rate cereals according to their lysine content, and animal proteins according to their methionine (plus cystine) content. About this time, both Waddell (*30*) and Block and Weiss (*8*) indicated that in most commonly used foodstuffs the limiting amino acids were usually lysine, methionine, or the combination of methionine plus cystine. Because Block and Mitchell (*7*) showed that a close relationship existed between chemical score and nutritive value of protein as judged by PER values, the same relation was investigated, using what is

termed the simplified chemical score (S.C.S.), which is based on the determination of these three amino acids.

Table III illustrates the principle involved in the determination of S.C.S. The content of the three amino acids, lysine, cystine, and methionine, was determined and expressed in terms of the concentrations of these amino acids in whole egg. To reduce the variation inherent in the amino acid determination, an egg standard was used in all assays and results were expressed in terms of this standard. As recommended by Block and Mitchell, if lysine or methionine were the most limiting amino acid, it was the basis of the S.C.S. If the cystine content were the lowest of the three amino acids, the combination of methionine plus cystine content was used as the S.C.S., as methionine is convertible to cystine. All three amino acids were determined on 2-hour acid hydrolyzates of the food using *L. mesenteroides* in the procedure of Steele *et al.* (*28*) for methionine and lysine and the method of Horn and Blum (*18*) for cystine.

The simplified chemical score was calculated for a variety of foods and compared with PER values determined on the same samples by the procedure described earlier. Foods tested were separated into two categories: those which were limiting in lysine, or those limiting in methionine (+cystine). As shown in the top section of Figure 3, correlation was good between lysine concentration and PER in the first group and between methionine (+cystine) content and PER in the second group.

Although the relationships were different, a factor of 15 added to the SCS. of the latter group resulted in a good correlation of both sets of data with PER (Figure 3, middle). This adjustment indicated that it was possible to predict PER values reasonably well from SCS data knowing the limiting amino acid of the three tested.

The SCS was also applied to literature data. Block and Mitchell (*7*) listed amino acid and PER data for 18 foods which were deficient in either lysine or methionine (+cystine). SCS values were calculated and compared with their PER data (Figure 3, bottom). Although relatively few values were available, the data fell into two classes as before and the correlation in each group was reasonably good.

Data on the six other foods listed by Block and Mitchell are given in Table IV. Chemical scores have been calculated from their data indicating that these foods were limiting in threonine, isoleucine, or tryptophan. SCS was calculated on the assumption that they were limiting in lysine or methionine (+cystine). The agreement between the two sets of data is reasonably good and does not change the interpretation of the food as a source of good or poor quality protein. This would suggest that foods limiting in threonine, isoleucine, or tryptophan are also near limiting in lysine or methionine. Thus, even in those foods limiting in some amino acid other than the three used for SCS, this procedure may offer a useful indication of protein quality.

The method is rapid, reproducible, and particularly suited to control work. As there is good correlation with PER data, the criticisms mentioned by Grau and Carroll (*13*) of analytical difficulties, differences in absorption, and other effects do not appear to be too serious. As methods become more accurate it should be possible to dispense with the egg standard. While at the present time it is not proposed that the S.C.S. procedure should replace the animal assay, sources of variation in the latter enhance the attractiveness of amino acid analysis. In this connection it is interesting to note that Miller and Naismith (*22*) have pointed out that in many diets the cystine and methionine contents appear to be limiting rather than the lysine content and have based a method for protein quality on the total sulfur determination.

Protein Rating

Having decided upon the PER assay for evaluating protein quality, it was then necessary to set up criteria by which the value of various foods as protein sources might be described. By such criteria, the terms "high quality" or "excellent dietary source" would have a definite meaning. The food processor or manufacturer would know what experimental work would be necessary to establish claims regarding the protein content; the physician, nutritionist, and consumer would know that claims were uniformly and soundly based on acceptable experimental data.

Any claims for the protein content of a food must be made on the basis that, for the average individual, the food contributes a significant proportion of the total protein requirement when consumed in a usual or reasonable manner. This principle has been found most useful in Canada for governing claims which may be made for the vitamin content of natural foods—i.e., those to which no vitamins have been added. Under authority of the Food and Drugs Act and Regulations (*12*), a food may be described as a "good dietary source" or an "excellent dietary source" of any vitamin when the amount of that vitamin in a "reasonable daily intake" of the food furnishes more than certain specified amounts. It appeared reasonable to extend this principle to permit the description of a food as being an "excellent dietary source of protein" or a "good dietary source of protein."

Table III. Calculation of Simplified Chemical Score (SCS)

	Amino Acid Content, % of Egg			
Food	Lysine	Methionine	Methionine + cystine	S.C.S.
Whole egg	100	100	100	100
Beef	114	89	66	66
Peas	110	32	38	32
White bread	35	56	66	35

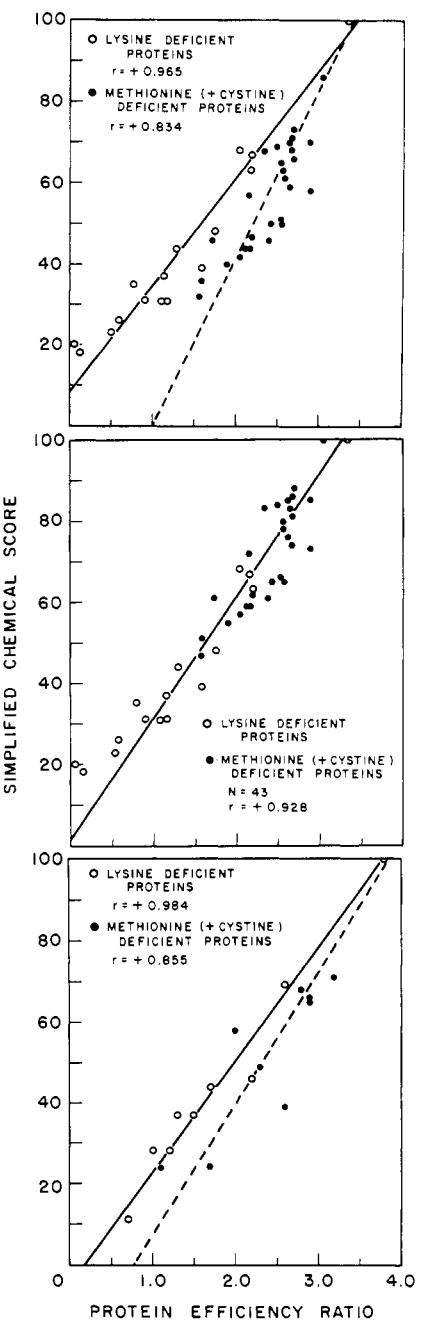

Figure 3. Correlation between SCS and PER values

Top. For 43 foods
Middle. For 43 foods after adjustment of methionine (+ cystine) scores
Bottom. For 18 foods [from data of Block and Mitchell (*7*)]

Table IV. Ratings by SCS and Chemical Score[a] of Foods Not Limiting in Lysine, Methionine, or Cystine

Food	Chemical Score: Actual limiting[b] amino acid	Chemical Score: Score	SCS: Apparent limiting[b] amino acid	SCS: Score
Egg albumin	Threonine	78	Lysine	90
Liver	Isoleucine	70	Methionine + cystine	71
Heart	Isoleucine	65	Methionine + cystine	68
Brain	Isoleucine	64	Methionine + cystine	74
Wheat germ	Isoleucine	38	Methionine + cystine	42
Gelatin	Tryptophan	0	Methionine + cystine	14

[a] Data taken from Block and Mitchell (*7*).
[b] Not necessarily the most limiting amino acid for growth of animals.

Table V. Protein Rating of Certain Foods

Food	Protein Content, %	Reasonable Daily Intake (Serving), Grams	Protein Intake, Grams	P.E.R.	Protein Rating[a]
		Literature Data (*7*)			
Cabbage	1.4	50	0.7	0.9	0.6
Wheat, whole	9.9	30	3.0	1.5	4.5
Oats, rolled	14.2	30	4.3	2.2	9.5
Bread, white	8.4	150	12.6	1.0	12.6
Soybean	34.9	30	10.5	2.3	24.1
Egg, whole	12.8	100 (2, eggs)	12.8	3.8	48.6
Beef	21.0	100	21.0	3.2	67.2
Milk, whole	3.5	708 (3 cups)	24.8	2.8	69.4
		Experimental Data			
White bread	8.4	150 (5 slices)	12.6	0.77	9.7
Whole wheat bread	10.5	150 (5 slices)	15.5	1.1	17.1
Gluten bread	16.9	150 (5 slices)	25.4	0.9	22.9
"Protein" bread	11.8	150 (2 slices)	17.7	1.3	23.0
Egg	12.8	100 (2)	12.8	3.5	44.8
Milk	3.5	708 (3 cups)	24.8	2.7	67.0
Oats	12.8	30	3.8	2.1	8.0
Oats plus milk	12.8 3.5	30 120	3.8 4.2	3.2	25.6
"Protein" cereal	20.0	30	6.0	0.03	0.2
"Protein" cereal plus milk	20.0 3.5	30 120	6.0 4.2	2.0	20.4

[a] These values do not take into account the known supplementary effect of animal proteins

Because protein requirements are met by combinations of amino acids, quantity as well as quality of protein must be considered. Three factors are involved: the amount of the food consumed in a reasonable daily intake, the per cent of protein in the food, and the biological value of the protein. As was pointed out by Hegsted (*17*) and others, if 20 grams of a protein with a biological value of 100 satisfied the protein requirement of an individual, it would take approximately 40 grams if the biological value is only 50. Within limits, therefore, protein requirements may be satisfied equally well by varying amounts of protein dependent on its biological value.

On this basis, and using the PER method as a measure of biological value, a protein rating was proposed which is the product of the PER of a protein multiplied by the grams of protein in a "reasonable daily intake" of the food. Table V lists representative foods with their protein content, protein efficiency ratio, and reasonable daily intake. All PER data in the upper part of the table are taken from Block and Mitchell (*7*). Reasonable daily intake is usually considered to be one serving of a food but may be more—for example, in the case of milk and bread. The protein ratings in Table V fall into two distinct categories. Meats, eggs, and milk have ratings varying from about 40 to 70, while cereals and vegetables have ratings of 12 or less. Soybeans are intermediate with a rating of about 24. Food with a rating above 40 is designated as an "excellent dietary source of protein." The protein contribution of an individual food with a rating below 20 is relatively insignificant and, therefore, it would seem misleading to attach any special significance to its protein content or to use it in any way as the basis of advertising claims. A food with a rating of 20 to 39 inclusive may be designated as a "good dietary source of protein." This category is one which could be reached by significant improvements in the quality and quantity of proteins in foods for which claims would not normally be permitted.

Platt and Miller (*26*) recently developed a somewhat similar system of rating diets using both quality and quantity of protein in the concept of "net dietary-protein value." There is no satisfactory way to allow for the supplementary effect of proteins at the present time although the work of Howard *et al.* (*20*), which appeared after this work was completed, offers definite possibilities in this regard.

To indicate how the technique may be used to classify foods, the protein ratings of some additional foods are also shown in Table V. These data were obtained by applying the PER assay procedure of Chapman *et al.* (*10*) to market samples. White and whole wheat bread are not "good dietary sources of protein." A new "protein" bread now appearing on the market in Canada and containing more and higher quality protein would be given a rating of 23 and hence might be referred to as a "good dietary source of protein." A sample of gluten bread also meets this requirement. The protein ratings found for white bread, eggs, and milk are close to the values calculated from the literature in the upper part of the table.

It has been argued that because cereals and milk are invariably consumed together, they should be evaluated as a combination rather than separately. If mixtures of 1 ounce of cereal with 4 ounces of milk are treated in this manner, it will be seen from Table V that some cereal plus milk combinations rate as good dietary sources of protein while others do not. The supplementary effect of milk tends to make the combinations more similar to each other than were the original cereals alone.

In proposing a single criterion to describe protein value, it was not possible to allow for the many points of view on this subject. Nevertheless, in view of the commercial interest in stressing the importance of protein in various foods and the claims being made at present, it was felt that some yardstick must be set up and a beginning made. It was hoped that the procedure recommended might not only serve a useful purpose but also encourage the development and adoption of better and more uniform criteria as more information is obtained. The protein rating procedure described has not been made the subject of regulations under the Food and Drugs Act but is being used administratively at the present time for the evaluation of the protein content of foods sold in Canada.

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PROTEIN QUALITY AND SUPPLEMENTATION

Effect of Amino Acid Supplements, Vitamin B_{12}, and Buffalo Fish on the Nutritive Value of Proteins in Sesame Seed and Meal

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A study of sesame seed and meal was undertaken, because of their importance to human nutrition and the animal feed industry. Addition of lysine, lysine and threonine, and buffalo fish benefited growth and the protein efficiency of sesame seed and meal. Sesame meal supplemented milled, white corn meal, enriched wheat flour, and white milled rice. Lysine and threonine influenced the biological value and net utilization of sesame seed. Vitamin B_{12} failed to supplement the lysine-threonine additions. Data are presented on amino acid, vitamin, and mineral content. The nutritive value of sesame seed and meal warrants their use in the enrichment of diets for certain population groups and for continued use in poultry and swine rations.

Sesame is one of the oldest staple, vegetable oil crops and a source of vegetable fats in India, China, Egypt, and Latin America. It was introduced in this country in the late 17th century in South Carolina and since 1953 has been grown on a commercial basis in Texas. Twelve million pounds a year are imported, and used for oleo, bakery products, candy, and cooking fat. The oil has the remarkable quality of remaining fresh and sweet for long periods of time. Foods, confections, oleomargarine, and bakery goods made with sesame oil remain free from rancidity up to 10 times longer than some of the better known vegetable oils. Detailed information about sesame meal, its production, trade, yields, breeding, harvesting, processing, and use are given in a recent publication by Altschul (*2*). Sesame seed is valuable to the diets of sections of the world's population and sesame meal is important as an ingredient of poultry rations (*1*).

This paper reports results of growth and metabolism experiments with young rats fed diets composed of fat-extracted sesame seed and meal with and without supplements of lysine, threonine, and vitamin B_{12}. Results are also presented of studies on the content of all members of the B-complex vitamins, amino acids (including nonessentials), calcium, phosphorus, and iron in sesame seed and meal. Included is a study of the supplementary value of the proteins of sesame seed as compared to those of milled white rice. The supplementary value of the proteins of sesame meal over those of milled white corn meal and of enriched milled wheat flour is presented, along with the supplementary value of the proteins of buffalo fish over those of sesame meal.

Experimental Procedure

Raw Material. Commercial samples of sesame seed and meal were used for the determination of vitamins, minerals, amino acids, and growth value of the proteins. The samples were fat-extracted with petroleum ether for use in rations. Growth value was determined in studies using albino rats as experimental animals fed fat-extracted sesame seed and meal rations containing 9% of protein. Fat-extracted sesame seed and meal furnished the only source of protein

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Table I. Relative Efficiency of Proteins of Sesame Seed and Meal with and without Added Supplements

(Experimental period 10 weeks, 6 males and 6 females in each group. Average results per animal given. Protein in rations is 9%, except in ratio 11 it is 9.84%)

Ration	Type	Gains in Body Weight, G.	Gains in Body Weight, Increase, %	Protein Intake, G.	PER[a], G.	PER[a], Increase, %
1	Sesame seed	124	..	66.1	1.86 ± 0.05[b]	..
2	+ 0.2% L-lysine	129	4.0	69.8	1.94 ± 0.10	4.3
3	+ 0.2% L-lysine + 0.2% DL-threonine	186	50.0[c]	74.6	2.95 ± 0.12	31.7[c]
4	+ 0.2% L-lysine + 0.2% DL-threonine + 0.1 γ B_{12}	141	13.7	67.0	2.09 ± 0.08	12.3[c]
5	Sesame meal	94	..	69.3	1.35 ± 0.05	..
6	+ 0.2% L-lysine	123	30.8[c]	77.9	1.57 ± 0.05	16.3[c]
7	+ 0.2% L-lysine + 0.2% DL-threonine	156	60.0[c]	84.9	1.82 ± 0.09	34.8[c]
8	+ 0.2% L-lysine + 0.2% DL-threonine + 0.1 γ B_{12}	147	56.3[c]	83.3	1.76 ± 0.04	30.3[c]
9	Sesame meal[d]	68	..	54.9	1.24 ± 0.08	..
10	+ 1% buffalo fish	93	36.7[c]	62.1	1.50 ± 0.07	21.0[c]
11	+ 1% buffalo fish	116	70.6[c]	73.7	1.57 ± 0.06	26.6[c]

[a] Gains in body weight per gram of protein intake.
[b] Standard error.
[c] Significant for $P = 0.05$.
[d] From a new lot.

Table II. Influence on Growth and Protein Utilization of Addition of Sesame Meal with and without Supplements

(Average results per animals for a 10-week period; 6 males and 6 females in each group)

Ration	Type	Grain and Sesame in Ration, %	Gains in Body Weight, G.	Gains in Body Weight, Increase, %	Protein Intake, G.	Protein in Rations, %	PER[a], G.	PER[a], Increase, %
12	Milled white corn meal	88	19	..	26.0	6.86	0.60 ± 0.05[b]	..
13	Sesame meal	58.6 4.5	24	26.0	34.6	6.86	0.72 ± 0.06	12.0
14	Sesame meal + 0.1% L-lysine	58.6 4.5	91	379.0[c]	55.1	6.86	1.65 ± 0.06	175.0[c]
15	Sesame meal	44 6.7	36	89.4[c]	40.0	6.86	0.90 ± 0.07	50.0[c]
16	+ 0.1% L-lysine Sesame meal	44 6.7	112	489.4[c]	61.7	6.86	1.81 ± 0.03	201.6[c]
17	Enriched milled wheat flour	88	66	..	61.6	9.03	1.08 ± 0.04	..
18	Sesame meal	58.7 5.9	69	4.6	58.7	9.03	1.17 ± 0.06	8.3
19	Polished rice	87	71	..	35.4	5.42	2.01 ± 0.07	..
20	Sesame seed	62.3 3.0	92	29.6[c]	46.0	5.42	2.02 ± 0.04	0.5
21	Sesame meal	10.6	90	26.7[c]	41.7	5.42	2.17 ± 0.07	8.0

[a] Gains in body weight per gram of protein intake.
[b] Standard error.
[c] Significant for $P = 0.05$.

Protein in rations 13, 14: $^2/_3$ from corn meal, $^1/_3$ from sesame meal.
Protein in rations 15, 16: $^1/_2$ from corn meal, $^1/_2$ from sesame meal.
Protein in ration 18: $^2/_3$ from wheat flour, $^1/_3$ from sesame meal.

in these rations and were fed at levels to incorporate the necessary protein. They were fed with and without 0.2% of L-lysine, 0.2% of DL-threonine, and 0.1 γ of vitamin B_{12} daily. The sesame seed used in this investigation is of the Palmetto variety, grown in South Carolina.

Sesame Seed Processing. The variety of sesame seed that has been used to prepare the samples of sesame meal is a K-10 type. It is also known as Margo (Texas A and M College selections) and Renner I (Texas Research Foundation selections). The seed is obtained by a wet process using alkali in the bath to remove the hulls. The hulled seed is dried before crushing in an expeller. The seeds are cooked at low temperature not over 210° F. before pressing. There is very little additional heat created during extracting, only from friction. The temperature of the cake that comes from the press is not over 200° F.

Buffalo Fish Preparation. Dried, fat-extracted buffalo fish was used in the ration for the supplementary study. This fish meal was obtained by cutting frozen buffalo fish in small pieces, grinding it in a meat chopper, drying it at room temperature with the aid of fans, and extracting the fat with petroleum ether. The protein content of fat-free sesame seed was 43.3% (nitrogen × 6.25), of fat-free sesame meal 51.3% (nitrogen × 6.25), of milled white (polished) rice 6.24% (nitrogen × 5.95), and of fat-free buffalo fish 79.1%.

Experimental Rations. The composition of the rest of the rations was 4% of salt mixture No. 1 (*14*), 4% of hydrogenated vegetable shortening, 2% of cellu flour, 2% of cod liver oil, and 1% of wheat germ oil as sources of vitamins A, D, and E, and the rest as glucose (cerelose). Lysine and threonine were added at the expense of glucose in the ration. The following crystalline components of the vitamin B complex were administered daily to each animal, separately from the ration: 25 γ each of thiamine, riboflavin, pyridoxine, and niacin; 150 γ of calcium pantothenate, 3 mg. of *p*-aminobenzoic acid, 6 mg. of choline chloride, and 1 mg. of inositol. The animals—six males and six females in each group—were fed *ad libitum* for 70 days in the growth experiments. Each animal was weighed weekly and accurate weekly records of food consumption were kept. From the gains in body weight per gram of protein intake the protein efficiency ratios were calculated. In the study of the supplementary value of the proteins of buffalo fish over those of sesame meal, an equivalent amount of the proteins of sesame meal was replaced by the protein of 1% of buffalo fish, leaving the protein level at 9%. In another experiment, 1% of buffalo fish was added, which increased the protein level to 9.84% (Table I, rations 10 and 11). In the study of the supplementary value of the proteins of sesame meal over those of milled white corn meal (Table II), the protein content of the rations was 6.86%. In rations 13 and 14 (Table II), two thirds of the protein was from corn meal and one third from sesame meal. In rations 15 and 16, one half of the protein was from corn meal and one half from sesame meal. In ration 18, two thirds of the protein was from wheat flour and one third was from sesame meal. The protein content of corn meal was 7.8% (nitrogen × 6.25), of enriched milled wheat flour 10.26% (nitrogen × 5.70). The protein content of the ration was 9.03% in the supplementary study of sesame meal proteins for those of enriched milled wheat flour. In the study of the supplementary value of the proteins of sesame seed over those of milled white rice, the proteins of 3% sesame seed replaced an equivalent amount of proteins in polished rice

leaving the protein level of the rations at 5.42% by varying the rice content (ration 20).

A complete description of the experimental procedure for the testing of amino acids, vitamins, and minerals, and for the determination of the biological values of the proteins is presented (*8*).

Results

Results of growth and supplementary experiments are given in Tables I and II and those of metabolism experiments in Table III.

Effect of Amino Acid Addition. The results, expressed as average gain per animal (Table I), indicate that the proteins of sesame seed and meal can be improved by supplementation with lysine and threonine and that addition of vitamin B_{12} is without beneficial effect. Addition of 0.2% of L-lysine produced an average gain of 129 grams and a protein efficiency ratio (PER) of 1.94 grams in rations containing 9% of sesame seed proteins. This is a 4% increase in gain and a 4.3% increase in PER. Further addition of 0.2% of DL-threonine produced increases in gain of 50% and in PER of 31.7%, as compared to performance in ration 1. This is the combined effect of the two amino acids. It shows the beneficial effect of the threonine addition, when compared to ration 2. Daily addition of 0.1 γ of vitamin B_{12} (ration 4) produced diminished growth when compared to ration 3; vitamin B_{12} failed to supplement the lysine-threonine addition (Table I, rations 1, 2, 3, and 4). Animals fed sesame meal at the 9% level showed increased gain of 30.8% and 16.3% in PER upon supplementation of the ration with 0.2% of L-lysine. Further supplementation with 0.2% of DL-threonine produced increases in gain up to 60% and 34.8% in PER. Daily supplementation with 0.1 γ of B_{12} did not produce further increases (Table I, rations 5, 6, 7, and 8).

Effect of Buffalo Fish. Table I also indicates that when 1% of buffalo fish replaced an equivalent amount of sesame meal protein, an increase in body weight of 36.7% and in PER of 21% was obtained in animals fed sesame meal–buffalo fish rations at a 9% protein level. Addition of 1% of buffalo fish to a ration containing 9% of sesame meal further increased the gain to 70.6% and the PER to 26.6% and the protein content of the ration to 9.84%. The addition of buffalo fish in small amounts to sesame meal was accompanied by large increases in body weight and PER (Table I, rations 9, 10, and 11).

These differences were tested statistically and found to be significant for $P = 0.05$, except for the increases in ration 2 and the 13.7% increase in ration 4.

Table III. Biological Value of Sesame Seed with and without Supplements Determined by Nitrogen–Retention Method

(*Ad libitum* feeding. 12 animals on rations. Protein in rations 9%. Average result per animal given)

Ration	Type	Biological Value[a]	True Digestibility[b]	Net Utilization[c]
1	Sesame seed	83.9 ± 0.66[d]	87.0 ± 0.20	73.0 ± 0.74
3	Sesame seed + 0.2% L-lysine + 0.2% DL-threonine	88.3 ± 0.87	85.9 ± 0.46	75.8 ± 1.15
4	Sesame seed + 0.2% L-lysine + 0.2% DL-threonine + 0.1 γ vitamin B_{12}	84.1 ± 0.77	83.4 ± 0.33	70.1 ± 0.92

[a] Per cent absorbed nitrogen retained in animal body.
[b] True coefficient of digestibility obtained by subtracting nitrogen lost in feces from total nitrogen intake and dividing by 100.
[c] Obtained by multiplying true coefficient of digestibility by biological value and dividing by 100.
[d] Standard error.

The results of these experiments indicate that the proteins of commercial sesame seed and meal can be further improved by supplementation with lysine, threonine, and buffalo fish.

The response to lysine addition is in agreement with reports by Grau and Almquist (*4*) who found optimal chicken growth when 0.5% of L-lysine was added to an all-sesame protein diet. They also reported a mutual supplementation effect of the proteins of soybean and sesame meals in which the ratio of sesame-soybean protein approached 7 to 13 at a total protein level of 20%. The supplementary effect of perch to milled rice has been reported by Kik (*9*) and the influence on growth and protein efficiency of the addition of small amounts of defatted fish flour to several grains has been demonstrated by Sure (*15*).

Supplements to Milled White Corn Meal and Enriched Milled Wheat Flour. Table II indicates that when milled white corn meal was supplemented by sesame meal, the animals showed an increase of 26% in gain and of 12% in PER, in rations containing 6.86% protein, two thirds of which were derived from corn meal and one third from sesame meal (Table II, rations 12 and 13). Further addition of 0.1% of L-lysine produced increases of 379% in gain and 175% in PER. Similar results were obtained in rations 15 and 16, where the rations also contained 6.86% of protein; however, one half of the protein was derived from corn meal and one half from sesame meal. The increased gain was 89.4% and the increased PER was 50%. Addition of 0.1% of L-lysine further increased the gain to 489.4% and the PER to 201.6%. When enriched milled wheat flour (Gold Medal flour) was supplemented by sesame meal, the animals showed an increase of 4.6% in gain and 8.3% in PER, in rations containing 9.03% protein, two thirds of which were derived from wheat flour and one third from sesame meal (Table II, ration 18). This indicates that sesame meal can partially replace milled wheat flour without any change in protein value.

Results of some of these experiments are in agreement with those reported by Jaffé (*5–7*) who has reported that sesame and corn proteins favorably complement each other and found good growth with a mixture of 90 parts of corn, 8 parts of sesame, and 2 parts of peanuts. He prepared local corn bread by adding sesame meal.

Supplement to Milled White Polished Rice. The proteins of sesame seed at a 3% replacement level have a supplementary effect for those of milled white (polished) rice. This is shown in Table II, rations 19 and 20. The protein content of the rations is 5.42%. An equivalent amount of the proteins of polished rice was replaced by the proteins from 3% of sesame seed, leaving the protein level at 5.42%, with an increase in gain of 29.6% and in PER of 0.5%. An increase of 26.7% in growth and 8% in PER was found in animals fed ration 21, containing 5.42% of protein derived from sesame meal alone, indicating that sesame meal had better nutritive value than milled rice at that level of protein intake.

Biological Values. Table III shows the biological values, true digestibilities, and net utilization values of sesame seed supplemented with and without additions of L-lysine, DL-threonine, and vitamin B_{12}, as determined by the nitrogen retention method of Mitchell (*12*, *13*). The net utilization values are obtained by multiplying the true coefficient of digestibility by biological values and dividing by 100. The resulting values are somewhat higher for the proteins of sesame seed supplemented by amino acids and addition of vitamin B_{12} decreased the utilization. The small differences in net protein utilization be-

Table IV. Determination of Amino Acids

	In Fat-Free Sesame Seed, %, 43.3% Protein		In Fat-Free Sesame Meal, %, 51.3% Protein	
	In dry matter	In protein	In dry matter	In protein
Alanine	1.30	3.00	1.35	3.63
Arginine[a]	5.00	11.55	5.20	10.40
Aspartic acid	3.33	7.69	3.99	7.78
Cystine	0.52	1.15	0.60	1.17
Glutamic acid	4.21	9.72	3.80	8.20
Glycine	3.49	8.06	3.96	7.72
Histidine[a]	0.90	2.08	1.10	2.14
Isoleucine[a]	2.00	4.62	2.30	4.48
Leucine[a]	3.00	6.93	3.60	7.02
Lysine[a]	1.20	2.77	1.41	2.75
Methionine[a]	1.23	2.84	1.40	2.73
Phenylalanine[a]	2.52	5.82	2.71	5.28
Proline	1.60	3.69	1.92	3.74
Serine	1.42	3.28	1.80	3.51
Threonine[a]	1.60	3.70	1.71	3.33
Tryptophan[a]	0.75	1.73	0.70	1.37
Tyrosine	2.00	4.62	1.90	3.71
Valine[a]	2.70	6.23	2.65	5.16

[a] Nutritionally essential.

Table V. Vitamins and Other Constituents in Fat-Free Sesame Seed and Sesame Meal

Constituent	Seed, γ/G.	Meal, γ/G.
Thiamine	2.4	2.7
Riboflavin	2.5	2.0
Nicotinic acid	80	82.5
Pantothenic acid		
Total	9.5	12.0
Free	3.4	3.8
Biotin	0.06	0.07
Folic acid		
Total	0.20	0.23
Free	0.10	0.13
Pyridoxine	2.33	2.32
Inositol	740	830
Choline	1320	1590
p-Aminobenzoic acid	0.65	0.80
	%	%
Calcium	1.65	1.10
Phosphorus	1.60	1.25
Iron	0.0044	0.0037
Nitrogen	6.93	8.21
Protein, nitrogen × 6.25	43.3	51.3
Fat, in the original	39.3	34.5
Moisture	4.5	4.5
Ash	5.5	5.2
Fiber	6.3	5.7

tween rations 1 and 3, 1 and 4, and 3 and 4 were statistically significant.

The results of the metabolism experiments can explain the differences in growth-promoting values of the sesame proteins found in the growth experiments. The amino acid supplemented proteins were more efficiently utilized and promoted better growth as a result of the smaller losses of nitrogen during metabolism.

Amino Acid Content. Table IV shows the results of the amino acid determination in fat-free samples of sesame seed and meal expressed as percentages calculated on the basis of air-dried samples, and also expressed as the percentage in the crude proteins (N × 6.25). The content of the amino acids (essential) agrees well with that reported (*3*, *11*). The experimental results indicate that sesame meal is nutritionally inferior to sesame seed, which is not reflected in the amino acid composition data. However, the sesame meal used in these experiments is not produced from the same sesame seed. They belong to different varieties. Possibly, the sesame meal might have been slightly damaged in processing during the crushing operation. Heat promotes chemical linkage of lysine to other amino acids leading to reduced availability to the animals. Heat also produces a reaction of some amino acids with carbohydrates to combinations which prevent complete regeneration of these amino acids in the digestive tract (*2*).

Vitamins and Other Nutrients. A number of vitamins were determined by other methods (*8*). The results of these tests are given in Table V, which also contains data on calcium, phosphorus, iron, and other constituents. The values found for niacin are lower and for riboflavin are higher than those reported by Lease (*10*), while the calcium values are lower than those reported (*2*).

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Effect of Autoclaving on the Basic Amino Acids and Proteins of the Chick Pea

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The work reported grew out of an interest in the effects of heat on the nutritive quality of plant proteins. Lysine is the limiting amino acid in most rations in which cereals are the source of energy. Because the chick pea, a typically starchy seed, is rich in lysine and is an important food crop, interest developed in the effects of heat on the nutritive quality of its proteins in comparison with typical oilseeds. About 25% of the chick pea is protein, rich in lysine (6.5 to 6.7%). The lysine content is reduced about 10% when the seeds are heated in the autoclave to 121° C. for 30 and 60 minutes. The reduction is greater the less the moisture content of the seed.

USUALLY the nutritional quality of oilseed meal proteins is improved when heating of the seed during processing for oil is moderate (*9*), but amino acids, especially lysine, cystine, and arginine, are destroyed when heating is severe. Renner *et al.* (*14*) reported the loss of 15% of the lysine and 9% of the arginine in sunflower seed during the processing for oil. Other workers (*5–7*) reported substantial losses in lysine, cystine, histidine, tryptophan, and arginine on heating soybean meal. A loss of cystine and arginine, and a serious loss of lysine, may occur during the processing of cottonseed (*3, 11*). Lysine is lost in the processing of peanuts for oil (*2*), and loss is severe when peas are autoclaved (*8*).

A study has been made to compare the effects of heat on chick peas (*Cicer arietinum*) with previously noted effects on typical oleaginous seeds. In contrast with the latter seeds, the chick peas are characterized by a low oil content (usually 4 to 7%), a relatively high proportion of starch (in the neighborhood of 45%), and protein at a 20 to 30% level, depending on the variety and prevailing ecological conditions (*4*). Changes induced by heat in the chemical properties and in the basic amino acids of the proteins of two varieties of chick peas are reported in this communication.

Experimental

Two varieties were used: a large white seeded variety (Garbanza) used for human food in southwestern areas of the United States, Mexico, India, Pakistan, and several European countries; and forage chick pea (garbanzo porquero), a small seeded variety used in the central valleys of Mexico (Bajio area) mostly for feeding swine.

Flour was prepared from each type by cracking the undecorticated seed in a Bauer mill and grinding the seed fragments in a ball mill to pass through a 40-mesh sieve.

A crude protein preparation was obtained from the flour by aqueous alkaline extraction (pH 8 to 8.5). Twenty grams of the flour from each variety were dispersed in 100 ml. of 0.025*N* aqueous sodium hydroxide, and the resulting suspension was agitated mechanically for 10 minutes. The suspension was centrifuged, the supernatant liquid decanted, and the residue suspended in 80 ml. of 0.0125*N* aqueous sodium hydroxide. The residue obtained on centrifuging was further extracted with 80 ml. of water. The combined extracts were dried by lyophilization. The crude protein thus obtained was extracted with diethyl ether at room temperature to reduce its oil content.

Purified proteins were also prepared from the aqueous alkaline extract of each variety, by precipitating them at their isoelectric point. These proteins were dried by lyophilization and the oil was removed from the dried protein by extraction with 95% ethyl alcohol. The purified protein from the large variety was subjected to four successive washings with water prior to drying, in order to obtain as pure a protein as possible. The purified protein from the small chick pea variety was not washed.

Samples of the flour and protein preparations were adjusted to 10 and 50% moisture and heated in a steam-jacketed autoclave (contact between sample and steam avoided) for 1 hour at 121° C. The higher moisture was selected for observation because a precooked chick pea food is produced by autoclaving soaked chick peas. The samples with a moisture content of 50% were dried by lyophilization; the samples autoclaved with 10% moisture were stored over anhydrous calcium sulfate.

The moisture, nitrogen, starch, total sugar, ash, crude fiber, and oil contents were determined by the methods of the Association of Official Agricultural Chemists (*1*). Nitrogen solubility was determined by the method of Lyman, Chang, and Couch (*10*).

In the determination of the basic amino acids, the sample of flour or protein fraction was hydrolyzed under refluxing conditions for 24 hours with twice-distilled constant boiling aqueous hydrochloric acid. Two milliliters of acid were used for each milligram of protein. The hydrolyzate was taken to dryness under reduced pressure, and the last traces of hydrochloric acid were removed by repeated evaporation to dryness under reduced pressure after addition of small quantities of water. The residue was then taken up in water,

Table I. Per Cent Composition of Chick Pea Products[a]

	Large Seeded Variety			Forage Variety		
Constituent	Flour	Crude protein	Purified protein	Flour	Crude protein	Purified protein
Protein (N × 6.25)	26.5	60.1	93.5	21.3	54.3	88.0
Total sugar	4.2	12.2	...	3.2	9.5	...
Glucose equiv. of reducing materials produced on mild acid hydrolysis	46.6[b]	9.3	3.4	45.7[c]	12.8	6.4
Oil	6.4	0.0	0.1	5.3	1.8	0.1
Ash	3.1	7.4	1.2	3.3	7.7	2.8
Crude fiber	2.6	0.0	0.0	7.7	0.0	0.0

[a] Moisture-free basis. [b] Starch, 41.9%. [c] Starch, 41.1%.

Table II. Basic Amino Acids in Chick Pea Products

(Grams of amino acid per 16 grams of nitrogen)

Basic Amino Acid	Flour			Crude Protein			Purified Protein		
		Autoclaved 1 Hour at 121° C.			Autoclaved 1 Hour at 121° C.			Autoclaved 1 Hour at 121° C.	
	Control	50% moisture	10% moisture	Control	50% moisture	10% moisture	Control	50% moisture	10% moisture
					Large Seeded Variety				
Lysine	6.5	6.2	5.8	6.5	6.2	5.7	6.5	6.3	6.1
Histidine	3.0	3.0	2.9	2.9	2.9	2.9	3.0	2.9	2.9
Arginine	11.4	11.2	11.3	11.0	10.9	11.0	10.0	10.0	9.9
					Forage Variety				
Lysine	6.7	6.3	5.8	6.6	6.3	5.7	6.6	6.5	6.1
Histidine	2.9	2.9	2.9	3.0	2.9	3.0	2.9	3.0	3.0
Arginine	9.5	9.5	9.6	9.6	9.6	9.4	9.6	9.5	9.5

Table III. Effect of Autoclaving on Protein Solubility

	Nitrogen Solubility in 0.02N NaOH, %		
	Control	50% H_2O	10% H_2O
	Large Seeded Variety		
Flour	98	61	55
Crude protein	99	76	64
Purified protein	99	75	59
	Forage Variety		
Flour	98	39	39
Crude protein	95	54	39
Purified protein	98	55	38

filtered through a medium-porosity sintered-glass filter, and brought to a known volume (25 to 50 ml.) at pH 6. An aliquot of this solution was shaken with an equal volume of a 50% suspension of Dowex 2 resin for 45 minutes. The suspension was then filtered and washed, first with water and then with 0.04*M* acetate buffer at pH 3.7. The combined washings and filtrate were evaporated to dryness under reduced pressure, and the residue was taken up in a known volume of citrate buffer (25 ml.) at pH 4. An aliquot of this solution was placed on a 3 × 15 cm. Amberlite IR 120 column, and the basic amino acids were eluted, according to the procedure of Moore, Spackman, and Stein (*12*). The basic amino acids, which are resolved on the column, were determined by the photometric ninhydrin method of Moore and Stein (*13*).

Results and Discussion

The chick pea is used for food and feed by a large segment of the world population. Because it is a potential source of a high quality protein concentrate, compositional data recorded in Table I are of interest. The protein, oil, and total sugar contents of the flour from the large seeded variety are approximately 20% higher than those of the forage chick pea. The starch content of both varieties is approximately 42% of the weight of the seed. The crude fiber content of the forage variety, however, is about three times that of the large seeded variety.

The nitrogen content of the crude protein fraction from the large seeded variety was about 10% and the total sugar content about 20% greater than those of the forage variety. The total reducing materials produced on mild acid hydrolysis as determined by the Somogyi (*15*) method and calculated as glucose were 25% greater in the crude protein of the forage variety than of the large seeded variety. Comparable reducing materials in the purified protein fraction of the forage variety were about 50% higher than those of the large seeded variety.

The crude protein product of the large seeded variety represented 42% of the seed, and contained 94% of the total nitrogen, while the crude protein of the forage variety represented only 36% of the seed, and contained 93% of the total nitrogen.

The purified protein product of the large seeded variety represented 22% of the seed and contained 75% of the total nitrogen. The purified protein of the forage variety represented around 20% of the flour and contained 80% of the total nitrogen. The difference in carbohydrate content was due to the fact that the precipitated protein from the forage variety was not washed with water prior to lyophilization.

Data for the basic amino acids in the flour and in the crude and purified proteins of both varieties are listed in Table II. The lysine and histidine levels were about the same in both varieties; the arginine level was lower in the flour from the forage variety. The arginine contents of the forage chick pea flour and proteins remained constant; however, the crude and purified proteins of the large chick pea were, respectively, 3.5 and 12.3% lower in arginine content than the flour.

The destruction of lysine on autoclaving the products of the chick pea for 1 hour at 121° C. was not greater than 14% in the chick pea flours and crude proteins. This measured destruction was about 7.5% in the purified proteins, when the samples contained 10% moisture. The highest drop in the lysine value for the moist samples heated for 1 hour was 6%. Conkerton *et al.* (*3*) reported a reduction of 37% in the lysine content on autoclaving cottonseed meal for 2 hours. The reduction in the lysine level which occurred with peanuts cooked for 2 hours at 121° C. was 15% (*2*). Destruction of lysine by heat appeared more extensive in cottonseed than in peanuts or chick peas.

No measurable reduction was noticed for the arginine on autoclaving the chick peas. Destruction of this amino acid was reported after autoclaving cottonseed meal for 2 hours (*3*).

The data recorded in Table III show that the fraction of total nitrogen soluble in 0.02*N* aqueous sodium hydroxide decreases on autoclaving the flour, crude protein, and purified protein from both varieties of chick pea, but that the reduction is greater for the drier materials and is greater for the forage chick pea fractions than for those from the large seeded variety.

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LITERATURE AVAILABLE

(*Continued from page 250*)

eight pages gives specifications, describes use of Attagel 30, a colloidal grade of Attapulgite, as a suspending agent for plant nutrients in water. Six typical liquid formulations in which product has been used successfully are included. Technical Data Sheet T. I. 959 is available from Dept. A&F, MINERALS & CHEMICALS CORP. OF AMERICA, Essex Turnpike, Menlo Park, N. J.

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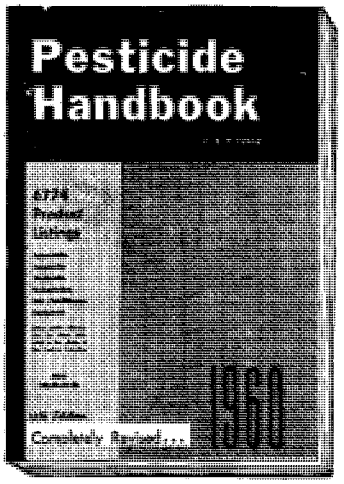

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Vibratory Equipment. New condensed catalog describes company's vibratory materials handling equipment and other lines. Among product lines covered are vibrators, car shakers and rappers, packers and jolters, hopper level switches, flow control valves, feeders, conveyors, spiral elevators, dry feeders and weigh-feeding equipment, vibrating screens, and many others. Catalog gives descriptions, data, and specifications, and lists company's representatives. Request Catalog 605 from Dept. A&F, SYNTRON Co., 1222 Lexington Ave., Homer City, Pa.

Filling Machines. Eight-page illustrated folder describes company's line of automatic and semiautomatic auger filling machines. Explains basic principle and inherent advantages of auger filling for powder, paste, and granular products. Chart lists speeds, weight ranges, and container sizes for 11 standard models. Copies are available from Dept. A&F, FMC PACKAGING MACHINERY DIVISION, 4900 Summerdale Ave., Philadelphia 24, Pa.

Seed Disinfectant. Data sheet describes new seed disinfectant and summarizes field experiments showing its effectiveness against loose smut of oats and bunt of wheat. Compound is only slightly volatile and is said to be less phytotoxic than other products. Request bulletin on Methylmercurypentachlorophenolate Compound "473" from Dept. A&F, Shepard Division, SOUTH

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Nutritional Research. New 60-page catalog contains a comprehensive selection of products for use by research workers in biology, microbiology, bacteriology, and nutrition. Included are a wide range of amino acids, peptides, carbohydrates, adenylates, nucleates, purines, pyrimidines, enzymes, hormones, prepared microbiological media, and prepared test diets for experimental animals. Catalog is completely indexed, and each page lists item, weight, and price. For copy, write Dept. A&F, GENERAL BIOCHEMICALS, INC., 49 Laboratory Park, Chagrin Falls, Ohio.

Aircraft Calibration. Handy pocket card, useful to aerial applicators, is plastic reproduction of USDA card giving formulas and tables for calibrating aerial application rates. One table gives number of acres in a swath of given width and length; the other shows the rate, in acres per minute, at which spray or dry material can be applied when swath width and speed of aircraft are known. Aerial applicators can secure free copies by writing Dept. A&F, TRANSLAND AIRCRAFT, Torrance Municipal Airport, 2600 West 247th St., Torrance, Calif.

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Arsenic Reagent. Bulletin details method for arsenic determination in parts-per-billion amounts. Based on new stable grade of silver diethyldithiocarbamate, colorimetric method is said to be sensitive and reproducible, simpler and more reliable than either the traditional Gutzeit method or the molybdenum blue method. Bulletin includes references. Write Dept. A&F, FISHER SCIENTIFIC Co., 308 Fisher Building, Pittsburgh 19, Pa.

Product Evaluation. Newly revised brochure describes company's broad scientific approach to biological and chemical research. Describes facilities and services for evaluation of chemical products in terms of their safety and effectiveness in the environments in which they will be used. For copy, send request to Dept. A&F, HAZLETON LABORATORIES, INC., P. O. Box 30, Falls Church, Va.

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Food Additives. Entitled "How to Proceed Under the Food Additives Amendment," 12-page booklet is first published section of a seven-part manual on food additives. It offers manufacturers a guide to legal and technical considerations presented by the Food Additives Amendment of 1958. In question-and-answer format, the booklet explains how to determine whether a substance should be classified as a food additive. Other sections describe procedures for filing petitions for approval, for filing objections to regulations, and for filing petitions for the U. S. Circuit Court of Appeals. Copies of the booklet are available for fifty cents each from Dept. A&F, MANUFACTURING CHEMISTS' ASSOCIATION, 1825 Connecticut Ave., N.W., Washington 9, D. C.

Agricultural Services. Brochure of 4 pages lists prices for argicultural services involved in Florida testing of agricultural chemicals. Among services offered are foliar and soil fungicide tests, seed treatment tests, insecticide, herbicide, and nematocide tests, residue tests, and horticultural tests. Booklet outlines planting schedules, material requirements, and reporting procedures. Request copy of "Florida Field Trials" from Dept. A&F, DR. G. R. TOWNSEND, Box 356, Belle Glade, Fla.

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White Oils and Petrolatums. Effect of the Food Additives Amendment on uses of white mineral oils and petrolatums is discussed in bulletin. Subjects covered include food grade specifications, ultraviolet absorbance, food uses and tolerances, and applications in the food and related industries in which less highly refined mineral oils and petrolatums have been used in the past. Request copy of the bulletin, *Current Topics* #144, from Dept. A&F, Industrial Research, SONNEBORN CHEMICAL AND REFINING CORP., 300 Park Ave. South, New York 10, N. Y.

Pharmacology for Business. Booklet discusses direct effect of new laws and indirect pressure of public opinion in such fields as foods, drugs and cosmetics, packaging, wearing apparel, and many types of household products. Trends in public demands for ever-increasing assurances of safety in consumer products are discussed from the point of view of the average business man; booklet attempts to show the scope of the problem and to suggest some economic solutions. Copies of "Your Products on Trial" are available from Dept. A&F, Client Relations, FOSTER D. SNELL, INC., 29 West 15th St., New York 11, N. Y.

Nutritional Status, USA. Interregional research publication gives data obtained throughout the United States in cooperative regional researches in nutritional status, 1947–59. Results reported in 178 publications from 39 states, and concerning more than 12,000 individuals, are compiled and interpreted as a whole. Sections of

(*Continued on page 424*)

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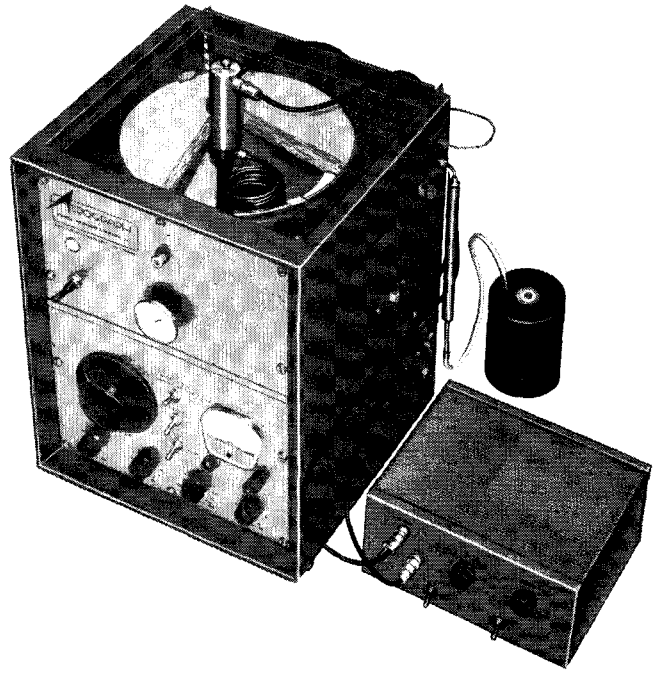

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Progress and Problems in Radiation Processing of Food

WALTER M. URBAIN
Swift & Co., Chicago, Ill.

The need for improvement of food preservation methods to aid in food distribution justifies interest in radiation preservation. Results of work to date on this process are reviewed. A look ahead is attempted. Governmental approval for the process is a first requirement for use. Secondly, products with acceptable or desirable characteristics must be made available at an allowable cost. Radiation-sterilized products must demonstrate a quality improvement over existing products. The use of less than sterilizing procedures will be governed more by economic factors, as quality improvement is unlikely.

DESPITE millennia of human effort on methods of food distribution, important problems still exist today, both economic and technical. Food preservation methods can contribute to the solution of food distribution problems by permitting a food to be saved from time of production to time of consumption and/or be transported from points of production to consumption. However, only when a desirable and useful balance of economic and technical factors exists is a particular preservation process usable. Clearly, particular foods have different requirements and, therefore, it is necessary or desirable to use a variety of preservative processes, each having some limitations in its applicability. Not all that we desire to do can be done. Therefore, there has been a continuing search for new preservation methods and, hence, a well justified interest in irradiation as another approach to food preservation to aid in solving distribution problems.

Today, after almost 20 years of research by countless workers, we can view radiation preservation of foods with a clearer perception of its capabilities and limitations. This paper reviews what has been learned and considers what may lie ahead.

Two comments are in order today with respect to the early views on irradiation: (1) The hope that irradiation would be a panacea was a vain hope, and (2) the opportunity for controlling spoilage without heating or without the use of additives which bring about concomitant changes in the foods was (and still is) a justifiable reason for interest.

Results of Work to Date

Radiation can destroy microorganisms responsible for spoilage. It can also control insect-caused spoilage and some other processes causing spoilage, such as sprouting.

With due allowances for differences in penetration and absorption, electron beams and gamma radiation or x-rays have similar if not identical action.

The amount of radiation required to control a particular spoilage agent varies with the agent over a wide range from about 10,000 to 4,500,000 rads. In control of spoilage by bacteria, two areas of application exist: destruction of all organisms present, and partial destruction, the former leading to essentially unlimited preservation and the latter to limited extension of product life.

The amounts of radiation to inactivate enzymes exceed by a substantial margin those needed for any other spoilage agent of a biological nature, and from a practical viewpoint are too great to allow the use of radiation as a means of control.

Radiation can change taste, appearance, and texture of a given food. The exact effect depends upon the food in question, the amount of radiation, and the conditions of treatment.

While inadequately evaluated at the present, consumer acceptance of foods irradiated at sterilization levels may be considered to be generally low. At less than sterilization levels, however, many foods are acceptable.

Certain levels of energy of both electron beams and γ-rays can induce radioactivity in foods; on the other hand, it is possible to select useful levels which avoid this effect. In this way, the question of the significance of radiation-induced radioactivity (which is of an amount comparable with the activity present from naturally occurring isotopes such as potassium-40) can be resolved most simply by controlling the process so as not to produce any.

On the basis of information available to date, wholesomeness—i.e., safety—of irradiated foods appears to be satisfactory.

The radiation-induced changes in nutrients are comparable with those which occur with other food preservation methods commonly in use and hence normally will not be of great importance.

Radiation sources are available, although not all requirements for large scale commercial operation could be fulfilled without further work. The control of sources through adequate dosimetric procedures, while well developed, may require additional work.

The economics of irradiation appear to fall within a reasonable range of requirements. They have not, however, been fully determined and undoubtedly each individual application will require its own appraisal.

Specific Accomplishments

Sterilization of Nonacid Low-Salt Foods. Complete sterilization of nonacid low-salt foods requires close to 4,500,000 rads, according to present estimates. This level will destroy *Clostridium botulinum*, which is the most radiation-resistant organism of spoilage significance. The precise value for destruction of *Cl. botulinum* in nonacid low-salt foods is not known and needs further definition. For foods containing high salt levels or having acidity adequate to make it unnecessary to provide for *Cl. botulinum* destruction, a significantly smaller figure for "sterilization" may be anticipated, but adequate information is lacking on this.

By combining radiation with heat,

a synergistic effect may be obtained. Apparently radiation can sensitize *Cl. botulinum* to heat. Thus with cooked beef, 1,200,000-rad pretreatment reduced fourfold the heat requirements (as measured by F_0 value) for sterilization (*4*). Such combination treatments may offer good approaches to more satisfactory sensory characteristics through reduced requirements for both heat and radiation.

For less than sterilization the requirements for a useful effect vary with the particular food, the conditions of storage, and the desired end result. In general, useful effects are obtained at levels between 10,000 and 500,000 rads. Table I lists a number of values reported for various foods.

Spoilage from Insect Infestation. Spoilage resulting from insect infestation can be controlled by approximately 25,000 rads (*5*). Sprout inhibition of onions can be obtained with 4000 to 8000 rads (*1*) and with potatoes at about 10,000 (*2*).

Sensory Changes. At sterilizing levels of radiation the following foods appear to undergo sufficiently small changes in sensory characteristics as to be generally considered acceptable: chicken, fresh (not cured) pork, oysters, and shrimp.

These are nonacid low-salt foods. Other similar foods show various changes which appear to be objectionable. Among these is a taste change associated with a flavor that seems characteristic of irradiation; it resembles but is not identical with scorching. Beef, for example, develops this off-flavor to a considerable degree. Milk products are especially affected. Eggs, cereals, and vegetables to varying degrees undergo flavor changes, which in some cases are not the typical irradiated flavor. The induction of flavor changes is probably the largest single obstacle to the broad use of irradiation as a preservation method.

Other sensory characteristics of foods may also be altered by radiation. Color is frequently altered, but the character of the alteration usually is specific to the food in question. At relatively low levels raw beef will be browned, but at higher levels (several million rads) the normal red color returns and may be intensified. Cooked beef when irradiated in the absence of oxygen turns pink, as does chicken. Cured meats are generally browned, especially in the presence of oxygen. The orange-yellow color of Cheddar cheese is bleached. Cereals are darkened. Vegetables display a variety of off-colors. Pink salmon is bleached.

Texture may likewise be altered. Loss of firmness is common with certain fruits and vegetables. Beef is "tendered" at several million rads. The thick white of eggs thins.

Functional properties may be harmed. The whipping properties of egg whites can be reduced. The ability of flour to yield normal volumes with baked goods such as cakes is reduced.

Irradiation Flavor Development. Studies have been carried out for the purpose of seeking an understanding of the chemistry of the irradiation flavor development. Much of this work has been directed toward isolating the flavor components.

From beef have been obtained sulfur compounds such as hydrogen sulfide and mercaptans. Work on proteins has suggested that the origin of these sulfur compounds lies with sulfur-containing proteins. Other compounds also have been obtained, including aldehydes, ketones, alcohols, and amines, but their significance seems to be less than that of sulfur compounds. Somewhat strangely, perhaps, the compounds isolated are the same ones found in unirradiated beef, but appear in larger amounts. No single compound has been found to account for the characteristic flavor.

Such work might be expected to give a clue as to the mechanism of the irradiation flavor formation. Results to date need to be extended before we can hope to understand the process and then be able to control it.

Mechanism of Flavor Formation. Even without an understanding of mechanism of flavor formation, attempts have been made to control it. These efforts have postulated that radiation produces free radicals in the food, for which there exists independent evidence. This approach has sought to provide free radical acceptors to compete with the compounds native to the food, which, when acted upon by the free radicals, form the unwanted flavor compounds. Sodium ascorbate is perhaps the best example of such a free radical acceptor. An alternative method freezes the food during irradiation. This method attempts to restrict the "sphere of influence" of the free radicals by irradiating in the solid state. In both approaches the presumed origin of the free radicals is the water natural to the food.

A third approach seeks to eliminate oxygen derived from the atmosphere. Radiation can convert molecular oxygen to active compounds which appear to account for some of the irradiated flavor. There seems little doubt about the desirability of irradiating in the absence of oxygen.

While the value of using these aids seems clear, neither alone nor in combination have they provided sufficient control of the irradiation flavor process.

Table I. Radiation Levels for Useful Effects with Less Than Sterilization Treatments

Food	Rads
Fresh beefsteak	100,000
Pork chop	100,000
Chicken	100,000
Bananas (*3*)	14,000
Oranges, lemons (*3*)	140,000
Grapes (Tokay) (*3*)	465,000
Apples (*3*)	50,000
Cabbage (shredded) (*3*)	47,000
Tomatoes (*3*)	279,000
Spinach (*6*)	1,500,000

What Lies Ahead

What lies ahead for food preservation by irradiation? With all the knowledge we possess about the process, why are we not using it? The answer is not simple, but we point out some problems which need solving, and perhaps in this way, we can get some picture of the future.

The commercial utilization of irradiation of foods cannot become a reality until its use is approved by government regulatory bodies. Under Federal Public Law No. 85-929 (Food Additive Amendment of 1958), the burden of proof of safety of a food additive or process is on the one who wants to use it, not the consumer and not a government agency such as the Federal Food and Drug Administration. In the atmosphere of this law and the attendant philosophy, no sketchy evaluation of food irradiation is acceptable. One can assume as realistic that industry, in the face of other uncertainties of the irradiation process, is not likely to undertake this proof. Fortunately, in the United States military interest in irradiated foods has been considerable and, in accordance with military objectives, a thorough program of evaluation is being conducted under the supervision of the Army Surgeon General in cooperation with the Food and Drug Administration. This program, when complete, should provide the information needed to pass on the safety of irradiated foods. Until it is completed, no commercial use of radiation on foods for preservation is likely to be permitted.

Setting aside the need for governmental approval as a consideration, the utilization of the process commercially depends upon two factors: the fulfilling of a useful purpose at an allowable cost, and the availability of products having acceptable or desirable characteristics. As of today certain irradiated foods appear to have met these requirements. One might include pork, chicken, oysters, and shrimp in this category even when completely sterile. Among the less than sterile foods may fall certain meats such as liver and pork sausage, frozen eggs, fruits such as strawberries, peaches, grapes, and citrus fruits, and vegetables such as cabbage, tomatoes, and potatoes.

In order to speculate as to whether such products would be produced commercially, one might attempt to determine what value could be ascribed to their availability. Complete sterilization of meats, fruits, and vegetables could yield products superior to those now available as fully preserved foods, specifically canned or dried products. Such quality improvements could justify added costs and make economics a secondary consideration. For example, the ability to sterilize a truly roasted piece of meat (dry-heat-cooked outside the storage container and then radiation-sterilized after packaging) would represent a substantial quality improvement over conventional thermal processing. Consumer acceptance of such an item might warrant its commercial preparation. Similarly, fruits and vegetables having fresh characteristics would be improvements over the usual canned products. While cost considerations would be significant, improvement of quality in shelf-stable products would be a factor of prime importance.

When products are treated only to extend their normal life, quality improvement, detectable by the consumer, may not result. The results are more likely to be reduction of spoilage losses, extension of the marketing period, and accessibility of hitherto unreachable markets. The value of such benefits is primarily economic and for this reason economics and not product quality improvement will determine their commercial preparation. On this basis one might conclude that the most likely applications will fall in the field of fruits and vegetables where distribution problems exist and losses today are large, and not in meats or poultry where losses are nominal. Which fruits and vegetables, will depend upon individual situations. At present, strawberries and potatoes afford some interesting possibilities.

To sum up the look ahead, we can list two points: Before any commercial use, we must have government clearance for the process, and for less than sterilized products, there must be favorable economics. For sterilized products there must be a clear-cut product quality improvement plus reasonable economics.

How do we proceed? The military program should determine whether government approval can be had. Assuming irradiated foods are cleared, then for less than sterilized foods we shall need suitable radiation sources, and beyond this little else from a technical point of view. For sterilized products, in addition to sources we shall need adequate controls to guarantee delivery of a minimum sterilizing dose for reasons of safety. But most of all for sterilized products we need quality improvement. Perhaps this is available now in pork, chicken, oysters, and shrimp. For other foods we need new knowledge in order to control the undesired sensory changes, particularly flavor. In view of the effort of the past years which has supplied little understanding of the mechanism of these changes, it is clear that what is needed is more basic research. This is a highly important area for the future.

There is one final aspect. Consumer or public attitude toward irradiated foods will be a large if not dominant factor in their success. Even with the assurance of safety based on government approval for irradiated foods, the public will act on its own confidence and understanding. Public consciousness of the hazards of potentially carcinogenic substances in foods, and its awareness of the dangers of radiation *per se* are bound to raise questions regarding safety. The possibilities of confusion between the effects of radiation on the human body and the consumption of irradiated foods will require education to clarify. The seller of irradiated foods will need to secure public confidence through an organized information program.

Prior events actually outside the area of irradiated foods will have great significance in the reaction of the public. We do not know what these will be and any counteraction of them will have to wait until we are ready to sell irradiated foods. It would appear, however, that we shall not be ready to offer irradiated foods for sale for some time to come.

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RADIATION PRESERVATION OF FOOD

Current Aspects of the Wholesomeness of Irradiated Food

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RADIATION PRESERVATION OF FOOD represents a rather radical innovation. It was first proposed nearly 15 years ago as a possible supplement to existing food-processing techniques. Its potential application has been under active investigation for over ten years. Throughout this period, the lay press, as well as the technical journals, has shown an exceptional interest in the research results as they were made available. As a result, food irradiation has been alternately heralded as a panacea for the world food problem and as a technological (or economic) impossibility. At present a fair interpretation would appear to be that radiation preservation of food will provide an important supplement to present food-processing practices but will by no means completely supplant any process now in use.

This conclusion is based upon two facets of the over-all problem of suitability: technological feasibility and the health-hazard potential (wholesomeness) of the treated food. Of these, technological development has presented the more formidable problems, particularly from the viewpoint of "commercially sterile" products. However, recent releases to the lay press have stressed the unsuitability of irradiated foods for human use from the whole-

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In order to speculate as to whether such products would be produced commercially, one might attempt to determine what value could be ascribed to their availability. Complete sterilization of meats, fruits, and vegetables could yield products superior to those now available as fully preserved foods, specifically canned or dried products. Such quality improvements could justify added costs and make economics a secondary consideration. For example, the ability to sterilize a truly roasted piece of meat (dry-heat-cooked outside the storage container and then radiation-sterilized after packaging) would represent a substantial quality improvement over conventional thermal processing. Consumer acceptance of such an item might warrant its commercial preparation. Similarly, fruits and vegetables having fresh characteristics would be improvements over the usual canned products. While cost considerations would be significant, improvement of quality in shelf-stable products would be a factor of prime importance.

When products are treated only to extend their normal life, quality improvement, detectable by the consumer, may not result. The results are more likely to be reduction of spoilage losses, extension of the marketing period, and accessibility of hitherto unreachable markets. The value of such benefits is primarily economic and for this reason economics and not product quality improvement will determine their commercial preparation. On this basis one might conclude that the most likely applications will fall in the field of fruits and vegetables where distribution problems exist and losses today are large, and not in meats or poultry where losses are nominal. Which fruits and vegetables, will depend upon individual situations. At present, strawberries and potatoes afford some interesting possibilities.

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Radiation processing potentially represents a new approach to food preservation. Recently, confusion has arisen concerning the suitability of irradiated foods for human consumption. This report describes wholesomeness testing and undertakes an analysis of the pertinent data now available. Sixteen foods have been found to be grossly nontoxic to either rats or dogs. Further work is needed to elucidate the heart rupture observed in mice fed highly irradiated foods. Vitamin A, B_1, B_6, C, E, and K are susceptible to extensive radiation destruction. These vitamin losses are dose-related and vary among foods. The resultant deficiency effects may be avoided by consumption of mixed diets of irradiated foods or by appropriate supplementation. No indications of toxicity or nutritional imbalance have been observed during short-term feeding trials with human volunteers.

someness viewpoint. Thus, it would appear timely to evaluate the available data to determine to what degree irradiated foods may be unwholesome.

Wholesomeness Testing

Before considering the research program concerning wholesomeness of radiation-preserved foods, it is necessary to examine the meaning and magnitude of the term "wholesomeness." Wholesomeness encompasses two major areas of interest: potential toxicity and nutritional adequacy. These two components are difficult to separate experimentally, inasmuch as there is no finite line which may be drawn between marginal toxicity and low-level nutritional sufficiency. The best approach available is to provide experimental animals with a diet containing all of the known nutritional requirements for adequate growth and development. To this ration may be added the material under question in as large a quantity as the animal will tolerate. Differences in response between animals fed the control and the experimental diets may then be ascribed to potential toxicity. In the case of food, of course, isolation of the offending compound(s) from the treated food must also be undertaken to establish conclusively the existence and identity of a toxicity syndrome. After it has been shown that a particular food is not toxic under these conditions in two or more species of animals, attention may be given to the question of nutritional adequacy. Here, standard techniques may be employed: growth; availability and utilization of the fat, protein, and carbohydrate components; and vitamin analyses. As long as no serious changes are discovered by these tests, it may be safely assumed that the treated food is wholesome.

The Army Research Program

The early experiments employing ionizing radiations for preserving food were very encouraging. The small samples employed did not undergo great alterations in color, texture, etc., so that the process held considerable promise for producing sterile products which, although raw, possessed remarkable keeping qualities. There was little reason to suspect that wholesomeness might be affected. The Department of Defense soon became interested in the potential application of radiation preservation to the serious problem of supplying front-line troops with attractive, nutritious, easily transported foods. A research program was therefore established to study the technological problems involved. It rapidly became apparent, however, that the large energies required for food sterilization caused demonstrable changes in the appearance and chemical composition of many products. Furthermore, it was possible that similar changes might also occur in the wholesomeness characteristics. Therefore, a special program was established to investigate this area simultaneously with the product development research.

It was readily apparent that the wholesomeness testing of radiation-preserved food items would be costly in both time and money. Although many private companies were interested in the results, the cost prevented them from undertaking adequate testing programs to establish wholesomeness. Therefore, the burden of such testing has, by necessity, fallen to the Government, particularly through the Office of the Surgeon General, U. S. Army. This program was initiated in 1953. In the succeeding years, it has been expanded to include 31 research organizations. By and large, most of these research contracts have been established with scientists in well-recognized universities throughout the United States. A few contracts have also been undertaken by private research foundations and government laboratories. During this period over $5,000,000 has been expended for wholesomeness testing alone.

The wholesomeness investigations have not been unilaterally undertaken by the Army. Throughout the planning of the national program, many interested government agencies were consulted. In particular, the Food and Drug Administration contributed greatly in establishing the research approach to be employed. As a result, it is believed that the resultant data may be used by FDA in considering the suitability of radiation-preserved foods for human consumption in the United States. In this way it is hoped that excessive duplication of effort will be avoided in making the transition to commercial application.

In undertaking research concerning the wholesomeness of gamma-irradiated foods, it must be kept in mind that blanket "clearance" of all foods may not be obtained by testing one individual irradiated food. The radiation-induced reactions in any system are dependent upon the components of that system. Many compounds preferentially absorb the incident radiation energy so as to protect the other components of that system. Because each individual food may be considered to be a unique chemical system, it is necessary to test each food (or at least a representative number of foods) for wholesomeness in order to obtain the desired answer. Furthermore, as the radiation dosage increases, the types of chemical products will change. It is conceivable that a product which is biologically unsuitable at one level might be harmless following treatment with a higher dosage of radiation because of the subsequent radiation destruction of the harmful by-product. Therefore, all investigations must include more than one radiation dosage. These have been selected on the basis of applicability to practical expectation. The lower dosage of 2.79 megarads (1 rad = absorption of 100 ergs of energy per gram; 1 megarad = 1,000,000 rads) was chosen as an estimate of the treatment likely to be necessary for commercial dosage; a second dosage twice as high (5.58 megarads) was included to accentuate radiation-induced changes. This was fortunate, inasmuch as present estimates suggest that 4.8 megarads may actually be necessary for commercial sterilization.

In the beginning, the studies were undertaken using individual foods stored frozen after irradiation to minimize storage-accentuated alterations. The growth of rats served as the index to toxicity. Subsequently, the program

was expanded to include 2-year feeding studies with cooked, irradiated food products which had been stored for 6 to 9 months at room temperature after irradiation. These experiments employ rats, dogs, mice, and monkeys as the test animals, with growth, reproduction, life span, and disease incidence as the primary criteria. Although the experiments are still in progress, they are in most cases sufficiently close to completion for tentative conclusions to be drawn. Additional research concerning radiation-induced vitamin destruction has been included in the program, as have extensive investigations concerning the possibility of increased cancer incidence (carcinogenicity) in animals fed the irradiated foods.

Once a food has been found to be wholesome in experimental animals, human testing may be undertaken. The latter utilizes the services of volunteer subjects who live under a controlled regimen. During the test periods, the volunteers consume irradiated foods first for 15-day periods and later for intervals up to 6 months.

When all of these investigations have been completed, the Armed Services will be in a position to evaluate the wholesomeness of irradiated foods for military use. The accumulated data will also be given to the Food and Drug Administration for its consideration. Thus, final conclusions concerning the wholesomeness of irradiated foods probably will not be completed for another three to five years.

Potential Toxicity

Short-term (12-week), single-food rat feeding tests have been completed employing a total of 38 frozen-stored irradiated (2.79 or 5.58 megarads) foods comprising 35% of the dietary solids in each case (*48, 58*). In these tests, only irradiated jello powder and irradiated peaches were found to exert a significant growth-depressant effect. In the case of the jello powder this was traced to the sucrose component of the product; it is possible that a similar phenomenon occurred with the peaches, as they were obtained from a commercial source and contained a sugar sirup. However, Bubl and coworkers (*7*) did not confirm the growth-depressant effect in a four-generation study using irradiated peaches stored at room temperature. In addition, a composite diet containing 20% irradiated peach solids (*49*) did not show adverse growth effects in four successive generations of rats.

Several long-term studies concerning the toxicological properties of irradiated foods have been reported. In the earliest of these, Da Costa and Levenson (*14*) found that consumption of an electron-irradiated semisynthetic ration decreased reproduction performance in rats. A later report by Poling and coworkers (*45*), in which irradiated (1.86-megarad) beef was fed as 45% of the solids, noted the same phenomenon but reported that oral supplementation with vitamin E overcame the problem. No other indications of toxicity or nutritional imbalance were observed. Similarly, no adverse effects were noted in 2-year rat-feeding trials with 2.79-megarad-treated mixed organ meats (*7*) or 1.55-megarad-irradiated butterfat (*4*) nor after one year of feeding a 2.79-megarad-irradiated commercial mash to chickens (*10*). A four-generation study utilizing growth, reproduction, life span, and disease incidence as the primary parameters has also been reported (*49*). No signs of toxicity were seen as a result of consumption of a diet compounded from nine individually irradiated frozen-stored food items.

More recent attention has been focused upon toxicity testing of foods stored at room temperature for 6 to 9 months following irradiation. Read *et al.* (*50*) found raw beef or pork treated with 2.79 or 5.58 megarads to be nontoxic for the rat following 12 weeks of feeding. Reber and coworkers (*52*), using dogs, reported no deleterious effects of feeding 37,200 or 74,400 rad-treated flour diets for 24 weeks.

Additional data are also available from the series of contracts sponsored by the Office of the Surgeon General. In these experiments, 22 foods are being tested as 35% of the dietary solids. All foods are fed to rats for 2 years (parent generation) and through three subsequent generations. Each food is tested at two irradiation levels with a nonirradiated vitamin supplement provided with the diet. Growth, reproduction, life span, and carcinogenicity are used as the indexes to toxicity. Using this approach, ten foods have been fed to rats with no grossly toxic manifestations: bacon (*31*), green beans (*53, 55*), chicken (*53, 55*), codfish (*1*), dried fruit mixture (*31*), evaporated milk (*15*), sweet potatoes (*1*), white potatoes (*6*), peaches (*9*), and pork loin (*8*). Excepting green beans, chicken, and white potatoes, the histopathology studies that accompany these feeding studies have not been completed.

The counterpart experiments employing dogs as the test animal assess toxicity by analysis of 2-year growth data plus reproductive data from two breeding trials. At the end of the 2-year feeding period, the animals are sacrificed for histologic examination. In experiments of this type, 13 foods have been successfully fed with no gross toxic manifestations: bacon (*18*), green beans (*25*), beef (*51*), carrots (*17*), chicken stew (*29*), codfish (*1*), flour (*51*), mixed dried fruits (*25*), evaporated milk (*15*), sweet potatoes (*1*), pork loin (*29*), shrimp (17), and tuna fish (*29*). Again, the histopathology phase of these studies is still in progress.

To augment these experiments, additional carcinogenicity studies have been undertaken with inbred tumor-susceptible strains of mice in order to assure that radiation treatment does not produce cancer-inducing substances in the foodstuffs. In one of these experiments bacon treated with 5.58 megarads was fed to mice for 2 years (*30*). No significant differences in rate of growth or cancer incidence were found between the irradiated and control diet groups. Additional studies are now in progress using mice fed composite diets of five or six foods from the list under investigation with other animals. None of these experiments has progressed far enough to permit evaluation of the tumor-incidence data. However, an interesting phenomenon completely divorced from carcinogenicity has been observed by Monsen (*40*). Vitamin-supplemented irradiated and control diets compounded from equal proportions (dry solids) of pork loin, chicken, evaporated milk, white potatoes, and carrots have been fed to mice for approximately 20 months. Of the 377 animals started on the irradiated diet, 66 have died of enlargement and rupture of the left auricle of the heart. None of the Cb animals fed the control diet showed this lesion. Furthermore, only seven Strong A mice exhibited these symptoms after consuming the irradiated diet. Subsequent attempts to reproduce the disease with new animals fed the same diet with or without vitamin supplement have failed. However, 77% of the Cb mice fed cooked irradiated milk (with vitamin supplement) showed this lesion within 141 days on the diet, whereas only 16% of the animals fed uncooked irradiated milk were afflicted. Thus, these observations have not yet been explained but the investigations are continuing. This disease has not been reported in long-term dog and rat experiments using diets containing 35% evaporated milk (*15*).

Nutritional Adequacy

The question of nutritional adequacy of irradiated food has received considerable attention. As a result, the literature in this area is voluminous, so that complete discussion of the subject is beyond the scope of the present paper. Comprehensive reviews have been prepared by Read (*47*) and Johnson (*19*). Nevertheless, a general review with a discussion of the most pressing current problems is of interest.

Under the influence of irradiation the carbohydrates are oxidized or depolymerized to form smaller molecules. The degree to which this occurs is relatively slight, so that little or no measurable effect upon the nutritive

value of simple sugars, starches, cellulose, etc., has been observed, with either purified compounds or the naturally occurring materials found in foods.

The effects of irradiation upon proteins and amino acids are somewhat more complex. In the case of the amino acids, the primary reactions appear to be deaminations and decarboxylations. This might be anticipated to have considerable nutritional significance, were it not for the fact that protein-bound amino acids are apparently protected from extensive radiation damage. Protein molecules undergo extensive molecular rearrangement, splitting, and/or polymerization, however. These changes may affect the allergenic properties of the treated protein. Thus, gamma irradiation has been found to alter the antigenicity of ovalbumin (*26*) and milk proteins (*21*, *22*). In the latter case, the change in biological properties was accompanied by radiation-related changes in sulfhydryl content, much as was reported by McArdle and Desrosier (*28*) following gamma irradiation of casein solutions. On the other hand, biological assay has shown little change in the nutritional value of irradiated protein. Using the nitrogen balance technique, Calloway, Cole, and Spector (*12*) report that irradiation of turkey with dosages up to 9.3 megarads did not affect the digestibility or biological value of turkey protein. Detailed studies of the digestibility and biological value of pea, lima bean, wheat, corn, milk, and beef protein have also been reported (*33*, *34*, *39*). As may be seen in Figure 1, lima bean protein gave a higher biological value following heating than either the raw or irradiated material; this probably was due to the heat destruction of the trypsin inhibitor in the lima beans. No explanation can be given for the loss in biological value of pea protein following irradiation. However, the similar loss following irradiation of milk protein was stated to be due to the radiation destruction of cystine. These small changes in utilizability of irradiated proteins presumably would be masked during the feeding of a mixed diet of irradiated foods. Such has been shown to be the case in studies employing a diet composed from nine frozen-stored irradiated foods (see Table I).

The irradiation of lipides results in the formation of small amounts of peroxides, polymers, and carbonyl compounds. As is true with proteins, the irradiation of natural food fats yields a smaller amount of these materials than might be anticipated from studies with purified fatty acids. Nevertheless, they exert some effect upon the digestion of irradiated foods. Schreiber and Nasset (*56*) have shown that, in the dog, irradiated lard passes through the stomach and the intestine more slowly than does nonirradiated lard (see Table II). Moore (*42*), using an in vitro technique, has demonstrated that the ability of lipase to hydrolyze lipide emulsions was decreased as radiation level increased (Table III). A tentative explanation is that the emulsions are not as stable following irradiation, so that lipase activity is physically impaired. Recent data by Monty (*41*) indicate that both these effects may be mediated through radiation-produced carbonyls but that the total mechanism is probably more complex than a simple physical effect upon lipide emulsion stability. Thus, the rate of digestion of lipides may be somewhat impaired following radiation sterilization but, as has been repeatedly shown by others, the net nutritive value to the intact host is not altered.

In general, it may be concluded that radiation treatment of food does not seriously alter the nutritional value of the major components (see Tables I and IV). The situation with the vitamins is different, however.

Most of the water-soluble vitamins are sensitive to ionizing radiation when irradiated in pure aqueous solution but the radiosensitivity is considerably less when food items are irradiated, undoubtedly because of the presence of protective compounds in the food. The percentage destruction differs from vitamin to vitamin, thiamine being the most sensitive and niacin the least sensitive. Furthermore, the percentage destruction of a specific vitamin also varies markedly between different foods (see Table V). Because of this variability, consumption of a mixed diet of ir-

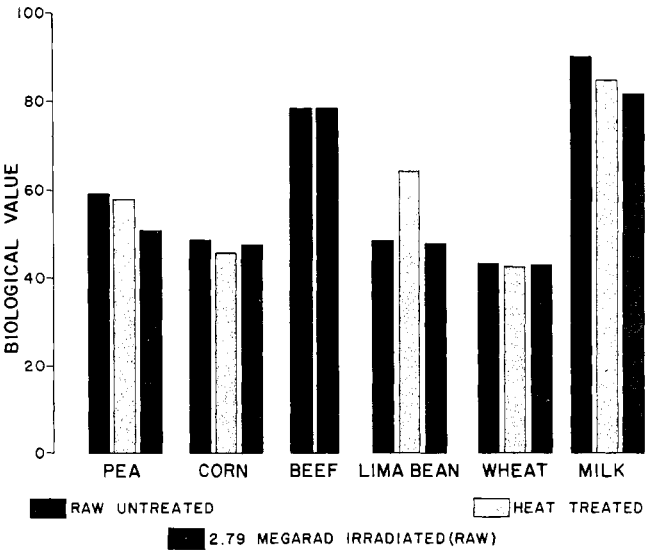

Figure 1. Biological value of proteins treated with heat or ionizing radiations (*33*, *34*, *39*)

Table I. Availability of Macronutrients in Diets Composed of Nine Frozen-Stored Foods (*11*, *62*)

	Nonirradiated, %	Irradiated (5.58 Megarads), %
Protein	85.9	87.2
Fat	93.3	94.1
Carbohydrate	87.7	87.9

Table II. Digestion and Absorption of Lard Fed to Dogs (*56*)

(Dogs sacrificed 3 hours after feeding of test meals)

	Non-irradiated, %	Irradiated (5.58 Mega-rads), %
Stomach contents	47	65
Small intestine contents	11	8
Absorbed	42	27

Table III. Effect of Substrate Irradiation upon in Vitro Lipase Activity (*42*)

Substrate Emulsion	Incubation Time, Hr.	Radiation Dose, Megarads: 0	2.79	5.58	9.30
		Units of Lipase Activity			
Olive oil	4	35	33	10	..
Corn oil	4	13	10	3	..
Lipomul	5	76	..	66	46

Table IV. Metabolizable Energy Value of Macronutrients Following Radiation Treatment (20)

	Calories per Gram	
	Nonirradiated	Irradiated (2.79 megarads)
Casein	4.56 ± 0.30	4.51 ± 0.22
Lard	8.82 ± 0.31	8.87 ± 0.39
Carbohydrate[a]	3.87 ± 0.20	3.78 ± 0.30

[a] Mixture of sucrose, starch, and dextrin.

Table V. Vitamin Destruction in Foods Exposed to Varying Doses of Gamma Radiation (63)

(Doses stated in megarads)

	Per Cent Destruction					
	Thiamine		Riboflavin		Niacin	
Food	2.79	5.58	2.79	5.58	2.79	5.58
Bacon	..	93	..	7	..	0
Beef	76	85	5	4	2	1
Haddock	68	76	0	4	14	9
Ham (fresh)	87	96	0	0	2	2
Turkey	76	77	27	50	7	0
Beets	52	75	14	10	0	10
Milk (powdered)	0	0	0	0	35	20
Peaches	94	98	0	0	48	56

Table VI. Comparative Effects of Radiation and Heat Treatment on Vitamin Content of Foods

	Per Cent Destruction	
Vitamin	Heat	Irradiation (2.79 megarads)
Thiamine	60–70	55–65
Riboflavin	18–22	6–10
Pyridoxine	28–32	24–25
Niacin	30–35	0–14
Choline	?	0
Folic acid	35	0
Inositol	?	0–5
Vitamin A	20	31–70[a]
Vitamin E	?	61[a]

[a] Dosage. 440,000 rads in dairy products.

Table VII. Ascorbic Acid Destruction in Orange Juice and Strawberries (13, 46)

	Per Cent Destruction	
Dose, Rads	Orange juice	Strawberries
93,000	4	..
279,000	22	63
396,000	26	81
465,000	35	..
698,000	50	..
930,000	59	..

Table VIII. Reproductive Performance of Dogs Fed Irradiated or Nonirradiated Beef Diets (29)

Radiation Dose, Megarads	% Conception	% Weaned
0	82	68
2.79	53	63
5.58	61	50

radiated foods might be anticipated to provide adequate levels of most vitamins, with the possible exception of the most radiosensitive vitamins—e.g., thiamine and pyridoxine. Table VI compares the relative destruction of vitamins by ionizing radiations and cooking. The data have been drawn from a variety of sources and represent only an approximation in both cases. It is readily apparent that, in general, radiation destruction is equal to or smaller than that resulting from cooking. However, the combination of irradiation plus cooking may result in serious losses in vitamin content, requiring vitamin supplementation. Surprisingly, in a study employing baby beef liver, Williams, Yen, and Fenton (*60*) found that subsequent cooking did not result in further destruction of thiamine.

In order to answer these questions more fully, an experiment in our laboratory utilized a composite diet of 15 irradiated foods stored at room temperature after irradiation (*61*). The resultant cooked or raw diets were fed to male rats for 12 weeks. A progressive decrease in growth rate was observed as the radiation level increased. For a given radiation treatment, the animals fed the cooked or the raw diets did not differ significantly in growth. Supplementation with a complete B-complex vitamin premix provided equal growth in all groups. Subsequent tests have shown that thiamine and pyridoxine are the limiting vitamins in this diet; of these, pyridoxine would appear to be the most important. Because the rat does not require ascorbic acid (vitamin C), these experiments did not permit an evaluation of its destruction or availability in irradiated products. This vitamin is one of the most sensitive in aqueous solution; fortunately, it is less sensitive in foods. Nevertheless, ascorbic acid is appreciably destroyed in fruits at even low doses of radiation (Table VII). It is somewhat more stable in milk (*24*), particularly evaporated milk, and in various vegetables (*23, 43*). There is considerable evidence (*57*) that irradiation of ascorbic acid produces dehydroascorbic acid. The latter, while readily reconverted to biologically active ascorbic acid, is easily oxidized to biologically inactive 2,3-diketogulonic acid. Analogous transformations occur in foods preserved by other methods. Thus, vitamin C destruction during radiation processing varies between food items but, in general, parallels the loss encountered during heat processing. Unlike the other water-soluble vitamins, however, the destruction may be sufficient to be of concern with some fruits now suggested for radiopasteurization.

Considerable information is also available concerning the fat-soluble vitamins A, E, and K. All of these are more sensitive to ionizing radiation than are the majority of the water-soluble vitamins, probably because of their antioxidant properties. For example, vitamin A and carotene are extremely sensitive to radiation in either pure solution or foods. This sensitivity undoubtedly explains the recent report of lower accumulation of vitamin A in the livers of rats fed irradiated carrot diets (*59*).

It was recognized early that vitamin E was extremely labile under the influence of irradiation (*4, 14, 45*). As a result, most subsequent experiments have employed an oral supplement of vitamin E administered separately from the diets. In general, this practice has obviated any difficulties in vitamin E nutrition among animals fed irradiated foods. However, it has been reported by McCay (*29*) that dogs fed diets containing irradiated beef show a reduced conception rate and lower weaning percentages than animals fed nonirradiated beef diets (Table VIII). Oestrus was normal in all animals. These symptoms are strikingly similar to those observed in vitamin E–deficient rats. As has been shown with other meat diets (*45*), the vitamin E incorporated in McCay's diets at the time of mixing may have been destroyed during subsequent storage of the diet. Although the symptoms observed by McCay do not parallel precisely those reported for severely E-deficient dogs raised on processed milk diets, the latter cases were apparently complicated by a simultaneous vitamin K deficiency (*2, 3*). In support of the E-deficiency interpretation is the fact that another dog-feeding experiment using beef, in which vitamin E was provided orally weekly, has not shown similar reproduction failure (*51*). The beef feeding experiments are now being repeated with larger numbers of animals in order to determine the fundamental mechanism involved.

The study of vitamin K in irradiated foods has proved to be most interesting and important. In a series of studies by Richardson and coworkers (*53, 54*), it has been shown that, in general,

natural vitamin K is stable to ionizing radiation in vegetables and some synthetic rations. However, Metta, Mameesh, and Johnson (*37*) have shown that male rats fed a vitamin K–deficient diet containing irradiated beef die of massive hemorrhage. We have confirmed this observation in our laboratory (Table IX) (*61*) and have also demonstrated that the same phenomenon occurs when cooked, nonirradiated pork is fed to male rats under these conditions (Table X). Irradiation of the pork increases the incidence of hemorrhagic diathesis.

The deficiency state created under these conditions is apparently complex. The fact that some control animals (20%) fed the nonirradiated beef also die of hemorrhage indicates that beef is probably a poor source of vitamin K; irradiation of the beef undoubtedly worsens this situation. Synthetic vitamin K_3 is destroyed when it is added to the irradiated beef diet (*38*), much as is seen with nonirradiated pork (Table VI) and as has been observed for vitamin E (*45*). Whether a similar situation also occurs with the naturally occurring forms of vitamin K is not known. However, rats fed a K-deficient diet have been shown in the past to obtain sufficient vitamin by recycling the feces (coprophagy) so as to recover the vitamin synthesized by the gut microflora. Consumption of the irradiated beef does not alter the gut micropopulation (*61*), but the rats apparently do not practice coprophagy (*36*). It is possible that this may be due to the carbonyls produced during irradiation, since *n*-decylaldehyde by itself will achieve the same effect (*35*).

Other factors also are involved. Table V shows that supplementation with bile acids will decrease the incidence of hemorrhagic death, suggesting that lipide emulsification and/or absorption from the intestine may be impaired; with this would go impaired absorption of vitamin K. This possibility is in keeping with the data of Schreiber and Nasset (*56*), which indicate a decreased rate of absorption of irradiated lard in dogs. It has also been shown that vitamins E (*32*) and A (*16*) are involved in the total syndrome. Supplementation with large quantities of either vitamin hastens the onset of hemorrhage; omission of either almost eliminates the appearance of the deficiency. Female rats do not become hemorrhagic on any of these diets (*37*). This is probably due to differences in growth rate, metabolism, and vitamin requirement between male and female rats and probably does not represent a direct protective effect of female sex hormones.

Inasmuch as vitamin K destruction is slight in vegetables, it might be anticipated that consumption of a mixed diet would prevent the appearance of the hemorrhagic disease. This has been shown to be the case in the 15-food composite diet study mentioned earlier, in which normal prothrombin times (12 to 13 seconds) were observed in all animals after 12 weeks of feeding.

Table IX. Deaths of Male Rats Fed Vitamin K–Deficient Diets Containing Cooked Irradiated (5.58-Megarad) or Nonirradiated Beef (*61*)

	Nonirradiated		*Irradiated (5.58 Megarads)*		
Supplement					
Oral K_3, γ/day	0	0	0	0	4
Bile acids,[a] mg./day	0	83	0	83	0
Number of rats	15	10	10	10	10
Deaths in 70 days	3 (20%)	0 (0%)	10 (100%)	5 (50%)	0 (0%)
Prothrombin times, av. in sec.	30	15	166	60	14

[a] Equal mixture of cholic, deoxycholic, and lithocholic acids.

Table X. Deaths of Male Rats Fed Vitamin K–Deficient Diets Containing Cooked Nonirradiated or 5.58-Megarad Irradiated Pork (*61*)

	Nonirradiated			*Irradiated*	
Daily vitamin K_3 supplement, γ					
In diet	0	20	0	0	0
Oral	0	0	20	0	20
Number of rats	20	10	10	14	10
Deaths in 70 days	12 (60%)	8 (88%)	0 (0%)	13 (93%)	0 (0%)
Prothrombin time, av. in sec.	85	75	13	140	12

From the experiments which have been completed with the vitamins, it is apparent that radiation destruction does occur. At least for the water-soluble vitamins, the extent of destruction at sterilization doses roughly parallels heat-induced vitamin losses and is probably not further affected by cooking the irradiated product. The vitamin losses may be overcome by supplementation in a manner analogous to present-day practices in other food-processing industries. The extent of vitamin destruction is also related to radiation dose. Thus, for all vitamins except possibly vitamins A, E, and C, no nutritional problem is now anticipated with radiopasteurized products treated with less than 0.5-megarad doses. In all cases, the significance of the losses will depend upon the type of diet consumed: whether it is a completely irradiated diet such as is envisioned for military survival rations or consists of a mixture of irradiated and nonirradiated foods, much as will be utilized by the average housewife.

Human Feeding Studies

As soon as specific food items have been shown to be grossly nontoxic in 3- to 6-month animal feeding trials, human experiments may be undertaken. The subjects are ten male volunteers who live under closely supervised conditions. Balance studies are performed for nutritional evaluation of the diets as well as a battery of clinical tests to determine the presence of any toxic manifestations. By techniques such as these, 42 frozen-stored irradiated (2.79-megarad) foods comprising successively 35, 60, 80, and 100% of the dietary calories have been fed to the subjects for 15-day periods with no ill effects (*27*). In addition, 23 food products stored at room temperature following irradiation have also been found to be nontoxic during 15-day feeding periods (*5*, *45*). Throughout all these experiments, the per cent availability, energy value, and nitrogen balance data have also indicated that irradiation has no adverse effects from the nutritional viewpoint.

The extrapolation of the hemorrhagic diathesis seen in rats fed irradiated meats to a similar situation in humans is not readily made. The vitamin K requirement is not known for the human nor is the significance of the intestinal micropopulation in satisfying K needs clearly established. However, at no time in any of the human tests did the prothrombin time deviate from normal. This is particularly significant in the one experiment where the subjects were fed 35% pork solids for the 15-day period (*44*). In male rats, a 15-day period is sufficient to induce hemorrhagic death in 10% of the animals and to increase prothrombin times in the remainder.

With the completion of the animal screening program, the lengths of time irradiated foods may be fed to human volunteers may be extended. Present plans indicate that these tests may be extended to 3 to 6 months as soon as sufficient foods are available to permit development of a nonmonotonous diet.

Summary and Conclusions

Wholesomeness is a term denoting health-hazard potential. It encom-

passes two major areas of research: potential toxicity and nutritional adequacy. The Office of the Surgeon General, U. S. Army, is sponsoring an extensive research program concerning the wholesomeness of radiation-preserved foods. Although final evaluation of all the data will not be completed for three to five years, the present report represents an analysis of the data now available.

Animal tests have uncovered few, if any, indications of toxicity of individual irradiated foods. In general, growth, reproduction, life span, and cancer incidence have not been significantly altered as a result of consumption of the treated product as long as adequate vitamin supplementation has been provided. Further research is required, however, to elucidate the heart rupture problem occurring in mice fed composite diets of irradiated foods.

The nutritional value of irradiated foods appears to be similar to that of cooked foods. Fats, carbohydrates, and proteins are not significantly altered in so far as nutritional properties are concerned. As with cooking, thiamine and pyridoxine are susceptible to radiation destruction. Vitamin C also is labile. The fat-soluble vitamins E and K are particularly susceptible to destruction by radiation, but the resultant problems in reproduction and bleeding may be overcome by supplementation with vitamins E and K, respectively. Consumption by animals of mixed diets of irradiated foods largely eliminates the problem of vitamin destruction in individual foods.

Interpretation of the data with respect to human consumption is difficult, particularly because human requirements for some vitamins (notably E and K) have not been clearly established. However, short-term (15-day) feeding trials with human subjects have shown no wholesomeness difficulties arising from consumption of diets containing up to 100% of the calories from gamma-irradiated foods. Vitamin supplementation, such as is practiced with other processing methods, would further decrease potential problems. Similarly, consumption of a mixed diet of irradiated and nonirradiated foods would provide an adequate nutritious diet. The latter is most nearly analogous to the anticipated practice of the average housewife, inasmuch as not all foods may be satisfactorily preserved with ionizing radiations.

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RADIATION PRESERVATION OF FOOD

Commercialization Technology and Economics in Radiation Processing

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PRESERVATION METHODS which potentially yield commercially sterile products or substantially extend the shelf life are worthy of investigation. During the last ten years extensive research has been conducted in the United States and Europe on a new concept in food technology, using ionizing radiation. To date, the conventional methods of processing have their limitations. Thermal treatment of food, for example, cannot preserve the product in the raw state and whereas canned food still represents a large volume of the market, such products differ markedly from "garden fresh" fruits or vegetables and fresh meats. To attain the desired degree of bacterial inactivation canned products are frequently overcooked. Freezing, on the other hand, permits the extended storage of foods with retention of original characteristics of texture, flavor, and nutritional properties. In meats, however, there are some color change, "freezer burn," and exudation of fluid (drip). Logistically, in military feeding freezing imposes problems in distribution and economical operation.

Radiation preservation has certain advantages, such as the prospect of preserving meat for prolonged periods, a process that is adaptable for continuous processing, the processing of large and small items because of deep penetration of the ionizing radiation, and the possibility of using new transparent plastic film packages because high temperature processing is avoided.

Although Food and Drug Administration clearance on irradiated foods awaits completion of the wholesomeness studies, the technological and economic realization for advancement of this process depends on how competitive this method is with existing methods of

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RADIATION PRESERVATION OF FOOD

Commercialization Technology and Economics in Radiation Processing

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PRESERVATION METHODS which potentially yield commercially sterile products or substantially extend the shelf life are worthy of investigation. During the last ten years extensive research has been conducted in the United States and Europe on a new concept in food technology, using ionizing radiation. To date, the conventional methods of processing have their limitations. Thermal treatment of food, for example, cannot preserve the product in the raw state and whereas canned food still represents a large volume of the market, such products differ markedly from "garden fresh" fruits or vegetables and fresh meats. To attain the desired degree of bacterial inactivation canned products are frequently overcooked. Freezing, on the other hand, permits the extended storage of foods with retention of original characteristics of texture, flavor, and nutritional properties. In meats, however, there are some color change, "freezer burn," and exudation of fluid (drip). Logistically, in military feeding freezing imposes problems in distribution and economical operation.

Radiation preservation has certain advantages, such as the prospect of preserving meat for prolonged periods, a process that is adaptable for continuous processing, the processing of large and small items because of deep penetration of the ionizing radiation, and the possibility of using new transparent plastic film packages because high temperature processing is avoided.

Although Food and Drug Administration clearance on irradiated foods awaits completion of the wholesomeness studies, the technological and economic realization for advancement of this process depends on how competitive this method is with existing methods of

Editor's Note

A fourth paper presented before the Radiation Processing Section, Nuclear Congress, New York, N. Y., April 1960, "Radiation Technology for Nonbiological Materials" by A. J. Restaino, was published in the August 1960 issue of *Industrial and Engineering Chemistry* (page 683).

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This study deals with technical and economic factors in the commercial application of radiation processing to foods, agricultural commodities, and hospital supplies, including specific applications of machine and gamma sources. Currently, low energy source radiation (cobalt-60) is preferred over high energy sources which potentially induce radioactivity. Design and description of radiation facilities for treatment of one or several products are presented. Low-dose treatment at 3×10^5 rads of fish, chicken, and prepackaged, refrigerated meats, at an estimated cost of 0.45 cent per pound, is of considerable interest to less developed countries for control of food spoilage. Trichina destruction in pork at 2×10^4 rads has an estimated cost of 0.15 cent per pound. Sterilization cost for food irradiated at 3 to 4 $\times 10^6$ rads is estimated at 2 cents and medical supplies at 2×10^6 rads at 0.96 cent per pound.

preservation such as freezing, thermal processing, and dehydration. Reduced costs of radiation and efficiency of utilization in machine sources and in nuclear sources, the availability and low cost per curie for the radioactive isotope, as well as minimal costs for installed radiation facilities, are key factors in commercialization of radiation processing.

Sources of Radiation Energy

For all practical purposes only two general sources of radiation need be considered — electron beam radiation from high energy machines and gamma radiation from radioisotopes. X-radiation obtained when the electron beam strikes a target and a portion of the electron energy is converted to electromagnetic radiation need hardly be considered. When the electron beam energy is low, the conversion efficiency is too low for economic results and when the electron beam energy is high, satisfactory penetration can be obtained without such a conversion. However, high energy electron beams introduce a serious problem—induced radioactivity in the product. The theoretical threshold of such induced activity is 2.2 m.e.v. (the reaction threshold of the γ,n reaction of deuterium), but for practical purposes it is probable that electron beams of up to 8 m.e.v. will be considered to be free of induced activity affects. Even considerably above this energy, the induced activity levels are small and arise from short-lived isotopes. However, such minor effects can be serious, particularly with respect to irradiation of food. Present Food and Drug Administration legislation (the Delaney Food Additives Amendment) precludes the use of a process deemed to induce any radioactivity in the food, on the basis that radioactivity is carcinogenic and no additive of a carcinogenic nature is permitted (*9*).

The penetrating power of electrons is small. For energies from 1 to 5 m.e.v., the effective thickness of a product of unit density for radiation treatment is 0.13 inch per m.e.v. for single-sided irradiation and 0.31 inch per m.e.v. for two-sided irradiation.

Even a 10,000,000-volt machine can be used only on packages a little over 1 inch thick with irradiation from one side. On the other hand, efficiencies of electron machines are attractive. About 80% of the electron beam energy will be absorbed in a material with a thickness 2/3 that of the maximum electron range (*4*, *12*). Considerably higher doses are received in the interior of the sample relative to the surface, so that the net absorption efficiency is about 60%. A further loss of efficiency (even assuming a scanning system designed to fit a given package area) due to geometrical losses and scanning overlap will reduce the net efficiency to approximately 50%. With two-sided irradiation the net efficiency can be raised to about 65% and the package thickness for a 10-m.e.v. beam increased to 3 inches. Two-sided irradiation, of course, creates additional complexity and expense in both the accelerator and the package conveyor.

Available Types of Machine Sources

Cascade generator (or Cockroft Walton machine)	For energies below 5 m.e.v.
Resonant transformer	
High frequency charging series capacitors	
Van de Graaf generator	For higher energies
Linear accelerator Linac	

The low energy machines are capable of large power outputs at low cost per kilowatt, while the high energy machines are substantially more expensive in both capital and operating cost.

The characteristics of radioisotope sources have been reviewed many times. Aside from the use of spent fuel rods, which are generally considered to be inconvenient and expensive except when they can be used in close proximity to the reactor from which they came, three isotopic sources merit serious consideration: Co^{60}, Cs^{137}, and Zr^{95}-Nb^{95}.

One useful figure of merit is a measure of the watt-hours available per curie (the energy per disintegration times the half life). For Co^{60} this is $2.5 \times 5.2 = 13$, and for Cs^{137} it is $0.61 \times 26.6 = 16$. However, it is doubtful if an industrial processor would, at this time, be prepared to amortize his Cs^{137} based on a 26.6-year half life. Consequently it is more practical in considering such a figure of merit to assign to Cs^{137} the same half life as Co^{60}, whereby the figure for Cs^{137} becomes 3.15 and, hence, the relative value of Co^{60} to Cs^{137} is 4.1 to 1. The number of curies required for a plant of a given throughput is in the inverse ratio 1 to 4.1 and the relative initial capital charge for the source is the same ratio multiplied by the ratio of the costs per curies.

Current prices for both Co^{60} and Cs^{137} are $2.00 per curie (unencapsulated); hence Cs^{137} sources cost four times as much as Co^{60} for the same plant throughput. The raw material for cesium sources is the waste fission products from spent reactor fuel rods and the cost per curie is the processing cost for recovery, concentration, extraction, and encapsulation and hence is very dependent on the quantities in demand. It is difficult to expect at any time a price below $0.25 per curie for Cs^{137}. With Co^{60}, on the other hand, the cost depends on the cost of the neutrons used and when Co^{60} can be made from waste neutrons—e.g., as a by-product in power reactor operations—the cost as in the case of Cs^{137} is very dependent on the quantities in demand. It is anticipated that the price per curie for Co^{60} will decrease in the future at least as rapidly as Cs^{137}. In addition, the greater penetrating power of Co^{60} (in the ratio 4 to 3) is an advantage in most large installations. It is therefore concluded that Co^{60} is a more economical source of radiation than Cs^{137} and likely to remain so.

The case of the fission product pair Zr^{95}-Nb^{95} is of special interest. Zr-Nb has a half life of only 65 days but is about 100 times more plentiful than Cs^{137} in fresh spent fuel rods. Consequently, it is anticipated that Zr-Nb

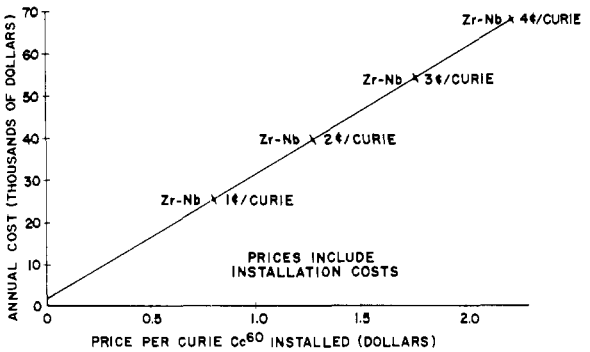

Figure 1. Annual source cost for Co^{60} and Zr^{95}-Nb^{95} considered on basis of plant operating continuously

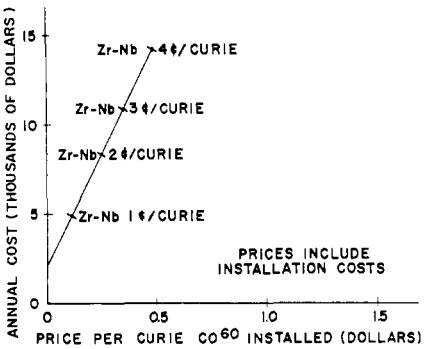

Figure 2. Annual source costs for Co^{60} and Zr^{95}-Nb^{95} considered on basis of plant operating seasonally

65 days, installed capacity 2 kw.

can be made available at a few cents per curie. A particular area of interest for such a short-lived source is in the processing of seasonal products such as fruits and vegetables, whereby a source would be installed for the harvesting season of a given product and permitted to decay to a negligible value during the remainder of the year.

It is instructive to compare the source costs for Co^{60} and Zr-Nb for a typical installation of, say, 2-kw. capacity (140,000 curies of Co^{60}) considered, first, as a plant operating continuously throughout the year and, secondly, as a plant operating for a season of 65 days. The results are shown in Figures 1 and 2. Figure 1 applies to a 2-kw. plant operating continuously, whereas Figure 2 applies to a seasonal operation of 65 days. For a given price per curie for Co^{60}, the annual cost is made up of the number of curies required per year for source replenishment times the price, together with shipping and installation charges and an interest charge of 6% on the capital investment. On the same basis the annual cost for Zr-Nb is computed assuming prices of 1, 2, 3, and 4 cents per curie. Hence, for example, 2 cents per curie Zr-Nb requires the same annual expenditure as Co^{60} at $1.27 per curie in continuous operation and as Co^{60} at $0.26 per curie for the seasonal operation. There have been estimates on Zr-Nb sources from $0.02 to 0.04 per curie (*10*). On this basis Zr-Nb is not expected to be competitive with Co^{60} for continuous operations (assuming Co^{60} prices to become $0.50 to $1.00 per curie) but Zr-Nb shows a distinct advantage for seasonal operations. Another advantage of Zr-Nb is the low capital investment to the user, where it holds a decided advantage even over machine installations.

Potential Areas of Radiation Processing Hospital Supplies, Agricultural Products, and Foods

Concomitant with the extensive research and development work on foods have been the important investigations on radiation processing of pharmaceuticals, hospital supplies, and nonfood agricultural products. As many important developments have evolved in this area with the process proved technically and economically feasible, commercialization on such products may precede that of food applications. In addition, the requirement for extensive wholesomeness clearance studies on foods with ultimate Food and Drug Administration approval does not provide for as early marketing of irradiated foods as might be the case, for example, in radiotreatment of surgical supplies, cotton, hemp, or tobacco.

Radiotreatment of Thermolabile Drugs, Hospital Equipment, and Exported-Imported Agricultural Commodities. Parenteral pharmaceutical products such as antibiotics, hormones, steroids, multivitamin preparations, sulfonamides, alkaloids, and blood plasma have been radiosterilized successfully. However, clinical trials and toxicity tests must be applied, because these products are essentially new, demanding a careful study of properties of the irradiated product. Antibiotics, for example, show no loss of potency on storage after irradiation. Antibiotics are labile to thermal processing. Bone and tissue grafts can be satisfactorily radiation-sterilized, stored, and successfully used in surgery, but this application does not represent an important large use (*5*). Horne (*6*) has suggested that radiomodified drugs might find an important use, for example, in inactivation of virus vaccine such as Salk poliomyelitis virus, which is now inactivated with formaldehyde. By current methods, formol inactivation of virus results in at least 95% loss in antigenicity or vaccinogen power as the infectivity is destroyed. Primarily because of radiosensitivity of the nucleic acids, infectivity of viruses would be destroyed but there would also be substantial loss in antigenicity. It is possible that future successes may come in this area by combination of radiation treatment with chemicals or by operating at low temperatures.

Of more immediate application is the radiosterilization of surgical materials such as surgical sutures, tubing for intravenous feeding, bandages, forceps, hypodermic needles, and talc. Some of these products are now marketed as radiation-sterilized materials—for example, sutures sterilized by radiation have a substantial portion of the market in this country, while catheters similarly sterilized are on the market in the United Kingdom. Packages sealed ready for shipment and clinical use are guaranteed sterile, because radiation-sensitive tapes affixed to packages indicate that the product has passed through the sterilization chamber. Pathogens such as staphylococcus have been reported, for example, in talc which escaped commercial steam sterilization, which could not occur in radiation processing. Indeed, commercial radiation sterilization of surgical supplies now being utilized has been shown to be competitive with steam sterilization.

Continuous sterilization of hospital equipment such as blankets, linens, and mattresses is being considered. Because of the incidence of staphylococcus infections in hospitals through general contamination of furniture and equipment, it is envisioned that a gamma-radiation facility which would also accommodate large items might be an important advance in central hospital sterilization operations.

Methods for treatment of agricultural commodities, foods and nonfoods, are continually being developed for control of parasites and insect pests in imported, exported, and domestic products. Radiation treatment with radiation doses below 50,000 rads has been recommended for these applications (*3*). The opening of the St. Lawrence Seaway has stressed the need for more and better facilities and methods for deinfestation of imported and exported products such as cotton, hemp, broom corn, and tobacco, to name but a few items of interest.

Table I. Effect of Chemical Treatment and Gamma Sterilization on Insect Population

[Chemical treatment that induces 90% sexual sterility and gamma-radiation sterilization where sterile males dominate a natural male population in ratio of 9:1. (*8*)]

Generation	Constant Release of Gamma-Sterilized Males	Chemical Sterilization
Parent	1,000,000	1,000,000
F_1	500,000	50,000
F_2	131,578	2,500
F_3	9,535	125
F_4	50	5
F_5	1	1

Table II. Radiation Processing of Seafoods

Items	Recommended Radiation Treatment, Rads	Average Days of Shelf-Life Extension over Untreated Controls at 35° F.[a]
Cod, butterfish	Raw at 2.5×10^5	35
Haddock, pollock	Raw at 6.0×10^5	30
Crabmeat	Raw at 5×10^5	50
Crabmeat	Cooked at 4×10^5	40
Oysters	Blanched, breaded, 5×10^5	60

[a] Shelf life of refrigerated raw fish 7 to 10 days.

Certain types of radiation facilities have been designed for radiation treatment of grains in silos or at the dock side, among which a gravity flow process with Co^{60} source or an electron beam machine on an air stream within the discharge chute appears feasible. A mobile source has many advantages for this purpose, as it can handle a variety of products at different locations with shift labor being involved due to seasonal demands. A short-half-life radioisotope such as Zr^{95}-Nb^{95} with low cost per curie built into a megacurie course that can handle a throughput of at least 200 tons per hour has been proposed because of reduced amortization on a low cost investment. Thus far, however, chemical fumigation with methyl bromide, cyanide, etc., appears to have a slight financial advantage. The fact that reinfestation may occur in warehouses or silos, which would necessitate repeated radiation treatments, imposes a cost which swings the advantage again toward the use of chemicals. The same criteria can be applied to treatment of spices.

In the exporting or importing of some products such as tobacco, cotton, hemp, and broom corn special fumigation facilities are maintained at ports of entry and embarkation for control of crop pests. It is believed that radiation facilities in new locations for radiotreatment of these commodities would be feasible economically; otherwise replacement of the capital investment in old fumigation equipment in existing sites would be prohibitive.

Whereas direct irradiation of products —for example, destruction of cigarette beetles in tobacco—has been proposed for elimination of this insect in stored tobacco, an indirect method based on the release of irradiated sterile male insects has come under consideration. The very successful elimination of the screw worm fly on the island of Curaçao and the gulf states of this country has been described by Knipling (*8*). Christenson (*2*) has had some initial success in his investigations on the irradiation of the fruit fly in Hawaii and Mexico, which again involves the continuous release of millions of irradiated sterile male fruit flies, with the end result being control and/or disappearance of subsequent generations of insects which cause heavy crop losses. It is hoped that future research will establish this unique procedure as effective in control of crop-damaging insects and insects that are vectors of diseases such as the mosquito. The effectiveness of the sterile male method is illustrated in Table I.

Horne, Turner, and Willis (*7*) have demonstrated that gamma radiation at a minimum dose of 2 megarads can successfully inactivate *B. anthracis*, which is a problem in the importation of goat hair. Baled goat hair imported into countries such as Australia and Great Britain constituted a health hazard from this pathogen for factory workers where goat hair was used in manufacture of carpets and other fabrics. Recently a factory in Australia has installed a gamma-radiation facility with a source strength 150,000 curies for processing goat hair and wool. This is the world's first commercial installation of radiation processing with Co^{60}.

Radiopasteurization and Radiosterilization of Foods. Extensive research and development work has been conducted for the past eight years relative to this new concept in food preservation. The program supported largely in this country by the Department of the Army has also stimulated work in Europe. Some major obstacles still prevail in the technology; the development of irradiated sterilized beef is not as yet satisfactory from a palatability standpoint. Pork and pork sausage, chicken, and fishery products respond favorably to radiation processing. Thermal processing yields products which are uniformly acceptable; however, radiation processing currently differs in this respect, in that palatability is related to specific chemical constitution of the food.

The technical reports that have been published thus far direct attention to radiation processing of foods that would be technically feasible. For commercialization, primary emphasis must be placed upon products which from a survey are considered important in so far as technical and economic feasibility is concerned. Pending final wholesomeness clearance of all foods tested, conceivably obtained at the end of 1960, the following irradiated foods might be suitable for marketing: fishery products (radiopasteurized and radiosterilized); chicken, radiopasteurized; fruits, radiopasteurized; eggs, radiopasteurized for Salmonella destruction; pork, radiopasteurized for shelf-life extension and destruction of trichina parasite; beef, radiopasteurized for shelf-life extension of refrigerated prepackaged meats and destruction of tapeworm parasite; frozen processed foods, radiopasteurized for bacterial control; pet foods, radiopasteurized; and "brown and serve" meats, radiosterilized.

Radiation processing of fishery products, particularly "low fat" type filleted fish and shellfish, is successful from the stand-point of consumer acceptance. Extension of refrigerated storage time by low dose radiation treatment of perishable products such as shelled shrimp, picked crabmeat, blanched oysters, and filleted fish such as cod, pollock, haddock, butterfish, and flounder is most promising. In Table II some fishery products are listed with the radiation dose required for processing and the days extension of shelf life at 35° F.

Fishery products do not present the problem of botulism, trichinosis, or spore-forming radiation-resistant bacteria. Pseudomonas group of microorganisms are the principal ones causing deterioration and off-odor, and these are readily destroyed at 30,000 rads. Hence, shelf life and acceptance of refrigerated fish can be extended several weeks by low irradiation. Fishery products have been treated with radiation doses ranging from 0.5 to 5 megarads, with no off-flavor development. Blanched oysters are of good quality at 5 megarads. Radiopasteurization of crabmeat may be the solution to the problem of maintaining minimal bac-

terial counts on canned iced product. Heat pasteurization is now resorted to in many cases to effect such control, with adverse effects on texture and flavor.

The shelf life of chicken can be extended as much as 21 days by radiopasteurization at 400,000 rads. The radiation-sterilized product at 4 or 5 megarads is acceptable.

Among the radiosterilized meat products "brown and serve" pork and chicken are superior to products that receive complete thermal sterilization. The increased cost on this product might be warranted on the basis of increased consumer acceptance.

The demand for "Salmonella-free" egg products necessitates processing methods which can easily and cheaply meet this requirement. While fresh eggs cannot be readily irradiated without adverse effects on flavor and texture, frozen and dried egg products can so be treated to eliminate the pathogens. Liquid eggs that are irradiated and subsequently spray dried are rendered "pathogen-free" and "radiation flavor" compounds are volatilized in the drying process. In Table III the recommended radiation doses are given for processing egg solids and liquid eggs to eliminate Salmonella.

Transcontinental shipments of fresh fruits and vegetables are becoming more common and low-dose radiation treatment of such products will contribute markedly to the retardation of rotting and spoilage. Perhaps the first commercial treatment will be centered upon strawberries, which are in great demand in the off-season. In Mediterranean countries radiation treatment of citrus fruits to arrest mold development will have commercial significance for exporting. Bananas, grapes, and tomatoes are treated satisfactorily at low radiation dose and have considerable promise for future marketing.

For public health reasons elimination of the trichina parasite in pork and tapeworm in beef by the economical process of low radiation dose treatment is important and conceivably will replace conventional methods — for example, freezing or smoking of pork for control of trichinosis. This process is on the threshold for early commercialization, and if the meat packers saw an immediate sales advantage in promoting it, this efficient and rapid method would become a reality. A radiation facility has been designed for accomplishing this objective with maximum economy and flexibility.

Processed foods and complete meals that are frozen and available for serving by quick heating present the problem of high bacterial counts. With more emphasis toward strict requirements for minimal bacterial counts on these frozen processed foods, low-dose radiation processing in combination with freezing should facilitate the attainment of low bacterial count.

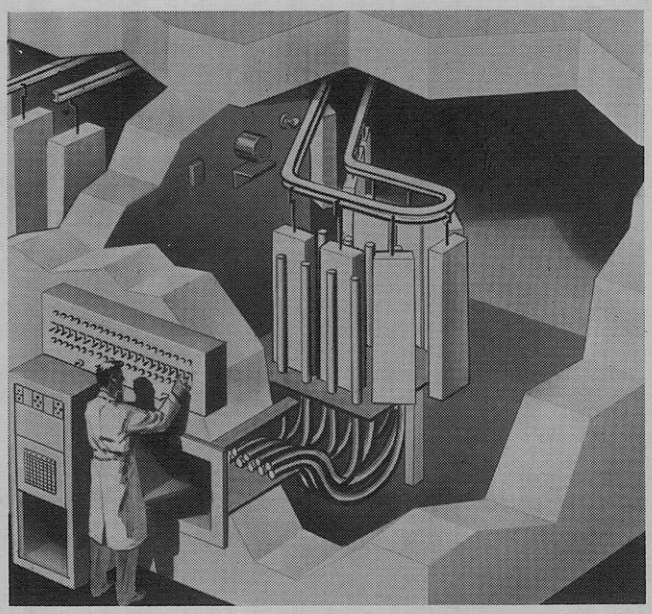

Figure 3. Multi-array gamma irradiator

Table III. Recommended Radiation Dose for Pasteurization of Egg Products[a]

(Radiation dose at 10^5 rads)

	Egg Solids			Liquid Eggs	
	Whole egg	Yolks	Whites	Whole eggs	Whites
S. Senftenberg	4.5	6.5	7.0	1.0	2.3
S. typhimurium	4.5	6.5	7.0	2.5	3.0

[a] For a 10^7-fold reduction in number of Salmonella.

Simple Design of a Gamma-Radiation Processing Facility

Designs for gamma-radiation facilities for the processing of pharmaceuticals, hospital supplies, agricultural products, and foods are numerous. A large scale gamma irradiator designed for the Department of the Army (the high intensity food irradiator) is an example of a multimegacurie Co^{60} source which could handle a minimum throughput of 6000 megarad pounds per hour, a short dwell time, and the nonuniformity in dose not to exceed 1.25 (*7*).

For smaller facilities a "multi-array gamma irradiator" presents the utmost in flexibility, because it has the ability to preset configurations of multiple individually projectable and retractable sources. This unique equipment, as shown in Figure 3, can handle kilocurie sources of radioactive isotopes.

With this MAGI equipment radiator tubes can be positioned in any configuration (as plaques horizontal or vertical, cylindrical arrangement). The gamma sources, which are movable and called "rabbits," are air blown from a storage cask through flexible transfer tubes to radiator tubes. When not in operation, the rabbits are retracted back through the transfer tubes to the cask by stainless steel cables. All these movements of the sources into the radiator tubes to provide a source arrangement with a specified field intensity can be controlled remotely from a panel outside the shielded radiation area by manipulating valves and switch controls.

The field strength will be determined by source strength of the rabbits and the number of rabbits projected into the field. Therefore, if each rabbit, for example, has 500 curies and there are 20 radiator tubes, the maximum source strength would be 10,000 curies. Increasing the specific activity and the size of the rabbit and the number of radiator tubes will increase the strength of the gamma source.

Batch or continuous irradiation can be used. By adding a conveyor (track conveyor shown in diagram) system, continuous processing of foods and biological products can be conducted. By allowing material to stand in the radiation cell, batch operations are carried out.

The MAGI with its shielding and conveyor equipment and the wide range in source strength and flexibility due to the unique physical arrangement of sources provides a versatile economic irradiator. This type of gamma ir-

Table IV. Processing Costs for Low-Dose (Pork) and High-Dose (Medical Supplies) Treatment

		Pork	Medical Supplies
A.	Production requirement, tons/yr.	25,000	25,000
B.	Radiation dose, rads	20,000	2,000,000
C.	Source strength, curies	20,000	2,000,000
D.	Capital costs, dollars		
	1. Source cost (assuming 50% efficiency)	40,000	2,000,000[a]
	2. Building–equipment costs	100,000	200,000
E.	Operating costs, dollars		
	1. Source costs	8,000	400,000
	2. Maintenance costs	1,000	2,000
	3. Amortization and interest on building and equipment	13,000	26,000
	4. Power	Negligible	Negligible
	5. Labor, dollars		
	4 operators at 18,000		
	1 supervisor 8,000		
	Overhead (100%) 26,000		
		$ 52,000	$ 52,000
	6. Total operating cost	$ 74,000	$ 480,000
F.	Total processing costs, cents		
	1. Per megarad lb. basis	7.4	0.48
	2. Per pound product basis	0.15	0.96

[a] Price per curie for large amounts of Co^{60} = $1.00 and for small amounts $2.00/curie.

radiator is well suited for radiopasteurization of food with the requirement of low dose radiation treatment. A mobile MAGI has been designed which is practical for the irradiation of seasonal crops at different locations.

Economics of Radiation Processing

In radiation treatment as in any other process the operating costs are made up of materials consumed, amortization of capital investment, interest charges, maintenance, and labor. In the case of electron machines the material consumed is negligible and the interest charges are small but the maintenance and labor costs are relatively high. With Co^{60} the converse is true. Cobalt-60 is consumed at the rate of 12.4% per year and the interest charges are high because of the larger initial investment, but maintenance and labor are smaller. It is reasonable to assume a 5-year straight-line amortization rate for machines and a 10-year rate on the remainder of an installation, since much of this consists of a concrete shielding structure. The interest rate assumed is 6% on the unamortized portion of the capital investment, since this represents capital borrowed against fixed assets. Because the decay loss on Co^{60} is made up annually, by the 12.4% material charge, the source is essentially a nondepreciating asset and the use charge is the 6% charge on capital investment. It is assumed, further, that sufficient space in the radiator is provided for annual addition of Co^{60}, so that Co^{60} is removed in less than 5 to 10 years. Allowance for such removal adds a 1 to 3% charge, so that the total annual charge for cobalt is 20% of the purchase price. The most difficult cost to estimate is the labor charge, because this depends on the products, the throughput, the location, and the equipment. However, for small to medium capacity installations the labor charge will be the dominating cost of processing. Actual costs can be estimated with reasonable accuracy only for a specific plant in a specific location with a specific product and throughputs. Only in such a context, also, does a comparison of machine costs and isotope costs become significant. Throughputs are defined as megarad pounds per hour, day, or year and, as implied, are the product of the minimum required dose and the number of pounds of material to receive this radiation dose. Radiation source costs are determined by the throughput and efficiency. The latter may be defined as the useful radiation energy absorbed in the product (the minimum dose required times the mass of product receiving this dose) as a percentage of the total radiation emitted by the source.

Cost Factors:

1. Capital costs
   a. Source cost isotope or machine
   b. Building and equipment costs
2. Operation costs
   a. Source costs (for isotopes, decay, and interest for machines, amortization, and interest)
   b. Maintenance cost (source equipment)
   c. Amortization (building, equipment, and interest)
   d. Power
   e. Labor

With this costing basis established, several interesting comparisons may be made—for example, the variation in cost with throughputs from 500 to 50,000 megarad tons per year using Co^{60} sources and the relative cost of Co^{60} and machine sources for different conditions.

The throughput variation or plant capacity may arise because of amount of material processed or the required radiation dose level for a specific application. For example, one might process 25,000 tons of pork per year at 20,000 rads (trichina elimination) or 25,000 tons of medical supplies for sterilization at 2,000,000 rads. The costs for these two radiation treatments are shown in Table IV.

It is obvious in the low-dose case that the labor costs dominate the cost picture and that the radiation source cost is less than 15% of the total cost. In such a case the choice of source—isotope or machine—will be determined by what is most suitable for the job and what will yield the lowest labor cost. The actual cost of the radiation energy is relatively unimportant. In most such cases, Co^{60} will be favored because, with it, product material can readily be handled in bulk form.

However, this is not the case for high dose with large quantities of product. Here the radiation energy cost dominates the cost picture. If, for example, the product were polyethylene sheet with 25,000 tons per year production, a low energy accelerator with a capacity of 30 kw. (or several lower capacity units) would suffice. The capital cost for source would be reduced to $300,000 to $500,000 (including standby units) and the annual operating cost for amortization and maintenance to about $120,000. However, it is assumed that the machines would require at least one machine operator per shift, which would add $48,000, and hence a total operating cost of $246,000 and a cost per pound of 0.49 cent.

Now let us consider an actual example of a proposed application of radiation processing. In choosing this example, every effort has been made to find situations well suited to radiation processing at this time. The example pertains to a radiation processing plant in a warm climate where refrigeration is lacking and transport facilities are inadequate. The extension of shelf life by as little as one day is economically significant. The primary products of interest are whole chicken and fish. The chickens, currently, pass through a central depot at the rate of 20,000 per day. Fish is available for irradiation in substantial quantities. Another product available to radiation processing and with a substantial sale in this area is "purified" bottled water. The purification process must eliminate the hazard from pathogens [particularly *Eberthella typhosa* (typhoid) or *Vibrio comma* (cholera)] and from parasites [*Edameba histolytica* (amebiasis) or liver flukes]. It is considered that a radiation dose of 5×10^5 rads is required for this purpose.

A radiation facility has been designed to handle these three products, simultaneously. A diagrammatic sketch is shown in Figure 4. In the first system, the chicken is carried by a conveyor above and below the plaque source with a radiation dose of 300,000 rads and an efficiency of about 35% (*11*). In the second system, the fish is carried by a conveyor past the ends of the plaque also with a radiation dose of 300,000 rads and an efficiency of 20%. The whole system is surrounded by a water jacket acting as a partial shielding and as a flow chamber for purified water. The water is radiated to a dose level of 500,000 rads with an efficiency of 20%. The total energy balance adds up as follows:

Chicken	35%	Total useful absorption	75%
Fish	20%	Self-absorption in source	10%
Water	20%	Overdose and escape to shielding	15%
Total useful absorption	75%		100%

Table V. Radiation and Production Data for Multifood Processing Plant

	Items Processed		
	Chicken	Fish	Water
Efficiency	35	20	20
Total dose, rads	3×10^5	3×10^5	5×10^5
Throughput, M rad lb./hr.	480	280	280
Capacity, lb./hr.	1600	930	560

Total processed (chicken + fish + water) = 3090 lb./hr.

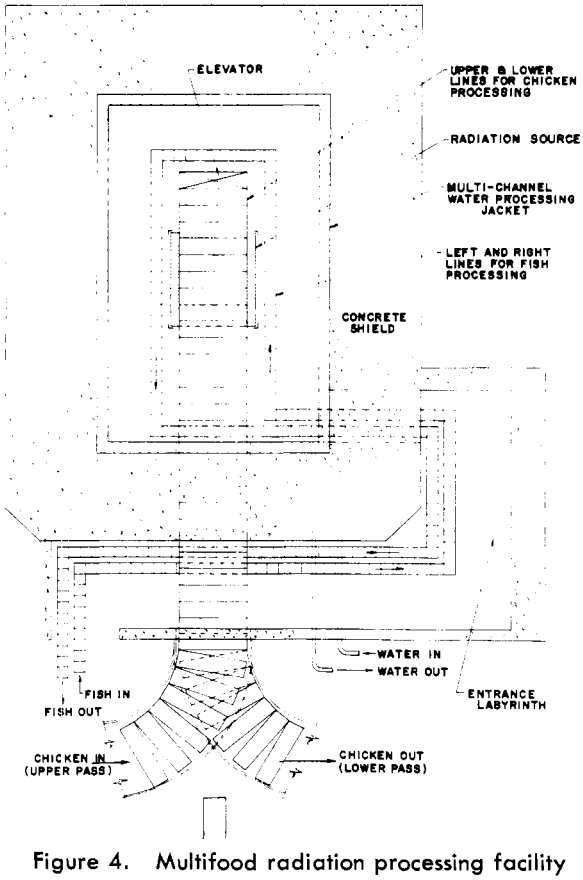

Figure 4. Multifood radiation processing facility

Table VI. Cost Analysis for Radiation Processing of Chicken, Fish, and Water

		Capital Cost	Operating Cost/Year
1.	Source cost at $1.40/curie installed	168,000	33,600
2.	Cost of building and equipment	150,000	29,580
3.	Labor		
	8 operators (low wage rate area)	16,000	
	Supervisor	8,000	
	Overhead (100%)	24,000	48,000
	Total annual cost		111,180[a]
4.	Annual production, all products in pounds		2.47×10^{7}[b]
5.	Cost per pound for processing		0.45¢

[a] Includes amortization and interest (6% on Co^{60} for 5 yr. and 3% on buildings and equipment for 10 yr.).
[b] Production = 160-hr. week × 50 weeks/yr. × 3090 lb./hr.

Therefore the net efficiency of the radiation is 75%.

The amount of Co^{60} required in this case is determined by the requirement to process 20,000 chickens or approximately 1600 pounds per hour. A useful reference figure is that 1 curie of Co^{60} will process 100 megarad pounds per year at 100% efficiency. Therefore at 35% efficiency and a throughput of 480 megarad pounds per hour the source strength required is 120,000 curies. The production and radiation data for this multifood radiation processing plant are shown in Table V.

The cost analysis for radiation processing of chicken, fish, and water is shown in Table VI.

The sale price of chicken in this product area averages about 35 cents per pound and fluctuates rapidly as much as 10 to 15 cents per pound. A quality improvement by better shelf life can easily return 1 to 5 cents per pound and provide a profitable operation, even allowing for much less favorable conditions than assumed above.

It is interesting to consider the alternative of applying an electron machine to this problem. Because the radiation must be carried out on whole chicken, at least a 20-m.e.v. machine and radiation from both sides are required. This immediately involves the touchy question of induced activity. Moreover, present experience indicates that such a machine would cost $500,000 to $1,000,000 and hence present a source expense of three to five times as much. The fact that a machine of such an energy would inevitably have a much greater throughput capacity than required is of no significance in this problem. In addition, maintenance of such a machine at this location would require a full-time resident electronics engineer at an additional expense (including overhead) of $20,000 per year. Hence, the net cost per pound for a machine source, if it could be considered feasible at all, is 1.3 cents per pound. Obviously the Co^{60} source is superior in this situation.

Low-dose radiation processing can be accomplished at reasonable costs. The processing cost for destruction of trichina in pork carcasses at a radiation dose as low as 15,000 rads would be as low as 0.05 cent per pound, according to Murray's data (*11*). Foods treated at sterilization doses of 3 to 5 megarads, however, would entail a higher processing cost above 2 cents per pound. Radiation sterilization, therefore, at the moment does not present the economic potentialities of those currently recognized in radiation processing with low dose treatment.

Acknowledgment

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FRACTIONATION OF CAROTENOIDS

Two New Solvent Systems for the Countercurrent Distribution of Carotenoids

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Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany 10, Calif.

Two new solvent systems for the countercurrent distribution of carotenoids have been discovered. A clean separation of the carotenoid diol, monoepoxide diol, diepoxide diol, and polyol fractions can be obtained in 100 transfers with the system petroleum ether and 73.5% methanol. A good system for the further fractionation of the carotenoid polyols consists of petroleum ether, acetone, methanol, and water (1.25 to 1.00 to 0.10 to 0.65 by volume, respectively). Determination of N_{100} values of individual polyol carotenoids with the latter system has considerable value in showing nonidentity, probable identity, or close relationship, as of stereoisomers.

Earlier work (*2*) shows that carotenoids can be fractionated by means of countercurrent distribution in a Craig apparatus. Two different solvent systems were used: I, petroleum ether and 99% methanol and, II, benzene, petroleum ether, and 87% methanol, 1 to 1 to 1.15 by volume.

More recent investigation has led to the discovery of two other useful solvent systems for the countercurrent distribution of carotenoids. By means of system IV (petroleum ether and 73.5% methanol) the diol-polyol fraction can be much more completely resolved into four fractions in 100 transfers than in 200 transfers with system II. System III (see below) can be used to fractionate further the very complex polyol fraction of fruits such as oranges (*4*) or cling peaches (*1*). Determination of N_{100} values with system III was especially useful in showing nonidentity or possible identity of various carotenoid polyols; with systems I, II, or IV, the N_{100} values are too close to zero.

Experimental

Solvent system IV consisted of petroleum ether, methanol, and water, 10 to 7.35 to 2.65, by volume. System III consisted of petroleum ether, acetone, methanol, and water, 1.25 to 1.00 to 0.10 to 0.65 by volume.

Countercurrent distribution runs were carried out in a 100-tube Craig apparatus in which the volume of the lower layer was 10 ml. The volume of the upper layer added in all cases was also 10 ml. The procedure used was essentially that previously described (*2*). At the end of the run, the contents of the various tubes were transferred to numbered test tubes by means of a glass syringe, and diluted with sufficient acetone to make them homogeneous and to a definite volume (50 ml. with systems III and IV). The depth of color was then measured in an Evelyn photoelectric colorimeter using filter 440. The results obtained were calculated as β-carotene by the use of a conversion table. (The resulting values are roughly approximate for most common carotenoids, and are used here mainly to show the positions of the various fractions.) In the case of some individual constituents, with absorption maxima at somewhat shorter wave lengths than 440 mμ, a 420 mμ filter was

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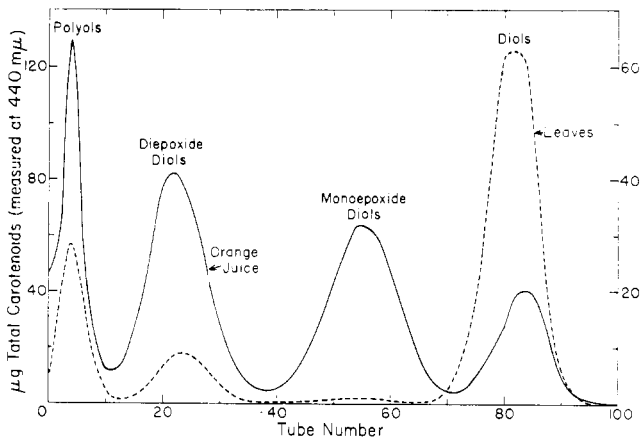

Figure 1. Countercurrent distribution of carotenoids in system IV, petroleum ether–73.5% methanol

Orange juice (scale on left) —— Pyracantha leaves (scale on right) - - - -

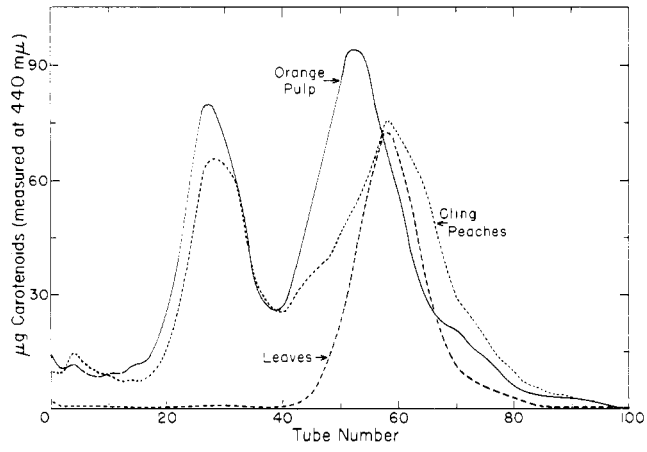

Figure 2. Countercurrent distribution of carotenoid polyols in system III, petroleum ether–acetone–methanol-water, 1.25 to 1.00 to 0.10 to 0.65, by volume, respectively.

Orange pulp —— Cling peaches - - - - Pyracantha leaves – – –

used in order to determine the position of the maximum in the Craig apparatus. Where the absorption maxima were below 420 mμ, the absorbance was measured in a Cary recording spectrophotometer at the wave length of the principal absorption maximum.

The carotenoid mixtures and fractions were obtained from orange juice or pulp (*4*), cling peaches (*7*), red bell peppers, or leaves (*3*). Much of this work was done during the winter months, at which time Pyracantha leaves were a convenient source; Strain (*9*) showed that the pigment mixture of leaves from various angiosperms is quite uniform. The diol-polyol fractions were obtained by countercurrent distribution with system I (or by distribution between petroleum ether and 95% methanol), the polyol fractions by means of system II. In some cases, further resolution of the polyol fraction was achieved by chromatography on magnesia, either Westvaco 2642 with an equal volume of filter aid, or Sea Sorb 43. The latter absorbant was used in columns 7 to 10 cm. in height and 14 mm. in diameter without a diluent. The bands were considerably sharper on Sea Sorb 43; the eluents used (*4*) were the same as on magnesia 2642.

Results and Discussion

Of the considerable number of solvent systems which have been investigated, many systems, some otherwise promising, were unsuitable because of the formation of persistent emulsions. Chlorinated hydrocarbons in combination with aqueous methanol usually formed an appreciable amount of acid, which is very undesirable because of the resulting isomerization of the carotenoid 5,6-epoxides to 5,8-epoxides. Cis-trans isomerization is also catalyzed by acids.

Table I. N_{100} Values of Various Carotenoids in System III—Petroleum Ether 1.25, Acetone 1, Methanol 0.1, Water 0.65, by Volume

No.	Constituent	Type of Epoxide	Source[a]	Spectral Absorption Maxima in Benzene, Mμ	N_{100}[b]
1	Diols	Non	O	...	97
2	Monoepoxide diols	Both	O	...	96
3	Diepoxide diols	Both	O	...	93
4	Capsorubin	Non	BP	521, 486, 459	78
5	Trollein *a*	Non	O	485, 456, 431	73
6	Trollein *b*	Non	O	484, 455, (431)	55
7	Trans-neoxanthin	5, 6	P	482, 450, 425	62
8	Neoxanthin	5, 6	L	476, 446, 421	61
9	Taraxanthin-like	5, 6	P	480, 452, 428	49
10	Trolliflor-like	5, 6	P	481, 450, 425	30
11	Unknown	Non?	P	480, 449, 422	4
12	Neochrome *a*	5, 8	L	459, 431, 406	72
13	Neochrome *b*	5, 8	L	458, 430, 405	70
14	Tarachrome-like	5, 8	O	458, 430, 406	58
15	Trolliflavin-like	5, 8	O	458, 430, 406	38
16	Sinensiaxanthin	5, 6	O	429, 404, 383	46
17	Sinensiachrome	5, 8	O	403, 380, 361	ca. 52
18	Valenciaxanthin	5, 6	O	402, 379, 361	50
19	Persicaxanthin	5, 6	P	401, 378, 360	39–40
20	Unknown	?	P	400, 377, 357	ca. 4
21	Valenciachromes	5, 8	O	375, 353, 336	ca. 54
22	Persicachromes	5, 8	P	373, 353, 336	48

[a] O: Valencia oranges; BP: bell peppers; L: leaves; P: cling peaches.
[b] N_{100}: tube number of maximum per 100 transfers.

System IV. The combined diol-polyol fraction can be separated into diols, monoepoxide diols, diepoxide diols, and polyols within 100 transfers on countercurrent distribution with systems containing petroleum ether, methanol, and water (70 to 85% methanol). The optimum separation was achieved at about 73.5% methanol (Figure 1). The N_{100} values of the polyol, diepoxide diol, monoepoxide diol, and diol fractions in this system were 2, 22, 55, and 83, respectively; the corresponding values with system II were 9, 39, 56, and 70 (*2*). With system IV a much better separation is obtained in 100 transfers than with 200 transfers with system II; considerably larger quantities of material can be used with the latter system.

A convenient method of determining the fractional composition of a carotenoid mixture is to make a 100-transfer run with system I. The diol-polyol fraction (or an aliquot thereof equivalent to 1 or 2 mg., as β-carotene) is then used in a 100-transfer run with system IV. With system I, the sample may be conveniently dissolved in 50 ml. of the upper layer, and five 10-ml. aliquots are added successively to tube 0, or initially to tubes 0 to 4. For system IV, it may be necessary to dissolve the sample in 50 ml. each of the upper and lower layers, and 10-ml. aliquots of each layer are added initially to tubes 0 to 4.

The curve for pyracantha leaf carotenoids (Figure 1) shows the preponderance of the diol fraction, mainly lutein, the

considerably smaller amounts of diepoxide diols and polyols, mainly violaxanthin and neoxanthin, respectively, and the very low content of monoepoxide diols, in good agreement with earlier work (*3*).

System III. A system containing petroleum ether, acetone, methanol, and water in the ratios 0.87 to 1 to 0.14 to 0.25, by volume, respectively, gave separations similar to those with system II. The water content of the former can be increased threefold or even more without excessive emulsion formation. A system in which the partition coefficient of the carotenoid polyol fraction of cling peaches was about 1 (system III) was petroleum ether 1.25, acetone 1.00, methanol 0.10, and water 0.65, by volume. Countercurrent distributions with this system of the carotenoid polyol fractions from Valencia orange pulp, cling peaches, and pyracantha leaves are shown in Figure 2.

The leaf polyols had only one maximum at N_{100} of 59. This is close to the N_{100} value of neoxanthin (Table I), the principal constituent of the polyol fraction of leaves. Neoxanthin was discovered by Strain (*9*) and is apparently a triol 5,6-epoxide (*3*). The curve shows the presence of very little carotenoids elsewhere, except for a minor bump at about tube number 73. The peach polyols had two major maxima with N_{100} values of 60 and 30, which are attributable to the presence of triols (such as neoxanthin) and tetraols, respectively, a minor maximum with N_{100} of 4 (pentaols or hexaols ?), and several minor inflections. The orange polyols also had a complex curve, with major maxima at 54 and 28, and several minor maxima and inflections. By the use of countercurrent distribution with system III, it is thus possible to separate the complex polyol fractions of fruits such as peaches and oranges into two major fractions, and perhaps two minor ones (by combining tubes 0 to about 17, and tubes 70 to 90). These subfractions are more readily resolvable by chromatography than is the entire polyol fraction. The curves in Figure 2 confirm the much greater complexity of the polyol carotenoids of cling peaches and oranges than of leaves (*1*, *3*).

Distribution of Individual Constituents in System III. Another means of fractionating the carotenoid polyols is to chromatograph the entire fraction, and then subject each of the separated bands to a countercurrent distribution with system III. Where the band is a single substance, only one maximum will appear with an N_{100} value characteristic of that substance, which will aid in its identification. In other cases, the distribution may show separation into two or more subfractions having different N_{100} values. The tubes representing these subfractions can then be combined, the samples recovered and dissolved in benzene, and spectral absorption curves run.

By this means, the polyol fractions of Valencia oranges, cling peaches, and leaves were separated into a considerable number of constituents. Eighteen of the more important ones are listed in Table I. For purposes of comparison, data are also included for the diol, monoepoxide diol (mainly antheraxanthin), and diepoxide diol (mainly violaxanthin) fractions of orange juice, and also for capsorubin, a dihydroxydiketocarotenoid obtained from bell peppers. The structure of none of the polyols listed in Table I is completely known.

A number of carotenoids have been found in nature which appear to contain three or more hydroxyl groups, including neoxanthin (*9*), taraxanthin (*5*), trollixanthin (*6*), trolliflor (*7*), fucoxanthin, myxoxanthophyll, and sulfatoxanthin; the complete structure is known only for trollixanthin. With the exception of neoxanthin, a universal constituent of green leaves, none of the above appears to occur as a major constituent in a readily available source. Hence, no authentic preparations of any of the above polyol carotenoids except neoxanthin were available. Trollein *a*, trollein *b*, valenciaxanthin, valenciachromes, sinensiaxanthin, sinensiachrome, persicaxanthin, and persicachromes were found by the present author in oranges or peaches.

In several instances, fractions which appeared to be cis-trans isomers were found to give closely agreeing N_{100} values in system III, such as neoxanthin and transneoxanthin. However, neochromes *a* and *b*, the 5,8-epoxide (furanoid oxide) isomers of neoxanthin, had N_{100} values higher by about 10 than neoxanthin. This behavior appears to be general, as in No. 9 and 14; 10 and 15; 16 and 17; 18 and 21; and 19 and 22 (Table I). This difference in the N_{100} values of 5,6- and the isomeric 5,8-epoxides was not observed in either systems I or II. The N_{100} value of 5,8-epoxide pairs showed no significant difference in the only instance observed, neochromes *a* and *b*.

There are a number of naturally occurring substances which have a spectral absorption curve and maxima which resemble closely those of violaxanthin (main part of No. 3). In Table I are Nos. 7, 9, 10, and 11, also lutein 5,6-epoxide and cryptoxanthin 5,6, 5′,6′-diepoxide (found in Meyer lemon peel). All of these substances are readily distinguished by their N_{100} values in either system II or III. Valenciaxanthin and persicaxanthin were shown to be distinct substances by their significant difference in N_{100} values (*1*).

Determination of the N_{100} value of carotenoid polyols with system III is thus of value in showing nonidentity in some cases, as in valenciaxanthin and persicaxanthin; in other cases identity of two substances may be indicated, or at least close relationship such as in cis-trans or other stereoisomers. The N_{100} values in solvent systems I, II, or IV are likewise useful in the identification of naturally occurring carotenoids containing fewer than three hydroxyl groups. Two substances having similar chromatographic behavior, closely agreeing spectral absorption curves, and N_{100} values (preferably not near either zero or 100) in a given solvent system are very probably either identical or closely related substances such as stereoisomers. Carotenoids differing in the position of functional groups, such as zeaxanthin and isozeaxanthin (3, 3′- and 4, 4′dihydroxy-β-carotenes, respectively), have distinctly different N_{100} values (in system II, 69 and 78, respectively), while isomers such as zeaxanthin and lutein (3, 3′-dihydroxy-α-carotene), which differ in the position of one double bond, have very similar N_{100} values.

N_{100} values are, of course, dependent on partition coefficients of the solvent system employed. If the distribution coefficient is 1.00, the N_{100} value should be 50. Petracek and Zechmeister (*8*) have recently published the partition coefficients of numerous carotenoids in hexane–95% methanol and hexane–85% methanol; their data show how the partition is affected by the presence of certain functional groups.

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ALFALFA HEMICELLULOSE

Constitution of the Hemicellulose of Alfalfa (*Medicago sativa*). Hydrolysis of Hemicellulose and Identification of Neutral and Acidic Components

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The work was carried out to determine whether there is a relationship between nutritional value and structure of the hemicelluloses of grasses and legumes. The hemicellulose of alfalfa (*Medicago sativa* var. Ranger) gives upon hydrolysis a mixture of sugars composed of L-arabinose (12.0%), D-xylose (67.3%), D-galactose (8.1%), D-glucose (8.1%), and L-rhamnose (4.5%), with a mixture of seven acidic components. Five of these acids are oxalic acid, D-galacturonic acid, 4-O-methyl-D-glucuronic acid, 2-O-(4-O-methyl-α-D-glucosyluronic acid)-D-xylose (I), and O-4-O-methyl-α-D-glucosyluronic acid-(1 → 2)-O-β-D-xylopyranosyl-(1 → 4)-β-D-xylose (Vb). The other two appear to be D-galactosyluronic acid D-xylose and D-galactosyluronic acid D-galactose. The results may have some bearing on the biochemistry of animal nutrition and be useful to the plant breeder.

CERTAIN FORAGE CROPS, notably the legumes, have long been known to be more nutritious than the grasses. Because differences in hemicellulose composition and structure may be responsible for some of the observed differences in feed value, a series of investigations has been initiated to ascertain the composition and structure of the hemicelluloses of certain selected grasses and legumes.

This communication deals with the preliminary results of studies on the constitution of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger), one of the major legume crops of the midwestern area of the United States.

Experimental

Unless stated otherwise, all evaporations were carried out in vacuo at 40° to 50° C. (bath temperature).

The solvents used in chromatography were A, pyridine–ethyl acetate–water (1:2.5:3.5); B,*n*-butyl alcohol–pyridine–water (6:4:3); C,*n*-butyl alcohol–acetic acid–water (2:1:1); D, butanone-water azeotrope; E, *n*-butyl alcohol–ethyl alcohol–water (4:1:5).

The cation resin, Amberlite IR-120 (H^+ form), and the anion resin, Duolite A-4 (OH^- form), were used throughout unless specified otherwise.

Isolation of Alfalfa Hemicellulose. Alfalfa hay (400 grams) was extracted with hot 80% aqueous methanol for 2 hours and then washed with distilled water. The wet residue was heated with 8 liters of water which contained 188 grams of sodium chlorite and 40 grams of acetic acid for 5 hours at 75° to 80° C. to remove the lignin (*27*). The white holocellulose was washed with distilled water until free of chloride ion and treated with 4 liters of 10% sodium hydroxide for 4 hours at 25° C. with occasional stirring. After filtration, the residue was heated with 2 liters of 10% sodium hydroxide for 3 hours at 80° to 90° C. and filtered again. The combined filtrates were acidified with acetic acid and 4 volumes of ethyl alcohol were added to precipitate the hemicellulose. The latter was washed successively with ethyl alcohol, diethyl ether, and petroleum ether (boiling point 35° to 60° C.) and dried in vacuo. The yield of crude hemicellulose was 49.6 grams or 12.4% of the original alfalfa hay.

Determination of Neutral Equivalent of Hemicellulose. A portion of the hemicellulose (1.5 grams) was dissolved in 5% sodium hydroxide; a small amount of insoluble residue was removed by centrifugation and discarded. The supernatant solution was acidified with 1*N* hydrochloric acid and dialyzed against distilled water until chloride ion was absent from the dialyzate. The hemicellulose was precipitated with ethyl alcohol and dried as described above.

Excess 0.02*N* sodium hydroxide (25 ml.) was added to 0.2946 gram of the hemicellulose and the excess alkali was back-titrated with 14.15 ml. of 0.0203*N* hydrochloric acid. A blank experiment carried out at the same time required 24.63 ml. of 0.0203*N* hydrochloric acid. Found: equivalent weight, 1384, 1380.

Hydrolysis of Alfalfa Hemicellulose. ISOLATION AND IDENTIFICATION OF NEUTRAL SUGAR COMPONENTS. The hemicellulose (10 grams) was heated with 100 ml. of 1*N* sulfuric acid on a boiling water bath for 9 hours. The solution was neutralized with barium carbonate, centrifuged, and passed successively through the cation and anion resins. The organic acids were absorbed on the latter. The solution containing the neutral sugars was evaporated to dryness, giving 8.2 grams of a thick sirup.

A portion of the mixed neutral sugars (1.8 grams) was resolved on a cellulose column with solvent B in the usual manner, the effluent from the column being collected (6 ml. every 15 minutes) in test tubes with an automatic fraction collector. The appropriate fractions were combined and the solvent was evaporated to give the five sugars listed in Table I, each recrystallized from methanol.

DETERMINATION OF RATIO OF NEUTRAL SUGARS. A portion of the neutral sugar mixture (22.7 mg.) was applied to Whatman No. 1 filter paper (8 × 22 inches). After irrigation with solvent A, three strips were cut from the paper (one in the center and one from each side) and sprayed with *p*-anisidine trichloroacetate in order to locate the position of the sugars. Each sugar was eluted from the appropriate section of the paper with water: the D-xylose with 10 ml., the L-arabinose with 10 ml., the D-glucose with 5 ml., the D-galactose with 5 ml., and the L-rhamnose with 10 ml. Each solution was filtered through glass wool. The D-xylose eluate was diluted with water 50 times, the L-arabinose 12.5 times, the D-glucose 12.5 times, the D-galactose 25 times,

Table I. Identification of Sugars in Hydrolyzate of Alfalfa Hemicellulose

Sugar	%	M.P. and Mixed M.P., ° C.	$[\alpha]_D^{25}$ (Equilibrium Value in Water)
D-Xylose	67.3	144–45	+18.3° (*c*, 1)[a]
L-Arabinose	12.0	158–59	+102° (*c*, 1)
D-Glucose	8.1	146	+52° (*c*, 5)
D-Galactose	8.1	166–67	+72° (*c*, 1)
L-Rhamnose	4.5	92–3.5 monohydrate	+8.0° (*c*, 5)

[a] Here and elsewhere *c* refers to concentration of solute in grams per 100 ml. of solution.

and the L-rhamnose 12.5 times. The concentration of each sugar was determined on 2 ml. of each of the diluted solutions by the phenol–sulfuric acid method (*6*). The results are recorded in Table I.

GRADED HYDROLYSIS OF HEMICELLULOSE AND ISOLATION OF MIXTURE OF URONIC ACID COMPONENTS. To improve the yield of the mono-*O*-methylaldotriouronic acid (Vb), 30 grams of the hemicellulose was heated with 0.5*N* sulfuric acid on a boiling water bath for 3 hours. The insoluble residue was removed by centrifugation and retreated with 0.5*N* sulfuric acid as before. Likewise the residue from this second hydrolysis was removed and heated with 1*N* sulfuric acid for 3 hours. All three hydrolyzates were combined, neutralized with barium carbonate, and centrifuged, and the supernatant solution was passed through the cation and anion resins. The latter was washed with water until free of neutral sugars (negative Molisch test). The acidic components were eluted from the anion resin with 1*N* sodium hydroxide and recovered by passing the alkaline eluate through the cation resin.

Paper chromatographic analysis of the mixture of uronic acids with solvent C indicated five components; the one having the greatest R_F value, 0.49, corresponded to 4-*O*-methyl-D-glucuronic acid, followed by a mono-*O*-methylaldobiouronic acid (R_F, 0.41), D-galacturonic acid (R_F, 0.31), a mono-*O*-methylaldotriouronic acid (R_F, 0.24), and a mixture of two oligouronic acids (R_F, 0.16).

Isolation of Oxalic Acid. The addition of calcium acetate to an aqueous solution of the acid mixture gave a small amount of white precipitate, which was removed by filtration and dissolved in dilute hydrochloric acid. Calcium ions were removed with the cation resin and the effluent was evaporated to dryness. A portion of the residue sublimed at 100° to 110° C. to give oxalic acid dihydrate, recognized by melting point and mixed melting point 100° to 101° C., and by the fact that it decolorized acidified (1*N* sulfuric acid) potassium permanganate at 60° C.

The amount of oxalic acid produced during hydrolysis of the hemicellulose was determined as follows: The hemicellulose (5 grams) from freshly harvested, air-dried alfalfa was isolated as described above and heated with 70 ml. of 1*N* hydrochloric acid on a boiling water bath for 6 hours. The solution was treated with just less than the stoichiometric amount of barium carbonate, followed by an excess of barium acetate. The white precipitate of barium oxalate was removed by centrifugation, washed with 10 ml. of water, and dissolved in dilute hydrochloric acid, and the barium ions were removed with the cation resin. The effluent was made alkaline with ammonium hydroxide and then acidified with acetic acid, after which an excess of 1*N* calcium chloride was added. The white precipitate of calcium oxalate was separated by centrifugation, washed with water, acetone, and diethyl ether, and dried; yield, 0.215 gram. This corresponds to 3% oxalic acid in the hemicellulose.

The calcium oxalate was dissolved in dilute hydrochloric acid and the free oxalic acid dihydrate was isolated by evaporation and sublimation as described above; melting point and mixed melting point 100° to 101° C.

Separation and Identification of Uronic Acids. An attempt to separate the uronic acid mixture by displacement of the acids from Amberlite IRA-400 resin (acetate form) with increasing concentrations of acetic acid (*2*, *5*, *25*) gave only a partial separation.

The uronic acid mixture was therefore separated on a cellulose column with solvent C using an automatic fraction collector adjusted to collect about 7 ml. per fraction every 20 minutes. Tubes 25 and 33 contained the 4-*O*-methyl-D-glucuronic acid; tubes 38 to 48, the mono-*O*-methylaldobiouronic acid (I); tubes 60 to 72, galacturonic acid; tubes 70 to 82, the mono-*O*-methylaldotriouronic acid (Vb); and tubes 140 to 155, the oligouronic acid components. The mono-*O*-methylaldotriouronic acid (Vb) crystallized spontaneously after evaporation of the solvent and exposure to the atmosphere.

IDENTIFICATION OF D-GALACTURONIC ACID. The uronic acid with an R_F value corresponding to that of galacturonic acid gave a brick red precipitate when heated with an aqueous solution of basic lead acetate (*7*).

The fraction crystallized spontaneously and after recrystallization from aqueous ethyl alcohol afforded D-galacturonic acid monohydrate; melting point and mixed melting point 156° C. (with decomposition) (during heating the crystals turned yellow at 115° and red at 130°), $[\alpha]_D^{22}$ + 95° changing to +51° after 24 hours (equilibrium value, *c*, 3.4 in water).

IDENTIFICATION OF 4-*O*-METHYL-D-GLUCURONIC ACID. The 4-*O*-methyl-D-glucuronic acid (28 mg.) obtained by column chromatography was refluxed with 1% methanolic hydrogen chloride for 5 hours, neutralized with silver carbonate, filtered, and evaporated to a sirup. A solution of the sirup in methanol was saturated with ammonia gas at 0° C. and allowed to stand overnight at 3° C., after which the solution was evaporated to a sirup at room temperature. Nucleation of the sirup with methyl 4-*O*-methyl-α-D-glucosiduronamide (*21*) provided crystals with melting point 195° to 205° C. Examination of the crystals under the microscope revealed the presence of both alpha and beta isomers, which are easily distinguished because the latter forms platelets and the former small needles. The methyl 4-*O*-methyl-α-D-glucosiduronamide was obtained by triturating the mixture with ethyl alcohol; melting point and mixed melting point 235° to 236° C. after recrystallization from methanol.

IDENTIFICATION OF 2-*O*-(4-*O*-METHYL-α-D-GLUCOSYLURONIC ACID)-D-xylose (I). The mono-*O*-methylaldobiouronic acid (I) (73 mg.), which showed $[\alpha]_D^{26}$ + 95° (*c*, 2.3 in water) and equivalent weight 350 (calculated for $C_{12}H_{20}O_{11}$, 340), was heated with methanol containing hydrogen chloride (8%) in a sealed tube at 110° to 115° C. for 11 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. The sirup was dissolved in methanol which was saturated with ammonia gas at 0° C. and the mixture was allowed to stand overnight at 3° C. After evaporation of the methanol in vacuo at room temperature, the amide crystallized. A small amount of ethyl alcohol was added and the sirupy mother liquor was withdrawn. The crystalline residue was triturated with ethyl alcohol and recrystallized from methanol to give methyl 4-*O*-methyl-α-D-glucosiduronamide, melting point and mixed melting point 235° to 236° C., $[\alpha]_D^{25}$ + 146° (*c*, 1.6 in water) (*21*).

The mother liquor from the previous experiment which contained the methyl xyloside was freed from solvent and heated with 1*N* sulfuric acid on a boiling water bath for 8 hours. The solution was neutralized with barium carbonate, centrifuged, and passed successively through the cation and the anion resins. Evaporation of the eluate gave a sirup which crystallized on trituration with

methanol. Recrystallization of the product from methanol gave D-xylose; melting point and mixed melting point 144° to 145° C., $[\alpha]_D^{25}$ + 19.9° (equilibrium value, *c*, 4 in water).

METHYL 2-*O*-(2,3,4,6-TETRA-*O*-METHYL-α-D-GLUCOSYL)-3,4-DI-*O*-METHYL-D-XYLOSIDE (III). The mono-*O*-methylaldobiouronic acid (I) (0.54 gram) was refluxed with 1% methanolic hydrogen chloride for 6 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to give methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside as a sirup.

A portion of the methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside (334 mg.) was shaken with 10 ml. of dimethylformamide, 3 ml. of methyl iodide, and 1 gram of silver oxide for 24 hours (*13*). The mixture was filtered and the residue was washed with 2 ml. of dimethylformamide and 15 ml. of chloroform. An excess of chloroform was added to the filtrate and the silver salts which precipitated were removed by filtration. The filtrate was washed with a small volume of aqueous potassium cyanide to remove the last traces of silver salts, dried over anhydrous magnesium sulfate, and concentrated to a sirup. The sirup was further methylated by refluxing with 8 ml. of methyl iodide and 0.5 gram of silver oxide for 18 hours, after which the mixture was filtered and the methyl iodide was evaporated, leaving 0.34 gram of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-methyl-D-xyloside (II) as a sirup.

A portion (0.20 gram) of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl) uronate]-3,4-di-*O*-methyl-D-xyloside (II), obtained above, was heated with 5 ml. of 1*N* sulfuric acid on a boiling water bath for 11 hours. The brownish solution was passed through the anion resin to remove the mineral acid and uronic acid. Concentration of the effluent provided only 26 mg. of sirup, which indicated that hydrolysis was not complete. The acid component was eluted from the anion resin with 1*N* sodium hydroxide, passed through the cation resin, and then neutralized with barium carbonate. The barium uronate was converted to the free acid with cation resin and the effluent was concentrated. The sirup was heated with 13% methanolic hydrogen chloride in a sealed tube at 110° to 115° C. for 12 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. The ester was saponified with 0.5*N* sodium hydroxide at 50° C. for 1 hour. The solution was passed first through the cation resin, then the anion resin, and the effluent evaporated to give 18 mg. of methyl 3,4-di-*O*-methyl-D-xyloside.

The methyl 2,3,4-tri-*O*-methyl-D-glucosiduronic acid was isolated from the anion resin in the usual manner and was refluxed with 2% methanolic hydrogen chloride for 6 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. A solution of the sirup in methanol was saturated with ammonia gas at 0° C. and allowed to stand at 3° C. for 24 hours. After evaporation of the methanolic ammonia, the amide so formed was recrystallized from ethyl alcohol–diethyl ether, giving methyl 2,3,4-tri-*O*-methyl-D-glucosiduronamide (*20*) as colorless needles; melting point and mixed melting point 184° to 185° C., $[\alpha]_D^{25}$ + 143° (*c*, 1 in water).

The methyl 3,4-di-*O*-methyl-D-xyloside was hydrolyzed with 1*N* sulfuric acid by heating in a sealed tube in a boiling water bath for 11 hours. The solution was neutralized with barium carbonate and centrifuged and the supernatant was evaporated to dryness. The 3,4-di-*O*-methyl-D-xylose extracted from the residue with chloroform was combined with that obtained by sulfuric acid hydrolysis of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)-uronate]-3,4-di-*O*-methyl-D-xyloside (II), purified by sheet paper chromatography using solvent D, and combined with the 3,4,-di-*O*-methyl-D-xylose obtained in the following experiment for the purpose of identification.

A solution of 0.14 gram of the methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-methyl-D-xyloside (II) in 5 ml. of dry tetrahydrofuran was added dropwise with stirring to a solution of 0.2 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran (*1*, *15*). The mixture was refluxed for 1 hour and cooled to room temperature. The excess lithium aluminum hydride was decomposed by the dropwise addition of ethyl acetate. After addition of 30 ml. of water, the mixture was acidified with dilute hydrochloric acid and extracted with chloroform (4 × 20 ml.). The combined chloroform extracts were dried over anhydrous magnesium sulfate together with a small amount of sodium carbonate to neutralize any trace of hydrochloric acid. The chloroform solution was filtered and evaporated to a sirup which was methylated twice with 8 ml. of methyl iodide and 0.2 gram of silver oxide, giving 120 mg. of methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) as a mobile sirup having $[\alpha]_D^{25}$ + 111° (*c*, 1.2 in methanol). Analysis. Calcd. for $C_{18}H_{34}O_{10}$, OMe 52.9. Found: OMe, 53.1.

A solution of methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) in 5 ml. of 1*N* sulfuric acid was heated on a boiling water bath for 11 hours. The solution was neutralized with barium carbonate, centrifuged, and concentrated to dryness. The residue was extracted with ethyl alcohol and, after treatment with a small amount of charcoal, evaporation of the extract gave 103 mg. of a sirupy mixture of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose, $[\alpha]_D^{23}$ + 64° (*c*, 2 in methanol).

The two sugars were separated on filter paper (Whatman No. 3MM) with solvent D as before. The component (29 mg.) with an R_F value corresponding to that of 3,4-di-*O*-methyl-D-xylose which had $[\alpha]_D^{22}$ + 22° (*c*, 3 in methanol) was refluxed for 4 hours with 5 ml. of ethyl alcohol containing 50 mg. of aniline. After evaporation of the ethyl alcohol and excess aniline, the product crystallized spontaneously. Recrystallization from ethyl acetate–petroleum ether (boiling point 35° to 60° C.) gave *N*-phenyl-3,4-di-*O*-methyl-D-xylosylamine, melting point 122° to 123° C. (*12*), $[\alpha]_D^{24}$ − 105° (*c*, 0.5 in ethyl acetate).

The mother liquor from the crystallization of the *N*-phenyl-3,4-di-*O*-methyl-D-xylosylamine was treated with charcoal in aqueous ethyl alcohol and filtered. A small amount of cation resin was added to the filtrate and the mixture was heated at 70° C. for 1 hour (*10*). The resin was removed by filtration and washed with ethyl alcohol. Evaporation of the combined filtrate and washings provided 15 mg. of sirupy 3,4-di-*O*-methyl-D-xylose, which was dissolved in 1 ml. of water and oxidized with 0.1 ml. of bromine in the dark at room temperature for 4 days. At the end of this time the excess bromine was removed by aeration and the solution was neutralized with silver carbonate, filtered, and passed through the cation resin. The acidic effluent was evaporated. Lactonization by heating at 85° C. in vacuo afforded 3,4-di-*O*-methyl-D-xylono-δ-lactone which readily sublimed; melting point and mixed melting point 65.5° to 66.5° C., $[\alpha]_D^{24}$ − 45° changing to −14.5° (equilibrium value *c*, 1.4 in water) (*11*).

The component derived from methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) with an R_F value corresponding to that of 2,3,4,6-tetra-*O*-methyl-D-glucose was treated with a small amount of charcoal in methanol. Evaporation of the solvent gave crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose; melting point and mixed melting point 86° to 88° C., $[\alpha]_D^{24}$ + 81° (equilibrium value, *c*, 2.3 in water) after recrystallization from diethyl ether–petroleum ether (boiling point 35° to 60° C.).

METHYL 2-*O*-[METHYL-(4-*O*-METHYL-α-D-GLUCOSYL)URONATE]-α-D-XYLOPYRANOSIDE TETRAACETATE (IV) (*24*). The methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xyloside (II) (200 mg.) was heated at 90° to 95° C.

with 0.4 gram of anhydrous sodium acetate and 15 ml. of acetic anhydride for 4 hours. The excess acetic anhydride was evaporated and the residue was shaken with a mixture of 20 ml. of chloroform and 20 ml. of water. The aqueous layer was separated and extracted four times with 20-ml. portions of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate and evaporated to give 288 mg. of a sirup which crystallized upon trituration with ethyl alcohol. Recrystallization from acetone–ethyl alcohol gave methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV) (*24*); melting point and mixed melting point 200° to 202° C., $[\alpha]_D^{26}$ + 98° (*c*, 1.4 in chloroform). Analysis. Calcd. for $C_{22}H_{32}O_{15}$: C, 49.25; H, 6.02; OMe, 17.3. Found: C, 49.50; H, 5.74; OMe, 18.4.

Identification of *O*-4-*O*-Methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (V). Reduction and Identification of Hydrolysis Products. Recrystallization of the crystalline mono-*O*-methylaldotriuronic acid (Vb) from aqueous methanol gave crystals with melting point 178° C., undepressed when mixed with the mono-*O*-methylaldotriouronic acid isolated from the wood of western hemlock (*Tsuga heterophylla*) (*8*), $[\alpha]_D^{23}$ + 45° changing to +52° (equilibrium value, *c*, 2.2 in water). Analysis. Calcd. for $C_{17}H_{28}O_{15}.3H_2O$: C, 38.8; H, 6.46; OMe, 5.89, equivalent weight, 526. Found: C, 38.95; H, 6.54; OMe, 7.6; equivalent weight, 540.

The mono-*O*-methylaldotriouronic acid (Vb) (108 mg.) was allowed to stand with 1% methanolic hydrogen chloride at room temperature for 1 week, after which time paper chromatography (solvent C; spray reagent, *p*-anisidine) indicated that glycoside formation was complete. The solution was neutralized with silver carbonate, filtered, and concentrated to give methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl)uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) as a sirup.

A solution of 62 mg. of VI in 10 ml. of tetrahydrofuran was added dropwise with stirring to a solution of 0.2 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran (*1*). After the mixture had been refluxed for 2.5 hours, the excess lithium aluminum hydride was decomposed with ethyl acetate and the reaction mixture acidified with acetic acid. The mixture was evaporated to dryness and the residue acetylated by heating at 85° to 95° C. with 0.2 gram of anhydrous sodium acetate and 8 ml. of acetic anhydride for 4 hours. The excess acetic anhydride was evaporated and the residue shaken with a mixture of 25 ml. of 0.5*N* hydrochloric acid and 25 ml. of chloroform. The aqueous layer was extracted four times with 15-ml. portions of chloroform and the combined chloroform extracts were dried over anhydrous sodium sulfate together with a small amount of anhydrous sodium carbonate. Evaporation of the chloroform gave 109 mg. of the acetylated trisaccharide, $[\alpha]_D^{26}$ + 44° (*c*, 2.1 in chloroform), which did not crystallize.

The heptaacetate obtained in the previous experiment was deacetylated (*28*) by dissolving it in anhydrous methanol which contained a trace of sodium and allowing the solution to stand overnight at room temperature. Evaporation of the methanol gave 62 mg. of the sirupy methyl glycoside of a trisaccharide which had $[\alpha]_D^{25}$ + 52° (*c*, 1.25 in water). The sirup was heated for 8.5 hours with 5 ml. of 0.5*N* sulfuric acid on a boiling water bath until a constant rotation of $[\alpha]_D^{25}$ + 33° (*c*, 1.25 in 0.5*N* sulfuric acid) had been reached. The hydrolyzate was passed through the anion resin and the effluent was evaporated to 53 mg. of a sirup.

The sugars were separated by chromatography on two pieces (9 × 22 inches) of Whatman No. 1 filter paper with solvent E. The component (18 mg.) with an R_F value corresponding to that of 4-*O*-methyl-D-glucose was heated at 85° C. with 2 ml. of water, 0.2 ml. of acetic acid, and 0.1 ml. of phenylhydrazine for 2 hours (*19*, *21*). The crystalline osazone which separated from solution on cooling was recrystallized from aqueous acetone to give 4-*O*-methyl-D-glucosephenylosazone; melting point and mixed melting point 157° to 158° C., $[\alpha]_D^{25}$ − 35° changing to ±0° (equilibrium value, *c*, 0.55 in ethyl alcohol).

The component (30 mg.) with an R_F value corresponding to that of xylose was dissolved in methanol and treated with a small amount of charcoal. Filtration, evaporation, and recrystallization from methanol gave D-xylose; melting point and mixed melting point 144° to 145° C., $[\alpha]_D^{27}$ + 18.4° (equilibrium value, *c*, 3.1 in water).

Methyl-*O*-[2,3,4,6-Tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl-(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII). The methyl ester methyl glycoside (VI) of the mono-*O*-methylaldotriouronic acid (Vb) (46 mg.) was methylated by the Kuhn technique (*13*) as described above for the mono-*O*-methylaldobiouronic acid. The sirupy product was methylated twice with 7 ml. of methyl iodide and 0.3 gram of silver oxide by refluxing overnight, methanol being added in the first treatment to dissolve the sirup.

The sirupy methylated product was dissolved in 5 ml. of tetrahydrofuran and the solution was added dropwise with stirring to a solution of 0.15 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran. The mixture was refluxed for 2 hours, cooled, and treated with 50 ml. of water, the water in the initial stage being added dropwise until the lithium aluminum hydride had been destroyed. The solution was acidified with dilute hydrochloric acid and the partially methylated trisaccharide extracted with chloroform (4 × 20 ml.). The combined chloroform extracts were dried in the usual manner and evaporated to a sirup. The latter was refluxed overnight with Purdie reagent (5 ml. of methyl iodide, 0.3 gram of silver oxide), filtered, and evaporated to give the nona-*O*-methyl derivative (VII) as a sirup which was heated with 1*N* sulfuric acid on a boiling water bath for 8 hours. The hydrolyzate was passed through the anion resin to remove the mineral acid and the effluent was concentrated to a sirup. Paper chromatography with solvent D and *p*-anisidine spray reagent revealed two spots, one corresponding to 2,3,4,6-tetra-*O*-methyl-D-glucose and the other to a di-*O*-methylxylose. Paper electrophoresis (*4*) (0.1*M* borate buffer and *p*-anisidine–phosphoric acid spray reagent) of the di-*O*-methylxylose component, separated from the 2,3,4,6-tetra-*O*-methyl-D-glucose by paper chromatography using solvent D, revealed two components, one giving a deep purple color corresponding to 2,3-di-*O*-methyl-D-xylose and the other a somewhat less intense pinkish red color corresponding to 3,4-di-*O*-methyl-D-xylose. The mixture of the two di-*O*-methylxyloses was boiled for 3 hours with 1 ml. of ethyl alcohol containing 20 mg. of aniline, and the solution was treated with a small amount of charcoal, filtered, and evaporated to a sirup. A drop of ethyl acetate was added to the sirup and the mixture nucleated with the aniline derivative of 2,3-di-*O*-methyl-D-xylose. Recrystallization of the product from ethyl acetate gave *N*-phenyl-2,3-di-*O*-methyl-D-xylosylamine, melting point and mixed melting point 125° to 127° C. $[\alpha]_D^{26}$ − 173° (*c*, 0.2 in ethyl acetate).

Methanolysis of *O*-4-*O*-Methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose. The mono-*O*-methylaldotriouronic acid (Vb) (82 mg.) was refluxed with 3% methanolic hydrogen chloride for 20 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup, which was saponified with 0.5*N* sodium hydroxide at 50° C. for 1 hour and passed through the cation and anion resins. The effluent containing the neutral component was evaporated to give methyl β-D-xylopyranoside; melting point and mixed melting point 155° to 157° C. (after recrystallization from ethyl alcohol).

The acid component was removed from the anion resin with 0.5*N* sodium hydroxide and converted to the free acid with the cation resin. Evaporation

of the effluent gave 51 mg. of a sirup which was refluxed with 2% methanolic hydrogen chloride for 4 hours; the solution was cooled, neutralized with silver carbonate, filtered, and evaporated to give methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside as a sirup. This methyl ester, methyl glycoside of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), was acetylated with acetic anhydride and sodium acetate by the procedure described above for the mono-*O*-methyl-aldobiouronic acid to give methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl) uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV), melting point and mixed melting point 200° to 201° C. (after recrystallization from ethyl alcohol), $[\alpha]_D^{24}$ + 95° (*c*, 1 in chloroform).

Discussion

The alfalfa hemicellulose has been shown to give, upon acid hydrolysis, a mixture of L-arabinose (12.0%), D-xylose (67.3%), D-galactose (8.1%), D-glucose (8.1%), and L-rhamnose (4.5%), with a mixture of seven acidic components. Five of these acids have been identified as oxalic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid which probably corresponds to the methoxyglycuronic acid previously reported (*9, 18*), 2-*O*-(4-methyl-α-D-glucosyluronic acid)-D-xylose (I), and *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb). The other two appear from preliminary studies to be D-galactosyluronic acid-(1→?)-D-xylosyl-(1→?)-D-xylose and D-galactosyluronic acid (1→?)-D-galactose.

The presence of 4.5% of L-rhamnose in the hemicellulose was of interest, in view of the fact that little or no rhamnose had been found in the hemicellulose of brome grass (*Bromus inermis*), timothy (*Phleum pratense*), or prairie grass (*Buchloe dactyloides*). The relatively high content of L-rhamnose appears to be a characteristic of the legumes, as the hemicelluloses of sweet clover (*Melilotus alba*), red clover (*Trifolium pratense*), alsike clover (*Trifolium hybridum*), bird's-foot trefoil (*Lotus corniculatus*), and Dutch clover (*Trifolium repens*) all contain a considerable proportion of L-rhamnose (*17*). Other studies (*17*) have shown that the hemicelluloses in the leaves of the elm (*Ulmus americana*), honey locust (*Gleditsia triacanthos*), maple (*Acer saccharum*), and oak (*Quercus alba*) contain significant amounts of L-rhamnose.

The alfalfa hemicellulose was isolated as a white amorphous powder ($[\alpha]_D$ − 35° in 1*N* sodium hydroxide) from alfalfa (*Medicago sativa* var. Ranger) hay, harvested in Minnesota, in the usual manner by chlorite delignification followed by extraction with dilute sodium hydroxide. Acid hydrolysis of the hemicellulose followed by the use of exchange resins afforded the mixture of the neutral sugars referred to above—L-arabinose, D-xylose, D-galactose, D-glucose, and L-rhamnose—all of which were obtained crystalline, and a mixture of the seven acidic components. The oxalic acid, the source of which is unknown, was separated as the calcium salt and identified as the dihydrate. The remaining uronic acid mixture was resolved by chromatography on a cellulose column and shown to consist of D-galacturonic acid, 4-*O*-methyl-D- glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb), and two unidentified oligouronic acids.

The D-galacturonic acid, which was identified as the crystalline monohydrate (*14*), does not appear to be derived from pectin, because the two unidentified oligouronic acids themselves were shown to contain galacturonic acid and because the method used for isolating the hemicellulose precluded the possibility of contamination with pectin.

The 4-*O*-methyl-D-glucuronic acid was converted with methanolic hydrogen chloride into the methyl ester methyl glycosides which upon treatment with ammonia afforded the characteristic crystalline α- and β- forms of methyl 4-*O*-methyl-D-glucosiduronamide (*21, 26*).

The 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), which was liberated by prolonged hydrolysis of the hemicellulose, was identified by reason of the fact that, upon methanolysis, it yielded methyl (4-*O*-methyl-α-D-glucosid) uronate, identified as the corresponding amide (*21, 26*), and methyl D-xyloside, identified after hydrolysis as crystalline D-xylose. Moreover, methylation of the methyl ester methyl glycoside of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose, prepared by boiling with 1% methanolic hydrogen chloride, followed by lithium aluminum hydride reduction (*1, 15*) and methylation, afforded methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III). Acid hydrolysis of III gave crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose, the latter identified as the crystalline lactone (*11*). The high positive rotation ($[\alpha]_D$ + 95° in water) of I indicates that the biose linkage is of the α type. Further proof of the identity of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I) was provided by the fact that acetylation of the methyl ester methyl glycoside of this monomethylaldobiouronic acid as described previously (*16, 21*) provided the crystalline methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosid) uronate]-3,4,-di-*O*-acetyl-α-D-xyloside (IV), which proved to be identical with a specimen prepared from white birch wood (*24*).

The crystalline *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb), isolated by controlled hydrolysis of the hemicellulose, was found to have the same properties ($[\alpha]_D$ + 52° in water, melting point 178°, OCH_3 7.6, and equivalent weight 540) as those displayed by the mono-*O*-methylaldotriouronic acid isolated from western hemlock (*8*) and jute fiber hemicellulose (*23*). Reduction of methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl) uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) derived from *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb) by the action of methanolic hydrogen chloride at room temperature, followed by hydrolysis, yielded 1 molar proportion of 4-*O*-methyl-D-glucose and 2 molar proportions of D-xylose, thus proving that compound V was a mono-*O*-methylaldotriouronic acid. When the methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl) uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) was successively subjected to methylation, lithium aluminum hydride reduction, and remethylation, the fully methylated trisaccharide methyl *O*-[2,3,4,6-tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl-(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII) was produced. Hydrolysis of this trisaccharide (VII) gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-, and 2,3,di-*O*-methyl-D-xylose. These results indicated that the mono-*O*-methylaldotriouronic acid was either 4-*O*-methyl-D-G_pA-(1→2)-D-Xyl_p(1→4)-D-Xyl_p or 4-*O*-methyl-D-G_pA-(1→4)-D-Xyl_p-(1→2)-D-Xylp. (Gp = glucosyluronic acid; Xylp = xylopyranosyl).

A decision between these two formulations for the mono-*O*-methylaldotriouronic acid (V) was based on the observation that methanolysis of compound V and subsequent acetylation gave rise to methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV), thus establishing that the 4-*O*-methyl-D-glucuronic acid residue was linked to C_2 of one of the D-xylose residues. This D-xylose residue clearly corresponds to that which gave rise to 3,4-di-*O*-methyl-D-xylose when methyl *O*-[2,3,4,6-tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII) was hydrolyzed. The 2,3-di-*O*-methyl-D-xylose obtained from VII must therefore be derived from a 1→4 linked reducing D-xylose residue located in a terminal position.

On the grounds that most xylan polysaccharides contain β-linkages, a deduction reached from the fact that xylans display a relatively high negative rotation, it has been inferred (*8, 22, 23*) that

the 1→4 linkage between the two xylose units of the mono-*O*-methylaldotriouronic acid (V) is of the β- type and consequently would have the structure 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-D-Xyl. Using Hudson's optical superposition rules and specific rotations (in water) of +150° for methyl 4-*O*-methyl-α-D-glucosiduronamide (*21*, *26*), −50° for methyl 4-*O*-methyl-β-D-glucosiduronamide (*21*, *26*), +154° for methyl α-D-xylopyranoside, −66° for methyl β-D-xylopyranoside, +94° for α-D-xylose, and −30° for β-D-xylose (*3*), specific rotations were calculated for the four mono-*O*-methylaldotriuronic acids. The calculated specific rotations of +134° and +105° for 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-α-D-Xyl and 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-β-D-Xyl, respectively, are much higher than that determined experimentally (+45°). The calculated specific rotations of +65° and +33°, respectively, for 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-α-D-Xyl (Va) and 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-β-D-Xyl (Vb) are in fair agreement with the observed value of +45°, indicating that the linkage between the two D-xylose residues is of the β- type. The fact that the mono-*O*-methylaldotriouronic acid displays an upward mutarotation (+45°→+52°) requires that the terminal reducing D-xylose unit exist in the β- form. From these data the mono-*O*-methylaldotriouronic acid is tentatively designated *O*-4-*O*-methyl-α-D-glucosyl-uronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb) (*23*).

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RICE AMYLOSES

Molecular Weights of Crystalline Amyloses from Certain Rice Varieties

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Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge, La.

ALTHOUGH the composition of rice with regard to its major constituents has been studied extensively, the processing characteristics of any variety cannot be predicted on the basis of present compositional information. Certain helpful generalizations can be made regarding amylose-amylopectin ratios and probable cooking quality, but exceptions can always be found (*19*). Consideration of grain type is also useful, but not infallible. Fundamental research is needed in all phases of rice composition to facilitate a more rational interpretation of existing information.

Because the principal chemical constituent of rice is starch, any differences in the chemical and physical properties innately pertaining to the different types of starch present should be reflected in the properties of the rice itself. For this reason, it was desirable to study the physical properties of the amyloses of rices of widely different processing characteristics. The present report describes a study of the crystallization and molecular weight determination of amyloses from four distinctly different varieties: Rexoro, Zenith, Century Patna, and Caloro.

Experimental

Samples of Zenith, Century Patna, and Rexoro rices were obtained from Crowley,

the 1→4 linkage between the two xylose units of the mono-*O*-methylaldotriouronic acid (V) is of the β- type and consequently would have the structure 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-D-Xyl. Using Hudson's optical superposition rules and specific rotations (in water) of +150° for methyl 4-*O*-methyl-α-D-glucosiduronamide (*21*, *26*), −50° for methyl 4-*O*-methyl-β-D-glucosiduronamide (*21*, *26*), +154° for methyl α-D-xylopyranoside, −66° for methyl β-D-xylopyranoside, +94° for α-D-xylose, and −30° for β-D-xylose (*3*), specific rotations were calculated for the four mono-*O*-methylaldotriuronic acids. The calculated specific rotations of +134° and +105° for 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-α-D-Xyl and 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-β-D-Xyl, respectively, are much higher than that determined experimentally (+45°). The calculated specific rotations of +65° and +33°, respectively, for 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-α-D-Xyl (Va) and 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-β-D-Xyl (Vb) are in fair agreement with the observed value of +45°, indicating that the linkage between the two D-xylose residues is of the β- type. The fact that the mono-*O*-methylaldotriouronic acid displays an upward mutarotation (+45°→+52°) requires that the terminal reducing D-xylose unit exist in the β- form. From these data the mono-*O*-methylaldotriouronic acid is tentatively designated *O*-4-*O*-methyl-α-D-glucosyl-uronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb) (*23*).

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Experimental

Samples of Zenith, Century Patna, and Rexoro rices were obtained from Crowley,

The molecular weights of amyloses crystallized from several varieties of rice were determined to obtain basic compositional information which might be related to known processing behavior. The amylose molecular weights as determined by periodate oxidation were found to be: Century Patna 231, 29,000; Rexoro, 41,000; Zenith, 41,000; and Caloro, 48,000. These findings show a possible relationship to water absorption and alkali dispersibility of the varieties.

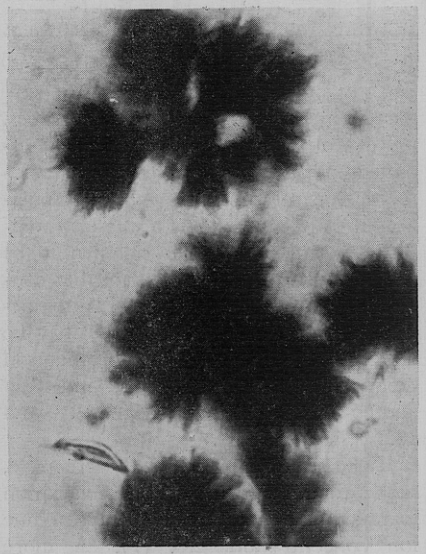

Figure 1. Rice amylose crystals from Rexoro variety

Precipitated with butyl and isoamyl alcohols and stained with iodine. Magnification, 1000 diameters

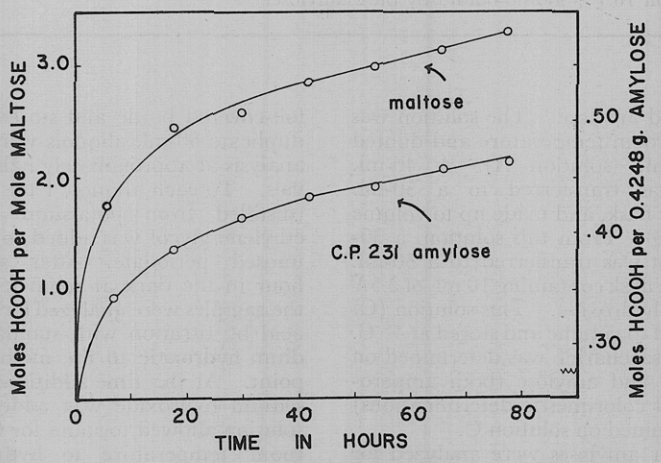

Figure 2. Total formic acid produced by periodate oxidation of maltose and Century Patna 231 amylose

La. The Caloro sample was obtained from Biggs Station, Calif. The rice was 1957 crop and the experiments were conducted during 1957–59.

Preparation and Purification of Crystalline Amylose. Well purified rice starch was prepared by the method of Hogan (*10*). The starch was fractionated by the method of Wilson, Schoch, and Hudson (*20*), modified to extend the cooling period. Approximately 48 hours in a Dewar flask was required for the solution to cool below 50° C. At this time a small amount of precipitated fraction was withdrawn, placed on a microscope slide, stained with iodine solution, and examined under the microscope. If amylose crystals were not present, the mixture was allowed to stand for a longer time. Crystals obtained at this stage were in the shape of rosettes or spherules and stained an intense blue with iodine (Figure 1).

The following method was found the most satisfactory for recrystallizing the rice amylose. The crude moist amylose fraction was dissolved in boiling water with vigorous stirring to give an approximately 0.2% solution. The hot solution was centrifuged as rapidly as possible to avoid cooling; the supernatant solution was decanted carefully and reheated to 100° C. Excess butyl alcohol was added and the solution vigorously shaken for 5 minutes, then shaken frequently for the next 2 hours. After standing overnight, the solution contained well-formed hairlike needles of crystalline amylose. The advantage of this method over the usual procedure of dissolving amylose in water previously saturated with alcohol lies in the ease of solution of the amylose in boiling water, as compared with alcohol-saturated water. No retrogradation occurs in the hot solution, so that the presence of butyl alcohol is not necessary during the dissolving process.

Drying amylose presents further problems. When rice amylose is dissolved in pure water or is dried without the removal of all moisture, it undergoes retrogradation very rapidly. Extreme care must be taken to replace the water content of the amylose by butyl alcohol. Room temperature or below is preferred for drying, because at high temperature the amylose may dissolve in the adhering solvent. Under the latter condition the final product will be a solid cake instead of a fine powder.

PROCEDURE. The amylose which had been stored in water saturated with butyl alcohol was collected by centrifugation. To the precipitate approximately 2 volumes of butyl alcohol was added and the suspension stirred vigorously with a magnetic stirrer for about 1 hour. The supernatant alcohol was decanted, another 2 volumes of butyl alcohol was added, and the suspension was stirred again. This treatment was repeated until the amylose became finely divided. During the alcohol treatment (fourth or fifth operation) the amylose stuck together in a gummy mass. The stirring and alcohol treatment were continued until the mass was dispersed and a very finely divided product obtained. During stirring, care had to be taken to keep all the amylose under the surface of the butyl alcohol. If the amylose dried on the walls of the beaker before its water content had been replaced by butyl alcohol, retrograded amylose was obtained. At the end of the drying treatment, the finely divided amylose was collected on a suction filter and dried in a vacuum desiccator over concentrated sulfuric acid.

Determination of Total Polysaccharide and of Amylose. Two possible sources of error existed in the periodate oxidation of the recrystallized amylose: the presence of insoluble retrograded amylose and traces of amylopectin. Because the amylose samples could not safely be assumed to be free of these contaminants, all samples, except the Caloro check sample, were analyzed for total soluble polysaccharide and amylose. A procedure was devised for preparation of one solution, from which aliquots were taken for total soluble polysaccharide, amylose, and molecular weight determination. All determinations were carried out in duplicate.

An amylose sample containing approximately 9 grams of hot water–soluble amylose was dissolved by heating in 700 ml. of 3.0% sodium chloride solution. When solution appeared to be as complete as possible, the solution was centrifuged while hot to remove

Table I. Amylose Analysis of Hot Water–Soluble Polysaccharide Solutions Prepared from Dried Crystalline Amylose

	Colorimetric Method		Amperometric Method			
Variety	Klett reading[a]	Amylose, %	Wt. of sample, mg.	Av. ml. 0.005N KIO_3	Iodine bound, %	Amylose,[b] %
Caloro	129	82.2	5.18	1.22	15.0	82.9
Rexoro	146	93.0	5.18	1.33	16.3	90.1
Zenith	146	93.0	5.25	1.42	17.2	95.0
Century Patna	157	100.0	5.30	1.51	18.1	100.0

[a] For soln. containing 1.0 mg./100 ml.
[b] Based on 18.1% iodine bound by pure amylose.

Table II. Determination of Molecular Weights of Crystalline Amyloses

Variety	Periodate Oxidation	Viscometry
Century Patna	29,000	...
Zenith	41,000	...
Rexoro	41,000	...
Caloro	48,000	...
Caloro check sample	56,000	44,000

retrograded amylose. The solution was cooled to room temperature and diluted to 720 ml. (solution A). A 40-ml. aliquot was transferred to a 50-ml. volumetric flask, and made up to volume (solution B). From this solution, a 30-ml. aliquot was transferred to a 50-ml. volumetric flask containing 10 ml. of 2.5*N* potassium hydroxide. This solution (C) was diluted to volume and stored at 4° C. Total polysaccharide was determined on solution B and amylose (both amperometric and colorimetric determinations) was determined on solution C.

The four amyloses were analyzed for their hot water–soluble polysaccharide content by the method of Fuller, Lampitt, and Coton (*6*).

Preliminary experiments with the potentiometric determination of amylose by the method of Bates, French, and Rundle (*4*) revealed that the end point was not sharp and that the method was very time-consuming. For these reasons the amperometric method of Larson, Gilles, and Jenness (*13*) was used and an additional check was made by colorimetric analysis, using the method previously published by this laboratory (*19*). For comparison both sets of data are given in Table I. The calculation of purity was based on the Century Patna 231 sample, which bound 18.1% iodine by amperometric titration. This value agrees with the report of Rao, Murthy, and Subrahmanya (*18*) that pure amylose binds 18.2% iodine.

Periodate Oxidation. The periodate oxidation methods described by Potter and Hassid (*16*) and Potter *et al.* (*17*) were tested in preliminary experiments. When the latter method was used, the rice amylose solution precipitated before the oxidation was complete. The concentration of the sodium metaperiodate was then adjusted so that only 2 or 3 days' oxidation was required.

Both free and total formic acid were determined as described by Wolff *et al.* (*22*). Pfanstiehl maltose monohydrate was used as a standard. Other chemicals were reagent grade.

The 680 ml. of solution A remaining after removal of sample for amylose and total polysaccharide analysis was combined with 170 ml. of sodium metaperiodate solution (8.0 grams per 100 ml.) after both solutions had been cooled to 6° C. The solution was placed in a foil-covered bottle and stored at 6° C.; duplicate 50-ml. aliquots were taken for analysis at approximately half-day intervals. To each aliquot, 1 ml. of purified (distilled from potassium hydroxide) ethylene glycol was added to react with unused periodate. After standing 1 hour in the dark at room temperature, the samples were analyzed for free formic acid by titration with standardized sodium hydroxide to the methyl red end point. At this time additional standard sodium hydroxide was added and the solution allowed to stand for 0.5 hour at room temperature to hydrolyze the formyl ester. The solution was then back-titrated with standard sulfuric acid. The reaction was assumed to be complete when 3 moles of formic acid had been produced per mole of maltose.

Oxidation curves for the maltose standard and the Century Patna 231 amylose are given in Figure 2.

Viscometry. The intrinsic viscosity was determined with an Ostwald-Cannon-Fenske viscometer according to the method of Wolff, Gundrum, and Rist (*21*). Separate solutions in 1.0*N* potassium hydroxide were prepared.

Results and Discussion

Total Polysaccharide and Amylose Determinations. Preliminary analyses of the four dried amylose preparations for total hot water–soluble polysaccharide gave 91.4, 98.5, 86.1, and 86.5% for Zenith, Rexoro, Caloro, and Century Patna 231, respectively. The insoluble material was judged to be retrograded amylose. Sample sizes were then adjusted to give solutions of approximately the same concentration of soluble polysaccharide. Total polysaccharide was then redetermined on the final solutions, which were free of insoluble material. With the exception of the second study on Caloro amylose, both amperometric titration and colorimetric analyses were used in determining amylose on the hot water-soluble fraction. Table I shows that the purity of the amyloses increased in the order: Caloro, Rexoro, Zenith, and Century Patna 231. The impurity was treated as amylopectin, for reasons stated below. Because of the low purity of the Caloro amylose, a check sample was prepared which was recrystallized three times.

Regardless of whether amylose is determined by the amperometric, potentiometric, or colorimetric method, calculations are always based on a standard well-purified sample which is assumed to be free of amylopectin. The fact that considerable variation is reported in the iodine-binding capacity of rice amylose may be significant. For example, the Anderson group (*1*), MacMasters (*15*), and Rao and coworkers (*18*) report 19.2, 19.8, and 18.2%, respectively.

If the samples in question were pure, such differences could arise from variation in pretreatment, pH, temperature, iodine ion concentration, etc. (*7*). A more disturbing explanation would be that such differences reflect true varietal differences in iodine binding. Inoue and Onodera (*12*) have reported that the intensity of the iodine coloration of amylose and amylopectin differs for each variety of rice, and therefore, the iodometric determination of amylose in a specific starch should be accompanied by a determination of the intensity of coloration of amylose and amylopectin samples initially separated from this same variety of rice. Bates, French, and Rundle (*4*) also reported that the iodine-combining capacity of amylose varies with the length of the starch chain. If these reports are correct, a completely accurate amylose determination by either the amperometric or colorimetric method would necessitate preparation of separate amyloses, absolutely pure, for each variety. As the varietal differences would probably be no greater than 5% (this being the range of variation in iodine binding for the different cereal amyloses), such a refinement in method would be ill advised for most routine work. In the study reported here, the iodine-binding differences reported for the samples in Table I had to be interpreted as varietal differences or purity differences. The latter explanation was chosen somewhat arbitrarily and the Century Patna 231 sample taken as standard, as it is customary to base analyses on one crystalline standard, the variety being disregarded. Whether or not significant varietal differences exist for the common American varieties

needs to be conclusively established, and until the matter is settled, such a possibility must be kept in mind in interpreting studies of the type reported here. The molecular weight determinations are reported in Table II after correction for amylopectin impurity on the basis of the amylose analyses given in Table I. The impurity present in the recrystallized amyloses was assumed to be amylopectin, because it is the only other hot water-soluble polysaccharide that could be present.

Periodate Oxidations. The periodate oxidation values were corrected for formic acid produced from such amounts of amylopectin, using the data of Halsall, Hirst, and Jones (*9*) as a basis for the calculation. The use of their data gave the correction: 3.125×10^{-4} mole of formic acid per gram of amylopectin.

The molecular weights of the amyloses were calculated by the formula:

$$\text{Corrected molecular weight} = \frac{A}{B - (C \times D/100 \times E)} \times C/100 \times F$$

where

- A = millimoles of formic acid per mole of maltose at 53 hours—i.e., 3000
- B = millimoles of formic acid per sample of amylose at 53 hours
- C = weight of sample, grams
- D = percentage of amylopectin impurity
- E = millimoles of formic acid per gram of amylopectin—i.e., 0.3125
- F = percentage of amylose in recrystallized preparation

The calculations were based on the colorimetric analyses, as they were in good agreement with the amperometric data and no advantage was to be gained from averaging the two.

The range of values reported in Table II exceeds the 10% error inherent in the periodate oxidation method. Errors involved in the other analytical methods employed are much smaller. In view of the preceding discussion on possible varietal differences in iodine absorption, the data of Table II indicate, in the light of existing information, that there are varietal differences in the molecular weights of the crystalline amyloses studied here.

Caloro Check Sample. Because the Caloro amylose contained almost 20% impurity, the formic acid correction was large, and a second batch of Caloro amylose was prepared. This sample was carefully recrystallized three times and was approximately 100% pure. Molecular weight was determined by periodate oxidation exactly as described for the above samples and also by viscometry. From the intrinsic viscosity the molecular weight was calculated by the modified Staudinger equation, using the newly evaluated constants for amylose in potassium hydroxide solution reported by Everett and Foster (*5*). This gives a viscosity-average molecular weight which shows surprisingly good agreement with the first periodate oxidation value. As viscosity measurements give a type of molecular weight that is always nearer to a weight-average molecular weight than a number-average (as obtained by periodate oxidation), however, it is felt that 56,000 is more nearly correct than 44,000 for Caloro.

Relationship to Other Varietal Studies. Assuming for the present that these corrected values represent real varietal differences in amylose molecular weight, it has been of interest to relate these findings to studies on varietal differences in response to chemical treatment. Unfortunately, the scarcity of such studies makes possible only a few comparisons. A more serious difficulty arises from the compositional differences in the starch of these four varieties. Amylose analyses (*19*) of typical samples gave the following results for white milled rice: Rexoro, 23.6%; Zenith, 14.9%; Caloro, 14.3%; and Century Patna 231, 12.9%. These will vary slightly from sample to sample, but are representative. Because the amylose content of the Rexoro variety is so much higher than the other three, any difference in response of this variety to chemical treatment as opposed to the other varieties is probably due to its starch composition rather than to molecular weight differences in the amylose, if all factors except starch could be safely excluded.

With this point in mind, only Caloro, Zenith, and Century Patna 231 will be compared. Batcher, Helmintoller, and Dawson (*3*) made quantitative measurements of some physical and chemical characteristics of different varieties of rice and found that the amount of estimated starch in the residual cooking liquid increased in the order: Century Patna 231 < Zenith < Caloro. Batcher, Deary, and Dawson (*2*) conducted further studies on 26 varieties of milled white rice and obtained similar results for these three varieties. These workers also measured the water uptake ratio (the weight of the cooked rice divided by the weight of the raw rice) of different varieties and reported the order Caloro < Zenith < Century Patna 231. These differences are fairly large and may be a reflection of amylose molecular weight differences. The cohesiveness scores reported for cooked rice by Batcher, Deary, and Dawson place the varieties in the following order of increasing cohesiveness: Caloro < Zenith < Century Patna 231. The same order is also observed in the alkali test as reported by Little, Hilder, and Dawson (*14*). When scored for clearing and spreading in 1.7% potassium hydroxide, the varieties rank as follows: Caloro > Zenith > Century Patna 231.

Recently Hogan and Planck (*11*) studied the water absorption and amount of undissolved solids and dissolved material of cooked rice. Their study differed from those of Dawson in technique and in that the mechanical loss of rice due to cooking was taken into consideration. The percentage absorption was calculated as the weight of moisture in cooked rice divided by the weight for dry material in cooked rice. The varietal differences in the water absorption were reported in the order of Caloro > Zenith > Century Patna 231 when measured at 70° C. At 100°, results showed Caloro > Century Patna 231 (no data for Zenith). The water absorption of rices below their gelatinization temperatures has been related to the gelatinization characteristics of the rice by Halick and Kelly (*8*). The nature of the compositional factors which control the gelatinization temperature of a variety has never been delineated. Halick and Kelly have shown that it is unrelated to amylose content. Their data suggest that it is also unrelated to amylose molecular weight: Caloro, 67.5°; Zenith, 65.0°; Century Patna 231, 79.5°; and Rexoro, 73.5° C. Their water uptake numbers as measured above gelatinization temperatures, however, give the same order as the other studies cited: Caloro > Zenith > Century Patna. The signs are reversed from those in Dawson's study because of the method of calculation of values and possibly because of technique.

If the varietal differences reported above result from the behavior of the rice starch and not other components, it is possible that they are a reflection of amylose molecular weight differences, or distribution, as Caloro, Zenith, and Century Patna 231 varieties have approximately the same proportions of amylose to amylopectin. Of course, if differences exist in the molecular weights of the amylopectins, such speculation is futile. This is obviously a fertile area for continued research.

Acknowledgment

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STARCH SOURCES

Barley Flour Composition and Use for Starch Production

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Barley is a very inexpensive source of carbohydrate in certain areas in the West and thus a potential source of starch. The alkali process can be used on barley flour to produce a starch of good quality. Barley protein is readily dispersed in alkali and can be recovered in yields of 77 to 86% after removal of the starch by adjustment of the pH. Of the six varieties tested, Compana appears most promising as a starch source. Analysis indicated that the amino acids in two varieties of barley protein were different. The behavior on dispersion in alkali also indicated differences in the nature of the protein. The existence of a new "pectinlike" polysaccharide in barley flour is reported.

BARLEY is rapidly becoming to the Intermountain area what corn is to the Corn Belt—the cheapest source of carbohydrate. This immediately suggests the possibility of using this cereal for starch production.

Very little information has been published on the production of starch from barley. The wet milling technique was found unsatisfactory (*15*). The other logical method is the adaptation of the alkali process developed by Dimler *et al.* (*9*) for use with wheat and tried by him on one variety of barley. This process requires flour as the starting material. The present study was initiated to examine the composition of barley flour and to see if starch could be satisfactorily recovered from it by the alkali process.

Materials and Methods

Six varieties of barley with a known history, which were well adapted to Montana, were used for this study. Flours were prepared in an experimental Buhler flour mill equipped with 10 XX bolting silk. In one case the barley was pearled before milling.

Protein in the various products was determined by a modified Kjeldahl method (*2*), using 6.25 as the conversion factor to convert per cent nitrogen to protein. Starch was determined polarimetrically by the procedure of Clendenning (*6, 7*). However, on the "tailings fraction" it was necessary to use a modified procedure (*10*). Crude fiber and ash were determined by the usual method (*3*). To determine moisture, the samples were dried 16 hours at 110° C. under a vacuum of 28 inches of mercury. Fat in the flour samples was determined by ether extraction (*4*). Fat in the purified starches was determined by acid hydrolysis and extraction from the hydrolyzate, as suggested by Taylor and Nelson (*18*). Magnesium, potassium, and sodium were determined by a modification of the method of Pro and Mathers (*17*). Calcium was determined by the flame photometric procedure suggested by Cooley (*8*), and silica was determined photometrically by the procedure of Kerr and Trubell (*11*). Phosphorus was determined photometrically by the procedure of Allen (*1*). The amino acids were determined by the procedure described by Moore and Stein (*16*). The pentosans were measured by the AOAC procedure (*5*), the distillate being redistilled and the furfural precipitated with thiobarbituric acid. The "pectin" was determined by the evolution of carbon dioxide using the method of McCready, Swenson, and Maclay (*14*) on material isolated with an ammonium oxalate extraction and purified by the procedure described by Kertesz (*12*). The fermentable sugars were determined by suspending 10 grams of flour, which had been autoclaved in a dry state to inactivate the β-amylase, in 100 ml. of water containing 5 ml. of an active distiller's yeast. The loss in weight after 24 hours was used to calculate sugars after making corrections for the fermentable material present in the inoculum.

The alkali process (*9*) developed for the production of starch from wheat was used in this study. This consists of dispersion of the flour protein in a dilute aqueous alkaline solution and removal

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Table I. Analyses of Flour Sample

(All data on moisture-free basis)

Strain of Barley	Lb./Bu.	Milling Yield, %	Starch Content, %	Protein Content, %	Ash, %	Crude Fiber, %	Fat, %	Fermentable Sugar, %	Pectinlike Polymer, %	Pentosan, %
Betzes	50.6	45	73	11.5	1.57	1.25	1.2	3.3	1.41	1.14
Carlsberg	47.3	51	67	11.0	1.98	1.92	0.9	3.8	1.48	1.57
Compana	51.4	58	80	8.4	1.23	1.03	1.2	2.5	1.49	1.06
Pearled Compana[a]	...	57	79	9.9	...	...	...	...	...	...
Hannchen	50.5	66	74	12.3	1.54	1.51	2.2	3.1	2.04	1.16
Vantage	48.8	43	79	12.9	1.27	1.23	1.5	2.8	...	1.11
Ymer	48.6	48	74	11.3	1.43	1.54	1.0	3.2	1.42	1.07

[a] A different sample of Compana. Milling yield was based on whole barley before pearling.

Table II. pH Determination for Maximum Protein Extraction

	Normality													
	0.015		0.020		0.025		0.030		0.035		0.040		0.045	
Strain of Barley	pH	% ext.	pH	% ext.	pH	% ext.	pH	% ext.	pH	% ext.	pH	% ext.	pH	% ext.
Betzes	10.0	93.3	10.5	94.5	10.8	96.6	11.0	99.1	11.1	100.2	11.2	100.0	11.3	100.2
Carlsberg	9.8	83.8	10.4	90.4	10.7	96.0	10.9	95.3	11.1	95.6	11.3	95.6	11.4	95.6
Compana	10.2	92.6	10.6	97.8	10.8	98.3	11.0	100.1	11.1	100.1	11.2	100.3	11.3	100.1
Pearled Compana	10.1	93.2	10.6	100.0	10.9	100.0	11.1	100.0	11.2	100.0	11.3	100.0	11.4	100.0
Hannchen	9.6	76.0	10.3	90.7	10.6	94.6	10.8	94.9	11.1	95.7	11.3	96.9	11.4	97.2
Vantage	10.1	86.6	10.6	91.4	11.0	96.3	11.1	98.9	11.2	99.6	11.3	100.0	11.3	100.0
Ymer	9.8	89.5	10.3	92.4	10.6	96.6	10.8	100.3	11.1	100.0	11.2	99.6	11.3	100.0

of the starch by centrifugation. In our operations a physical separation was made by scraping off the loose top layer of the centrifuge cake. This was referred to as the "tailings" fraction. The alkaline protein solution was then acidified and the precipitated protein was removed by centrifugation. Although many bases and acids can be used, sodium hydroxide and sulfuric acid were used in this study.

Table III. pH Determination for Maximum Protein Precipitation

(Protein calculated as N × 6.25)

	Per Cent Protein Precipitated at pH						
Strain of Barley	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Betzes	77.4	80.0	82.5	80.2	69.6	44.4	38.0
Carlsberg	74.1	80.9	80.3	77.5	53.8	38.9	36.2
Compana	65.5	75.8	80.9	80.2	73.9	35.2	31.4
Pearled Compana	63.3	79.7	83.3	83.4	74.3	47.6	42.4
Hannchen	71.6	76.2	81.6	76.7	43.4	35.0	31.5
Vantage	79.3	84.4	85.7	83.9	72.3	38.3	14.8
Ymer	75.2	81.0	83.3	80.6	71.0	45.1	42.5

Results and Discussion

Effect of Variety on Yield and Composition of Flour. There were considerable differences in the ease with which the various barleys were milled. When Ymer was milled, the flour had a tendency to ball up. Carlsberg, on the other hand, milled readily, but a much greater percentage of hull was retained in the flour. In general, the large heavy kernels milled more readily and gave higher flour yields. Tempered barley had a tendency to ball up. For this reason, barley was milled just as it came from storage with an average moisture content of 6 to 9%. Pearling before milling did not improve flour yields. The flour yields and composition for six varieties are given in Table I.

The galacturonic acid polymer, which for want of a better name was called pectin, was isolated from the flour by first extracting with 0.5% ammonium oxalate at 75°C. This solution was centrifuged to remove starch and protein, cooled, neutralized, and then made up to 0.05*N* with hydrochloric acid. Two volumes of cold 95% ethanol were then added and the "pectin" was removed by filtration. All sugars are eliminated by this treatment, as no trace of free sugar could be detected with paper chromatography. Although this residue gave a sky-blue color with iodine, treatment with crystalline α- and β-amylase produced no detectable amount of maltose.

It gave the characteristic color reactions (*13*) with hydroxylamine and iodine and formed good films. On treatment with pectinase this compound decomposed, yielding galacturonic acid, but the rate of reaction was somewhat slower than that of commercial pectin used as a control. Furthermore, this pectin failed to form good gels with sugar. A chromatograph of the acid hydrolyzate indicated the presence of galacturonic acid, a small amount of glucose, and traces of arabinose but no xylose or galactose. This suggests that it is definitely not a hemicelluose and not a true pectin. The structure of this material is now being determined in this laboratory. From Table I it is observed that from 90 to 99.8% of the total constituents of the flour have been accounted for. Some of these flours gave a positive test for fructosans. Fructosans and glucosans might account for the balance of this material.

Dispersing Action of Alkali on Barley Protein. The solubility of barley proteins in alkali was strikingly different, as shown in Table II.

Only 95.6 and 97.2% of the protein was extracted from Carlsberg and Hannchen, respectively, in a 0.045*N* solution, whereas the protein in the other varieties was completely extracted at this alkalinity. This was not due to the buffering capacity of the flour, as indicated by the pH of these solutions. The fact that the proteins from different varieties of barley show different solubilities at the same pH would indicate distinct differences in their composition.

Effect of pH on Protein Recovery. The dispersed barley proteins were readily recovered on acidification (Table III).

In all cases the optimum pH for protein precipitation was approximately 5. The fact that protein recoveries varied with the variety again suggested that they were somewhat different. This would account for the variations

Table IV. Amino Acid Analysis of Barley Protein

(Values expressed as per cent of total protein)

Amino Acid	Barley Variety	
	Compana	Vantage
Alanine	1.8	3.4
Arginine	3.1	2.6
Aspartic acid	1.4	2.5
Glutamic acid	31.0	31.8
Glycine	1.3	2.0
Histidine	1.8	1.2
Isoleucine	2.9	2.9
Leucine	7.7	5.8
Lysine	3.0	2.2
Methionine	1.0	0.4
Phenylalanine	4.3	4.5
Proline	16.1	7.9
Serine	1.7	3.1
Threonine	1.4	2.1
Tyrosine	2.7	2.5
Valine	5.4	5.4

Cysteine, cystine, hydroxyproline, and tryptophan were not run by this procedure.

Table V. Yield of Starch and Protein from Various Varieties

(All data calculated on flour, moisture-free basis)

Strain of Barley	Crude Starch			Tailings Starch			Precipitated Protein		
	Yield, g./100 g.	Starch content, %	Protein content, %	Yield, g./100 g.	Starch content, %	Protein content, %	Yield, g./100 g.	Protein content, %	Protein recovery, %
Betzes	51	98.5	0.3	24	68	1.1	13.2	72.1	82.8
Carlsberg	47	99.7	0.2	24	80	1.2	11.7	72.7	77.4
Compana	63	99.7	0.1	16	57	1.7	11.6	58.9	81.3
Pearled Compana	60	99.7	0.2	20	69	1.4	11.5	71.9	83.5
Hannchen	56	97.3	0.3	18	77	1.3	14.1	68.9	79.0
Vantage	59	98.5	0.2	16	77	1.3	16.6	67.0	86.2
Ymer	43	99.7	0.2	30	82	1.1	16.6	56.8	83.4

observed in feeding different barleys to livestock. Because the greatest discrepancy in protein recovery, especially at the lower or higher pH values, was between Compana and Vantage, it was considered desirable to run an amino acid assay on these varieties. These data, presented in Table IV, verified the assumption that there were distinct differences in the composition of barley proteins.

Effect of Variety on Yield of Starch and Protein. The yield of starch and protein from six varieties is reported in Table V.

Compana appeared to be the most promising of the varieties tested. Starches of satisfactory purity were obtained by the use of this method. The "tailings fraction" probably could be reduced in amount, if suitable equipment were available. Good yields of high protein residue were obtained by this method.

Noncarbohydrate Constituents of Barley Starch Other Than Proteins. The separated starches were resuspended several times in distilled water, dried, and analyzed for inorganic constituents and "bound" fat. Analyses for P_2O_5, SiO_2, CaO, MgO, K_2O, Na_2O, and fat gave average values of 0.296, 0.013, 0.014, 0.023, 0.059, 0.123, and 0.78%, respectively. Hannchen and Vantage appear to be slightly different from the other varieties, as they contain approximately 25% more phosphorus and 30% more magnesium. In general, the inorganic constituents of barley seem to conform to the data for other common starches.

It would appear that starch of satisfactory quality can be prepared from barleys by the alkali method. Because Compana, the largest seeded variety tested, gave the most satisfactory results, it would appear that kernel size is very important for starch production. With this in mind a program is under way at Montana State College to develop a giant seeded variety of Compana for industrial use.

The physical properties of the barley starch from the various varieties were not determined because of lack of proper equipment. This work will be completed and reported on later.

Acknowledgment

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Relation of Interspace Relative Humidity to Growth of Molds and Heating in Feed Ingredients and Feed Mixtures

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The relation of interparticle relative humidity to the growth of molds and/or spontaneous heating was determined in various feed ingredients and mixed feeds stored at 10°, 21°, and 32° C. Increasing moisture or increasing temperature increased the relative humidity, but when the relative humidity was measured at the same temperature as the storage temperature, no feed ingredient or mixed feed became moldy or heated during 6-week storage at relative humidity of 72.0% or less. Safe relative humidity was less variable than safe moisture content; therefore, humidity can be used to determine safe storage conditions for feed ingredients and mixtures.

Most investigators agree that growth of molds is primarily responsible for the heating and accompanying deterioration in agricultural products during storage. Several recent reviews (*1,4,5,9,10*) summarize the literature in this field. Reports cited in these reviews show that the amount of available moisture or the relative humidity of the interparticle air is the most important condition that determines the growth of molds on stored grains. Comparatively few studies have been made to determine the conditions for the safe storage of mixed feeds or the ingredients used in mixed feeds. Snow, Crichton, and Wright (*11,12*) determined the growth of molds and the water uptake of oats, linseed cake, bone meal, and bran at different humidities. At the same relative humidity, wide variations existed in the moisture content of the different ingredients. Waite (*13*) showed that molds developed in 19 days on hay with 15.7% and in 280 days with 12.9% moisture. The critical moisture level of 32 feed ingredients was reported from this laboratory (*6,7*). The ingredients tested included grain by-products, protein concentrates, dehydrated alfalfa meals, bone meal, and distiller's dried grains. Generally the critical moisture level is referred to as the moisture content that is in equilibrium with a relative humidity of approximately 75% and for most cereal grains a moisture content of 13 to 15% is in equilibrium with this relative humidity. Similar studies on the nature of heating in simple and complex feed mixtures (*14*) showed that the moisture content of a mixture did not reliably indicate whether the feed was safe or unsafe from heating. Heating in a feed mixture depended on the moisture content and the concentration of each ingredient in the mixture.

In view of previous reports that the relative humidity of the interparticle air largely determines whether molds grow on stored grains, relative humidity was investigated as a measure of the moisture conditions for safe storage of mixed feeds and feed ingredients at 10°, 21°, and 32° C.

Materials and Methods

All feed ingredients were purchased locally and the moisture contents were below the critical level, so that no mold growth or heating occurred in the ingredients before they were used in the tests. In each test the ingredient was adjusted to a given moisture content by the addition of calculated amounts of water. The moist samples were thoroughly mixed and then allowed to equilibrate in a cold room at 7° C. for approximately 48 hours.

Equilibrium relative humidities of the feeds were measured with an electric hygrometer indicator and single humidity sensing elements. This equipment is essentially the same as that used previously for determining the relative humidity of corn (*3*) and the absorption and desorption isotherms of wheat (*8*).

Briefly, the following procedure was used in this laboratory. Approximately 150 grams of feed or ingredients were placed in a 250-ml. extraction flask immersed to the lip in a constant temperature water bath. A single narrow range humidity sensing element was inserted through the rubber stopper in the extraction flask to the surface of the feed. The sensing element was selected to cover the expected range of humidity as indicated from the moisture content. A constant hygrometer reading was obtained in approximately 2 to 5 hours. The exact time depended on the material and the temperature. In order to run relative humidities on several feeds in one day with only one hygrometer indicator, it was necessary to use 10 to 12 sensing elements and to allow the temperature of the feeds in the flasks to equilibrate with that of the water bath before the readings were started. In most of this work the flasks were allowed to stand overnight in the constant temperature water bath before any readings were made. Regardless of the preliminary treatment, the hygrometer reading which remained constant for three successive 15-minute intervals was used to obtain the relative humidity from the calibration charts supplied by the manufacturer. According to the manufacturer, relative humidity values obtained by this instrument are accurate to ±1.5%. A schematic diagram of the apparatus is shown in Figure 1.

Relative humidity was determined on each lot of feed or feed ingredient at 10°, 21°, and 32° C. (approximately 50°, 70°, and 90° F.). Heating properties of the same feeds were determined by the procedure described by Halick and Richardson (*6*). In these tests the inside of the heating apparatus was maintained at 32° C. and 70.0% relative humidity. Growth of molds in the samples stored at 10° and 21° C. was determined by visual inspection after a storage of 6 weeks. Moisture content was determined in triplicate by the official vacuum oven procedure for grain and stock feeds (*2*).

Results and Discussion

Storage Temperature and Interspace Relative Humidities of Feed Ingredients. The relationship of moisture content and interspace relative humidities of seven feed ingredients at three storage temperatures are summarized in Table I and Figure 2.

The data for milo gluten meal in Table I show that relative humidity is affected by both storage temperature and moisture content of the feed. When the temperature was constant, the humidity increased with increase in moisture

Table I. Relative Humidities of Milo Gluten Meal Containing Different Amounts of Moisture at 10°, 21°, and 32° C.

Moisture, %	Relative Humidity, %		
	10° C.	21° C.	32° C.
9.9	51.4	58.9	66.1
11.0	56.7	61.0	70.4
11.5	57.5	63.7	71.5
12.0	61.4	65.8	73.5
12.5	63.8	68.9	75.8
13.2	64.7	69.8	78.7
13.5	66.3	71.9	79.9
14.5	69.2	74.9	81.5
14.9	71.3	75.1	82.6
16.1	73.0	77.8	83.4
16.8	74.5	79.6	84.5
18.3	74.8	80.0	86.4

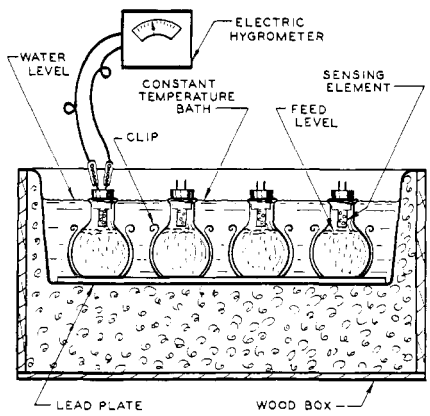

Figure 1. Apparatus for determining relative humidity of feed samples

and when the moisture was constant, it increased with increase in temperature. Similar results were obtained with other feed ingredients. Relative humidities of ground corn, ground milo, steamed bone meal, soybean oil meal, dehydrated alfalfa leaf meal, and cottonseed meal containing different amounts of moisture were determined at 10°, 21°, and 32° C. for each ingredient at moisture contents corresponding to humidities ranging from 45 to 95%. Sections of curves illustrating the moisture and relative humidity relationship are shown in Figure 2 for relative humidities ranging from 60 to 85%. This humidity is the range primarily involved in safe and unsafe storage conditions for feeds.

These curves show that the relative humidity at a given per cent moisture varies widely with different ingredients. The change in humidity per unit change in moisture was largest in steamed bone meal and least in dehydrated alfalfa leaf meal.

Relation among Moisture Content, Relative Humidity, and Growth of Molds and Heating in Feed Ingredients Stored at Various Temperatures. The relative humidity and moisture content of seven feed ingredients which were safe and unsafe from the growth of molds and/or heating when stored at 10°, 21°, and 32° C. (summarized in Table II) illustrate the wide variation between safe and unsafe moisture contents for different ingredients. Likewise, safe and unsafe moisture values differed widely for the same ingredient stored at different temperatures. On the other hand, differences between safe and unsafe humidity values were small regardless of the kind of feed or storage temperature, if the temperature for measuring humidity and storage were the same. No ingredient or mixed feed investigated so far has become moldy or heated in 6 weeks when the storage humidity was below 72.0%. In general, growth of molds or heating did not occur until the humidity was above 73.0%. On the basis of these data, the critical relative humidity for feed in storage for 6 weeks has been taken as approximately 72.0%. This critical relative humidity is shown graphically in Figure 2. This critical humidity may not represent the absolute minimum relative humidity at which some species of molds germinate and grow when a feed is stored for longer periods (*11,13*). In order to use humidity values to predict whether an ingredient or mixed feed will be safe during storage, it is necessary to determine the relative humidity at the maximum temperature which the feed is likely to encounter during storage.

Table II. Safe and Unsafe Relative Humidities and Moisture Contents of Feed Ingredients Stored at 10°, 21°, and 32° C.

Ingredient	Mold Growth or Heating[a]	10° C.		21° C.		32° C.	
		Moist., %	RH, %	Moist., %	RH, %	Moist., %	RH, %
Ground corn	−	16.2	67.7	15.0	71.0	13.7	71.6
	+	17.0	72.5	15.4	73.7	14.1	74.1
Ground milo	−	17.3	73.1	15.3	71.3	14.1	72.5
	+	17.9	75.0	15.7	73.8	14.3	73.6
Soybean oil meal	−	18.6	70.3	15.3	70.8	14.0	73.1
	+	20.6	72.5	16.8	73.0	14.4	74.2
Cottonseed meal	−	16.0	71.3	14.2	70.9	12.2	70.9
	+	17.6	73.0	15.1	72.9	12.5	73.2
Milo gluten meal	−	16.1	73.0	13.5	71.9	11.5	71.5
	+	16.8	74.5	14.5	74.9	12.0	73.5
Dehydrated alfalfa leaf meal	−	18.5	68.9	15.9	71.5	13.0	71.1
	+	21.3	73.7	17.0	72.8	14.6	74.3
Steamed bone meal	−	11.2	71.5	8.4	68.4	7.7	70.0
	+	12.6	77.3	9.4	72.6	8.1	75.3

[a] − No mold growth and/or heating. + Mold growth and/or heating.

Moisture Content of Ingredients with 72.0% Relative Humidity at Different Temperatures. The moisture content of each of seven feed ingredients with a relative humidity of 72.0% is given in Table III for 10°, 21°, and 32° C. These values were obtained from the data given in Table I and those used to plot the curves in Figure 2. As no feed with a relative humidity below 72.0% became moldy or heated, the moisture values given in Table III would correspond to critical moisture values for the ingredient at each temperature. It would be possible also by using the graphs in Figure 2 to obtain the approximate critical moisture value for an ingredient for any temperature between 10° and 32° C.

Table III. Moisture Content of Feed Ingredients with a Relative Humidity of 72.0% at 10°, 21°, and 32° C.

Ingredient	Per Cent Moisture at 72.0% RH		
	10° C.	21° C.	32° C.
Ground corn	16.9	15.2	13.8
Ground milo	17.0	15.4	13.8
Soybean oil meal	20.3	15.9	13.6
Cottonseed meal	16.3	14.6	12.4
Milo gluten meal	15.8	13.6	11.6
Dehydrated alfalfa leaf meal	20.7	16.4	13.9
Steamed bone meal	11.0	8.9	7.8

Relative Humidity and Rate of Heating. The time required for ground corn, cottonseed meal, and steamed bone meal with relative humidities above the critical level (72.0%) to heat when stored at 32° C. is summarized in Table IV. In general, heating started within two periods. Feeds with humidities ranging from 73 to 77% usually started to heat in about 3 weeks; those with humidities ranging from 77 to 85%, in less than 2 weeks. These data show that storage periods of at least 6 weeks should be used when the moisture and relative humidity conditions are determined for safe storage of feeds.

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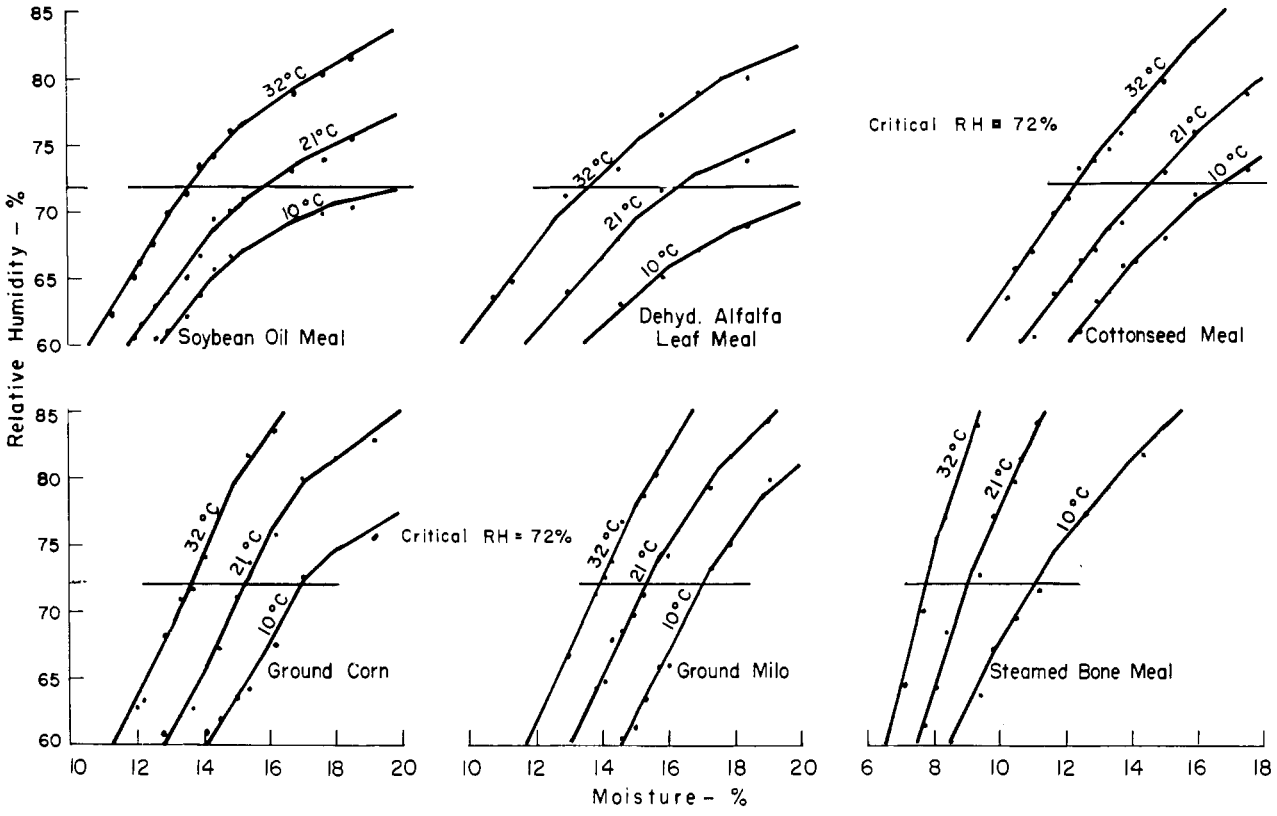

Figure 2. Relation of relative humidity to moisture content of feed ingredients at 10°, 21°, and 32°C.

Table IV. Time Required for Ground Corn, Cottonseed Meal, and Steamed Bone Meal with Different Relative Humidities to Heat When Stored at 32° C.

Ground Corn		Cottonseed Meal		Steamed Bone Meal	
RH, %	Heated, days	RH, %	Heated, Days	RH, %	Heated, days
72.0	..	72.0	..	72.0	..
74.1	38	73.2	35	75.3	36
77.2	23	74.5	20	77.0	23
81.6	9	77.4	10	83.8	4
86.6	5	86.0	4	86.3	5

Table V. Critical Moisture Level of Some Feed Ingredients

Ingredient	Critical Moisture Level, %	
	Reported previously[a]	From graph at 72% RH
Corn, ground	13.0	13.8
Milo, ground	13.0	13.8
Soybean oil meal	13.1–13.8	13.6
Cottonseed meal	11.5–12.8	12.4
Dehydrated alfalfa leaf meal	14.9	13.9
Steamed bone meal	8.7	7.8

[a] See Halick *et al.* (*6*, *7*).

Table VI. Relation of Relative Humidity and Moisture Content to Heating in Feed Mixtures Stored at 32° and 70% Relative Humidity

Mixture No.	Added to Basal Mixture[a]		Mixture		Heated, Days
	Supplement	%	Moisture, %	RH, %	
1	None	...	13.0	70.8	..
		...	13.4	73.2	31
		...	12.0	69.4	..
2	Corn fermentation[b] solubles	2.5	12.9	72.9	36
		5.0	13.0	72.9	29
		5.0	13.1	73.2	..
		5.0	13.7	76.2	21
3	Fish solubles[c]	2.5	13.1	74.2	24
		5.0	13.5	75.3	18
4	None	...	13.1	71.3	..
	Fat[d]	5.0	12.4	70.9	..
		10.0	11.7	71.4	..
5	None	...	13.5	74.1	34
	Fat	5.0	12.8	73.6	33
		10.0	11.9	74.2	30

[a] Basal mixture. Ground corn 69, soybean oil meal 25, steamed bone meal 3.0, dehydrated alfalfa leaf meal 2.5, salt 0.5%.
[b] Condensed corn fermentation solubles contained 45% moisture.
[c] Fish solubles contained 53% moisture.
[d] Animal feeding fat.

grade, the maximum moisture content of the whole grain must not be over 15.5%. If this corn is ground, then stored or mixed in feeds in normal amounts, the ground corn or mixed feed is susceptible to the growth of molds and heating. A maximum of 15.5% moisture for corn over a storage period of 6 weeks may be satisfactory for some areas, but ground corn or a feed containing ground corn with this amount of water is not safe in the Gulf Coast area during the warm season.

Critical Moisture Values of Feed Ingredients. The critical moisture content of 32 feed ingredients and the procedure used to determine the critical level have been reported (*6*,*7*). In general, these values represented a moisture content approximately 0.3% below the maximum moisture content tested which did not heat in 6 weeks. The values reported previously and those obtained from the graph at 72.0% relative humidity are given in Table V. The values by both procedures were

obtained at a storage temperature of 32° C.

In general, the moisture content of ingredients with a relative humidity of 72.0% was slightly higher than that reported previously. A maximum difference in moisture content obtained by the two methods was 1.0% each for dehydrated alfalfa leaf and steamed bone meal. From a practical viewpoint, less time is required to obtain the critical moisture level from the relative humidity measurement than by observing the time required for a feed to heat in the heating apparatus. The former procedure required only a few hours; the latter, at least 6 weeks.

Relation of Humidity, Moisture Content, and Spontaneous Heating in Complex Feed Mixtures. It is practically impossible to apply critical moisture levels in predicting moisture conditions that will be safe or unsafe for storage of feed mixtures. Before moisture contents can be used in judging whether a feed mixture will be safe from heating, it is necessary to know the composition, concentration, and critical moisture level of each principal ingredient. On the other hand, the data in Table VI show that all mixtures were safe for a 6-week storage period when the relative humidity was 72.0% or less. The unreliable nature of moisture contents for determining safe storage conditions is illustrated by two mixtures with and without animal feeding fat. In this instance, heating depended upon moisture content of the hygroscopic components of the mixture. When a nonhygroscopic ingredient was added, the total moisture was less but this did not change the ability of the feed mixture to support the growth of molds.

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FOOD ADDITIVES

Preservative Effect of Sorbic Acid on Creamed Cottage Cheese

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Commercially made creamed cottage cheese is a microbiologically unstable product because bacteria, yeast, and mold contaminants cause flavor changes. This deterioration is retarded, but not halted by low temperature storage, or by rigid sanitation. The incorporation of sorbic acid into this product arrests the growth of most contaminants, but not the group of microorganisms associated with flavor formation in dairy products. The efficacy of sorbic acid depended on its concentration, the storage temperature, and the intensity of the initial contamination. Levels of 0.05 and 0.07% sorbic acid in creamed cottage cheese of pH 4.9 increased microbiological and flavor stability.

Many factors affect spoilage in creamed cottage cheese. As a result, opinion varies among investigators in the field as to the type of microflora prevailing. Actually, 17 different organisms capable of causing spoilage were isolated and identified by Bonner and Harmon (*1*). They grew these isolates in laboratory media at three concentrations of sorbic acid and reported that concentrations up to 0.25% were not effective in completely destroying the organisms when the pH was not reduced. They also employed a 0.1% aqueous solution of sorbic acid for curd-washing and reported an adverse effect upon flavor. Geminder (*2*) showed that 0.075% potassium sorbate or sorbic acid in finished cottage cheese retarded the growth of yeasts, molds, and slime-forming bacteria. Stull (*5*) reported that sorbic acid at a level of 0.05% inhibited the growth of psychrophilic (cold-loving) organisms, thereby extending the shelf life of cottage cheese.

Materials and Methods

Portions of curd and cream dressing were taken from regular production in a large, independent dairy plant. The curd was made using the "short-set" method which entails culturing skim milk (of 10% total solids content) at 90° F. for 4 to 4.5 hours. The curd was cut and then cooked until a desirable consistency was attained. After draining, the curd was washed three times using water containing 5 p.p.m. of chlorine, the last wash consisting of 35° F. water. To this curd, salted cream was added as a dressing.

In these studies, sorbic acid (in amounts required to give levels of 0.05 and 0.07% in the finished product) was added to the pasteurized and cooled cream dressing (80° F.) and the treated cream was held at 40° to 45° F. until used.

obtained at a storage temperature of 32° C.

In general, the moisture content of ingredients with a relative humidity of 72.0% was slightly higher than that reported previously. A maximum difference in moisture content obtained by the two methods was 1.0% each for dehydrated alfalfa leaf and steamed bone meal. From a practical viewpoint, less time is required to obtain the critical moisture level from the relative humidity measurement than by observing the time required for a feed to heat in the heating apparatus. The former procedure required only a few hours; the latter, at least 6 weeks.

Relation of Humidity, Moisture Content, and Spontaneous Heating in Complex Feed Mixtures. It is practically impossible to apply critical moisture levels in predicting moisture conditions that will be safe or unsafe for storage of feed mixtures. Before moisture contents can be used in judging whether a feed mixture will be safe from heating, it is necessary to know the composition, concentration, and critical moisture level of each principal ingredient. On the other hand, the data in Table VI show that all mixtures were safe for a 6-week storage period when the relative humidity was 72.0% or less. The unreliable nature of moisture contents for determining safe storage conditions is illustrated by two mixtures with and without animal feeding fat. In this instance, heating depended upon moisture content of the hygroscopic components of the mixture. When a nonhygroscopic ingredient was added, the total moisture was less but this did not change the ability of the feed mixture to support the growth of molds.

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FOOD ADDITIVES

Preservative Effect of Sorbic Acid on Creamed Cottage Cheese

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Commercially made creamed cottage cheese is a microbiologically unstable product because bacteria, yeast, and mold contaminants cause flavor changes. This deterioration is retarded, but not halted by low temperature storage, or by rigid sanitation. The incorporation of sorbic acid into this product arrests the growth of most contaminants, but not the group of microorganisms associated with flavor formation in dairy products. The efficacy of sorbic acid depended on its concentration, the storage temperature, and the intensity of the initial contamination. Levels of 0.05 and 0.07% sorbic acid in creamed cottage cheese of pH 4.9 increased microbiological and flavor stability.

Many factors affect spoilage in creamed cottage cheese. As a result, opinion varies among investigators in the field as to the type of microflora prevailing. Actually, 17 different organisms capable of causing spoilage were isolated and identified by Bonner and Harmon (*1*). They grew these isolates in laboratory media at three concentrations of sorbic acid and reported that concentrations up to 0.25% were not effective in completely destroying the organisms when the pH was not reduced. They also employed a 0.1% aqueous solution of sorbic acid for curd-washing and reported an adverse effect upon flavor. Geminder (*2*) showed that 0.075% potassium sorbate or sorbic acid in finished cottage cheese retarded the growth of yeasts, molds, and slime-forming bacteria. Stull (*5*) reported that sorbic acid at a level of 0.05% inhibited the growth of psychrophilic (cold-loving) organisms, thereby extending the shelf life of cottage cheese.

Materials and Methods

Portions of curd and cream dressing were taken from regular production in a large, independent dairy plant. The curd was made using the "short-set" method which entails culturing skim milk (of 10% total solids content) at 90° F. for 4 to 4.5 hours. The curd was cut and then cooked until a desirable consistency was attained. After draining, the curd was washed three times using water containing 5 p.p.m. of chlorine, the last wash consisting of 35° F. water. To this curd, salted cream was added as a dressing.

In these studies, sorbic acid (in amounts required to give levels of 0.05 and 0.07% in the finished product) was added to the pasteurized and cooled cream dressing (80° F.) and the treated cream was held at 40° to 45° F. until used.

Table I. Effect of Sorbic Acid on Flavor and pH of Creamed Cottage Cheese

Sorbic Acid	Days at 50° F.			Days at 45° F.			Days at 35° F.	
	1	6	14	8	15	25	30	45
None								
Flavor	Good	Fair	Poor	Fairly good	Fair	[a]	Fair	Poor
pH	4.9	4.75	4.4	4.8	4.7	4.7	4.9	4.9
0.05%								
Flavor	Good	Good	Fairly good	Good	Good	Fairly good[b]	Good	Good
pH	4.9	4.85	4.9	4.8	4.9	4.9	4.9	4.9
0.07%								
Flavor	Good	Good	Fairly good	Good	Good	Good	Good	Good[c]
pH	4.9	4.9	4.85	4.9	4.85	4.9	4.9	4.9

[a] Visible growth, not submitted to panel.
[b] Off-odor noticed after 36 days.
[c] After 120 days judged as palatable.

The solubility of the sorbic acid in cream was enhanced by the 16% butterfat content. In actual plant practice this holding or "aging" period may extend from 1 to 3 days. During this time, psychrophilic bacteria may grow in untreated cream and become a source of contamination in the finished product.

After mixing the curd with cream at a 3 to 1 ratio, the creamed cottage cheese was packaged in waxed, 12-ounce cups, capped with plastic lids using a commercial filling and packing machine. Except for the presence of sorbic acid, all standards of identity for creamed cottage cheese were met. Fourteen cups of each series were held at 35°, 45°, and 50° F.

Sensory Evaluation and Diacetyl. Freshly made and incubated samples were scored by a flavor panel of five members. In a study to determine the effect of sorbic acid on flavor, sets of cheeses were made with and without diacetyl. Analysis for diacetyl was carried out using a modified nickel dimethylglyoxime test (*3*).

Microbiological Procedure. Both the surface and interior portions of the cheese were sampled at periodic intervals. For the surface examinations, 1-gram samples were taken (about 1/16-inch surface layer) and for the interior examination, 11-gram samples were used, each representing a composite of two or three containers. Sampling and preparation for plating were conducted under aseptic conditions. The first dilutions for plating were made in 2% aqueous sodium citrate solution by macerating the cheese, using a mortar and pestle and then disintegrating in an Osterizer blender (John Oster Mfg. Co.) using a pint jar in place of the regular container. Preliminary macerating of the cheese with 2% sodium citrate solution followed by 2.5 minutes at high speed in an Osterizer was necessary to obtain complete solubilization of this particular type of cheese. Serial dilutions were plated on nutrient agar (1.5% agar, 0.5% peptone, 0.3% beef extract) and acidified potato dextrose agar (Difco Laboratories). All plates were incubated at 72° F. for 6 days and pH determinations were made on composite samples using a Beckman Model G pH meter.

Microorganisms growing on nutrient agar in the form of pinpoint colonies following the plating of samples containing sorbic acid were subjected to identification studies.

The effect of sorbic acid on the microbiological stability of cream dressing was also studied; and a special experiment was conducted to determine the effect of 0.05% sorbic acid toward large numbers of contaminants in cottage cheese. Surface-growing isolates from spoiled, creamed cottage cheese were used as inoculum.

Results and Discussion

At the three temperatures studied, all control samples were microbiologically unstable. At the very low temperature of 35° F., the growth of contaminants in the controls was slow, but was not arrested completely. The encountered microorganisms grew most intensely on the surface portions of the examined samples. Molds were the slowest to grow; therefore the counts on potato dextrose agar were mainly those of yeast. Surface mold growth could be observed visually over the period of incubation—the samples with visible growth were not plated. Off-flavors (bitter) developed in the controls and after 45 days, total counts reached 9,000,000 per gram *vs.* less than 10,000 per gram in the 0.05% sorbic acid samples. The cheeses with 0.07% sorbic acid were still palatable after 120 days at this temperature and were free of visible growth.

At 50° F., off-flavors were noticed in the controls in 6 days with total counts of 5,000,000 per gram. After 14 days, sourness was pronounced and pH decreased from an initial of 4.9 to 4.4. Cheeses made with 0.05% sorbic acid were still acceptable flavorwise after 14 days of incubation (total counts were less than 10,000 per gram).

There was no change in pH in any of the other cheeses, regardless of the temperature or time of storage, as indicated in Table I.

Microbial growth retardation was accompanied with longer retention of fresh flavor. The least retarded, if any, were the microorganisms of noncontaminant origin which grew as pinpoint colonies on nutrient agar. The growth of these was noted in the cheeses containing sorbic acid after prolonged incubation, but never in the control samples.

The results of microbiological studies of cheeses held at 45° F. are seen in Figures 1 and 2. These are reported in detail, as 45° F. is considered more representative of actual retail storage conditions than are 50° and 35° F. Figure 1 shows the microbiological population in the 1/16-inch surface sample and Figure 2 that of the interior of creamed cottage cheese. The control was off in flavor in 15 days, while neither the 0.05 nor 0.07% sorbic acid samples had detectable off-flavors after 25 days. The slight rise in the curves indicating an increase in yeast count in the 0.05% sorbic acid samples is too insignificant to confer any defect in the cheese.

In both figures it will be noticed that the bacterial population increased during prolonged storage in the cheeses containing sorbic acid. The increase was due to the natural microbial flora associated with cultured dairy products, whereas in the control, the bacterial population was predominantly of the spoilage type—pinpoint *vs.* large colonies. Yeast and mold inhibition by sorbic acid is shown. Pinpoint colonies on nutrient agar were isolated and found to be cocci of the genus Leuconostoc. These were gram-positive and possessed the rather distinctive characteristics of being catalase-negative and producing slime from sucrose. In milk containing the pH indicator, bromocresol purple, they grew with a slight acid reaction.

Nutrient agar proved to be an adequate differentiating medium. Leuconostocs—i.e., noncontaminants—grew as readily recognizable pinpoint colonies,

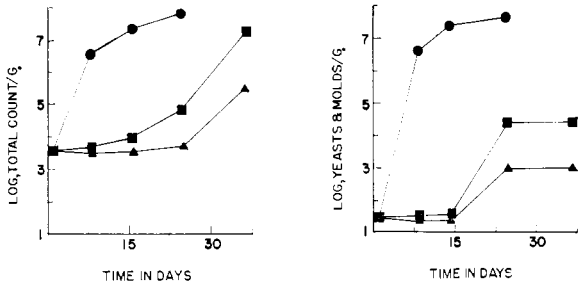

Figure 1. Microbiological population in 1/16-inch surface sample of creamed cottage cheese held at 45°F.

● Control ■ 0.05% sorbic acid ▲ 0.07% sorbic acid

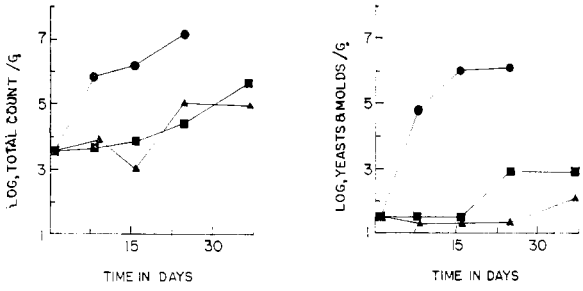

Figure 2. Microbiological population of interior sample of creamed cottage cheese held at 45°F.

● Control ■ 0.05% sorbic acid ▲ 0.07% sorbic acid

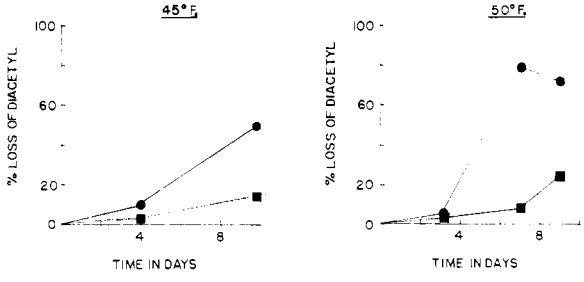

Figure 3. Per cent loss of diacetyl in creamed cottage cheese

● Control ■ 0.05% sorbic acid

while contaminants which were less fastidious were characterized by distinctly larger size colonies, among which bacteria as well as yeasts were encountered. Nutrient agar is a poor but not inhibitory medium for the growth of the latter contaminants. Thus, the bacterial population could be evaluated by subtracting the number of yeast colonies as counted on potato dextrose agar from the numbers representing the total count on nutrient agar. This applied to the counts as obtained on similar dilutions.

The changes of microbial population in the samples incubated at 50° and 35° F. were similar to those at 45° F. The difference was in the growth rate, being faster at 50° F. and slower at 35° F. The growth-retarding effect of 0.07% sorbic acid as compared with 0.05% was not significantly greater within the time limits indicated in the figures. However, a pungent odor developed in the cheeses containing 0.05% sorbic acid at 45° F. after 36 days which is beyond shelf life expectancy. The origin of the odor was not identified. The cheeses containing 0.07% sorbic acid were free of this defect.

Parker and Elliker (*4*) have reported that the loss of diacetyl—a natural flavor component in cultured milk products—in creamed cottage cheese is related to the growth of spoilage microorganisms.

The per cent loss of diacetyl in creamed cottage cheese is shown in Figure 3. Using 150 p.p.m. as the initial concentration, the loss of diacetyl at 50° F. in the control is rapid, with about 80% decrease in 7 days. The sample with sorbic acid shows less than a 10% loss after 7 days at 50° F. At 45° F. after 10 days, the control had decreased 50% *vs.* 10% in the sorbic acid sample.

The viscosity of cream dressing was not affected by sorbic acid added at a 0.2% level after aging at 45° F. and the cream dressing was microbiologically stable during the 4-day holding period. Controls were overgrown with rod-shaped bacteria, when held under these conditions. Experimentation with potassium and calcium sorbate suggested that these compounds are adequate preservers for creamed cottage cheese of pH 4.9 as administered at concentrations of 0.05% equivalent to sorbic acid. However, these compounds at 0.2% concentrations (calculated as sorbic acid) exerted no preserving effect on cream dressing during aging at 45° F. This, presumably, was due to the failure of the salts to lower the pH of this medium.

In earlier studies a deliberately inoculated series was included to determine the effect of 0.05% sorbic acid toward large numbers of initially present contaminants. Examinations had to be limited to visual observations and fairly rapid growth of spoilage organisms in all samples at three temperatures was obvious. No preservative should be a substitute for poor quality milk or unsanitary conditions, and for this reason a preservative should not be effective where initial contamination is large.

Acknowledgment

The authors express appreciation to C. M. Gooding for his encouragement and helpful advice in their work.

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The Fatty Acids and Glycerides of Cow's Milk Fat

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Milk fats contain a variety of individual fatty acids ranging in carbon chain length from 4 to 26 and of various degrees of unsaturation from monoenes to pentaenes. The unsaturated acids exist in many positional and geometrical isomeric forms. The composition of milk fats is influenced by diet and by the plane of nutrition of the animal. The general distribution patterns of fats have been studied, but no sound conclusions can be drawn about milk fats. Investigative tools are now becoming available whereby both the general distribution pattern and the specific distribution and location in glycerides of individual fatty acids can be determined.

Fats have always had a special place in the diet of man. They are important because of their high caloric value—2.25 times that of equal weights of carbohydrate or protein—but, perhaps more important are the noncaloric functions. These have been reviewed by Burr and Barnes (*4*) and include such things as vitamin-carrying functions as well as direct vitamin activity by the essential fatty acids. The property of improving the palatability of foods should not be overlooked. When properly distributed among the muscle fibers of meat, fats contribute to tenderness and also have a general lubricating effect for the passage of otherwise dry foods through the mouth. Certain fats such as butter also contribute appetizing flavors to many foods.

Because fats play such an important role in the human diet, it is essential to know their composition, structure, and properties for the understanding of their digestion, absorption, and metabolism as well as their technology and preservation. Research in the chemistry of fats has lagged behind that of other substances of biological importance. Far-reaching advances were made in the chemistry of proteins and carbohydrates in the 19th century, but it was not until well into this century that much interest centered on fats. Within the last 25 to 30 years, however, much progress has been made. This progress has been greatly accelerated in recent years by the great advances in instrumentation analyses. The development and adoption of countercurrent distribution separation, infrared and ultraviolet spectrometries, and gas-liquid partition chromatography as analytical tools make routine a study of complex substances that was impossible or impractical a few years ago.

The milk fats among the major food fats are of interest because of their intended use directly as food for the newly born animal, in contrast to the depot fats which are storage reservoirs. The fat of egg yolk is the only other animal fat intended solely for this purpose. The milk fats contain a great variety of fatty acids and are characterized particularly by their content of the shorter chain fatty acids—butyric, caproic, caprylic, and capric—and of those in the monoethenoid series from decenoic through octadecenoic. Most tables of composition for milk fats contain nine saturated and seven unsaturated fatty acids of sufficient quantitative importance to justify listing, whereas in the major depot food fats, three saturated and two unsaturated acids are the ones usually reported.

Fatty Acid Composition

Milk fat occurs as an emulsion in milk and contained within the complex are a number of lipide materials. These include triglycerides, free fatty acids, possibly mono- and diglycerides, phospholipides, and a heterogeneous group designated as unsaponifiable matter. The fatty acids present in the free and glyceride state are discussed here. They are available from three sources: ingested fat, depot fat, and in the case of ruminants, those resulting from rumen metabolism. Table I shows a comparison of the major fatty acid composition of milk fats from animals of different species. The quantities of each will vary depending on many factors, but the listed values are representative.

The amounts of the three shortest chain acids—C_4 to C_8—are small in the fats of the nonruminants, but are substantial in those of the ruminants. Is this because of different mechanisms of syntheses or because of different dietary needs of the young?

Factors Influencing Fatty Acid Composition. It seems reasonable that the dietary needs of the young are a major factor in determining the inclusion of the main fatty acid constituents. The most convincing evidence is the behavior of young calves, when fed rations containing milk fats and others containing fats from plant sources. The animals do not thrive on these foreign fats.

The values for the amounts of the different fatty acids in cow's milk fat in Table I are representative. However, there may be a considerable quantitative variation depending on several factors (*15*). The character of feed will influence the composition of the fat. Concentrate feeds rich in unsaturated fatty acids tend to produce milk fats with an increased unsaturation. Pasture grasses also increase the unsaturation of the milk fat produced from them. The type of feed also affects the total quantity of fat produced. Rations with little or no roughage produce a milk of substantially lower fat content than those with a liberal amount of roughage. Whether or not the composition of the fat is affected by the roughage content of the ration has not been determined fully, but preliminary observations indicate that along with a decrease in the amount of fat there is a pronounced increase in the degree of unsaturation, particularly in the polyethenoid acids.

The plane of nutrition has a significant effect on milk fat composition. Underfeeding results in a decrease in lower molecular weight volatile fatty acids and an increase in unsaturated acids. Other factors such as environmental temperature and stage of lactation have been reported to influence the

Table I. Major Fatty Acid Components of Milk Fats from Different Species

Animal Species	Component Acids, Weight %: Saturated C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	Unsaturated C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	Polyethenoid
Nonruminants																
Human (*3*)	0.4	0.1	0.3	2.2	5.5	8.5	23.2	6.9	1.1	0.1	0.1	0.6	3.0	36.5	7.8	3.3
Mare (*12*)	0.4	0.9	2.6	5.5	5.6	7.0	16.1	2.9	0.3	0.9	1.0	1.8	7.5	42.4	5.1	
Sow (*5*)			1.3			1.5	26.9	6.5					8.3	36.7	4.2	14.6
Ruminants																
Cow (*13*)	3.5	1.4	1.7	2.7	4.5	14.7	30.0	10.4	1.7	0.3	0.2	1.5	5.7	18.7	1.0	2.1
Goat (*12*)	3.0	2.5	2.8	10.0	6.0	12.3	27.9	6.0	0.6	0.3	0.3	0.8	2.6	21.1	0.2	3.6
Buffalo (*1*)	5.8	0.6	0.9	1.0	1.6	9.0	35.2	15.3	0.1	0.1	0.1	0.6	3.3	20.5	4.4	1.5
Sheep (*12*)	2.8	2.6	2.2	4.8	3.9	9.7	23.9	12.6	1.1	0.1	0.1	0.6	2.2	26.3	1.9	5.2

composition of milk fat. However, the effects reported cannot be distinguished from those resulting from a lowered plane of nutrition and no clear-cut conclusions can be drawn. Environmental temperatures above 85° F. cause a decrease in the volatile acids and an increase in the degree of unsaturation. Whether or not these changes are the result of temperature per se or of "hyperthermic undernutrition" cannot be stated. The latter seems the more probable.

Minor Saturated Fatty Acids. Improved analytical techniques and instruments have made possible the isolation and identification of a large number of fatty acids formerly thought not to exist in milk fat, and of some present in recently identified isomeric forms. The work of Shorland and Hansen (*10*, *21*) has been closely identified with the establishment of the presence of *n*-odd numbered acids, branched chain acids, and *n*-even numbered high molecular weight, saturated acids. Table II shows the reported occurrence of these acids.

The presence of these acids, particularly the odd-numbered acids and those with branched chains, is unexpected in the classical concept of fat composition and is of considerable theoretical interest. However, their significance to the physiologist, the biochemist, the nutritionist, and the technologist is not yet established.

The Unsaturated Acids. The kinds of unsaturated fatty acids present illustrate the compositional and the configurational complexity of milk fat. Table I shows the major groupings usually considered, but within these groupings are a large number of different individual acids, different with respect to the location of the double bonds and to their geometric configuration.

Smith, Freeman, and Jack (*22*) have confirmed earlier reports that the lower homologs of oleic acid occur in milk fat. The location of the double bond in these lower homologs generally has been reported only in the 9-10 position. However, Gupta, Hilditch, and Paul (*8*) and others have similarly confirmed the occurrence of vaccenic acid (*trans*-11-octadecenoic acid). The monoethenoid acids were all thought to be exclusively in the cis form until Smith *et al.* (*22*) showed the presence of trans acids. Backderf (*2*) found also a *trans*-16-octadecenoic acid. Thus, the monoethenoid acids exhibit both positional and geometric isomerism.

The polyethenoid acids, at one time thought to be mainly isomers of linoleic acid—*cis-trans*-9, 12- and *trans-cis*-9, 12-octadecadienoic acids—have been shown to be much more complicated. The length of the carbon chain of these acids extends to at least 22 carbons (*20*) —docosapentaenoic acid—with both conjugated and nonconjugated forms and all possible combinations of geometric isomers. Table III shows average values for the amounts of the polyethenoid acids in terms of their degree of unsaturation and their conjugation.

Table II. Some Minor Saturated Fatty Acids in Cow's Milk Fat

n-Even Numbered Acids	Weight %	Branched Chain Acids	Weight %
C_{22}	0.07	C_{13} (iso)[a]	0.05
C_{24}	0.05	C_{13} (anteiso)[b]	0.01
C_{26}	0.06	C_{14} (iso)	0.05
n-Odd Numbered Acids			
C_{11}	Trace	C_{15} (anteiso)	0.43
C_{13}	0.03	C_{17} (iso)	Trace
C_{15}	0.82	C_{17} (anteiso)	0.41
C_{17}	Identified	C_{18}	Trace
C_{19}	Identified	C_{18} (multibranched)	Trace
C_{21}	0.05		
C_{23}	0.06		

[a] The iso acids have the methyl group in the penultimate position.
[b] The anteiso acids have the methyl group in the antepenultimate position.

Table III. Polyethenoid Fatty Acids of Milk Fat

(Average values, weight %)

Conjugated			Nonconjugated			
Diene	Triene	Tetraene	Diene	Triene	Tetraene	Pentaene
0.89[a]	0.02[a]	0.003[a]	1.45[a]	0.83[a]	0.35[a]	0.02[b]

[a] (*22*). [b] (*20*).

Glyceride Composition

Milk fat contains a large number and a great variety of fatty acids.

Distribution Patterns. Two main proposals have been made to account for the distribution of fatty acids in the glyceride molecule. These are "even distribution" and "random distribution." Hilditch (*11*) has been the foremost advocate of the even distribution theory. According to this scheme, a fatty acid will not repeat itself in a glyceride until it exceeds 30 to 35% of the total acids and will not form a simple triglyceride until it exceeds 65% of the total acids. Longenecker and others (*6*, *7*, *16*, *17*), on the other hand, proposed that the fatty acids are distributed indiscriminately throughout the molecule, according to the mathematical rules of randomness.

Hilditch claims that the rule of even distribution holds for fats from plant sources, but requires modification for animal fats. He believes that even in the case of animal fats, including milk fat, which diverge somewhat from the general pattern the unsaturation is evenly distributed. Some investigations by the author (*14*) a few years ago, based on permanganate oxidation of the un-

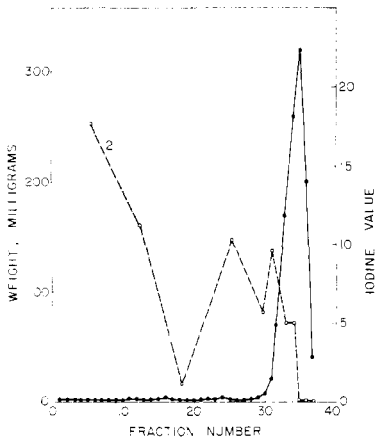

Figure 1. Countercurrent distribution of +4° C. precipitate

1. Weight of sample
2. Iodine value of sample

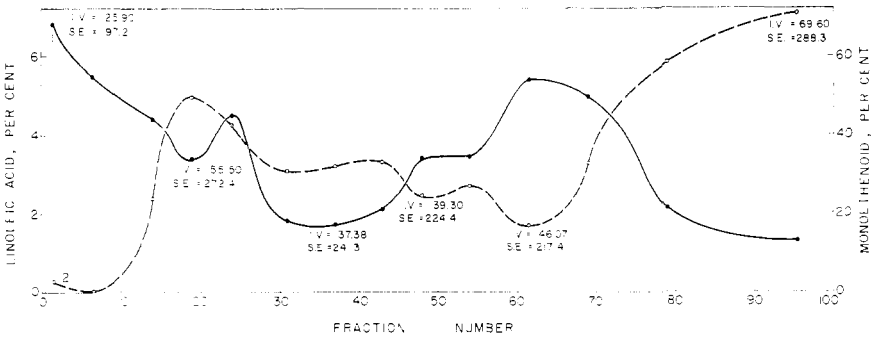

Figure 2. Countercurrent distribution separation of mono- and polyethenoid acids in −53° C. filtrate

1. Polyethenoid acids
2. Monoethenoid acids

saturated acids, supported Hilditch's thesis for this group of acids in milk fat.

The theory of random distribution also has been modified by most proponents and it is generally proposed, based on analytical data obtained, that the strict random pattern does not hold rigidly. As the investigations become more extensive and the analytical tools more refined, it becomes increasingly evident that some mechanism for selectivity is operative in the synthesis of most natural fats.

Countercurrent Distribution Separation of Milk Fat. Data obtained in the author's laboratory (*9*) using countercurrent distribution separation on fractions obtained by solvent precipitation provide evidence for some degree of selectivity in the synthesis of milk fat. The fractions chosen as most interesting for detailed study were the most saturated fraction precipitating at +4° C. and the most liquid fraction appearing as the −53° C. filtrate.

The countercurrent distribution of the +4° C. precipitate and the iodine values of the individual samples are shown in Figure 1. There is a considerable quantity of glycerides in this fraction that has an iodine value of practically zero. These completely saturated glycerides were converted to methyl esters and then analyzed by gas chromatography. The molar percentage of the esters were: methyl laurate, 0.2; methyl myristate, 3.6; methyl palmitate, 80.4; and methyl stearate, 15.7. This amount of methyl palmitate, 80.4 mole %, requires, therefore, that some of the glycerides from which the esters were prepared be tripalmitin.

The countercurrent distribution of the −53° C. filtrate shows an interesting separation of the monoethenoid and the polyethenoid acids (Figure 2). Early fractions are high in polyethenoid acids and low in monoethenoid; intermediate fractions are intermediate in amounts of each, while latter fractions are high in monoethenoid acids and low in polyethenoid. Study of the saponification equivalents of the samples in relation to the degree of unsaturation shows a definite relationship (Table IV) wherein the longer chain saturated acids are associated in the glyceride with the monoethenoid acids and a less definite relationship, but nonetheless a tendency for the low molecular weight acids to be associated with the polyethenoid.

Thus it can be said with some confidence that there is some specificity in the structure of milk fat glycerides.

Table IV. Distribution of Fatty Acid Types in −53° C. Filtrate Fraction

Sap. Equiv.	I_2 Value	Average Length Carbon Chain (approx.)	Mole % Satd. Fatty acids	Mole % Poly-ethenoid	Mole % Mono-ethenoid
288.3	69.60	18	28.6	2.4	69.0
272.4	55.50	17	40.9	5.1	54.0
241.3	37.38	15	70.5	3.0	26.5
224.4	39.30	14	75.9	5.3	18.8
217.4	46.07	13	78.1	9.5	12.4
197.2	25.90	12	91.1	7.1	1.8

Enzymatic Lipolysis Studies. Perhaps of greater importance than the general distribution pattern of the fatty acids is the specific location in the glyceride molecule of the different types and of the individual acids. Savary *et al.*(*19*) and others have developed a technique using controlled enzymatic lipolysis to split off the terminal fatty acids and leave β-monoglycerides. This technique has been applied to a number of food fats, with the result that it has been established that in most fats the unsaturated acids are mainly in the β, or internal, position, whereas in pig body fat the unsaturated acids are in the α or external position. Only one report has been published about the application of this technique to milk fat. Patron and associates (*18*) have suggested that the unsaturated fatty acids are in the external position corresponding in this respect to pig body fat.

When the total composition and the exact configuration of this important food are known, it will aid greatly in the better understanding of the physiology of milk synthesis, the biochemistry of its digestion and metabolism, and in the technology of processing.

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RICE PROTEIN SUPPLEMENTATION

Further Studies on the Nutritional Improvement of Rice

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The proteins of whole and milled rice can be effectively supplemented by those of whole milk solids, lactalbumin, and defatted whole egg. Data are presented on the content of amino acids (essential and nonessential), vitamins, and other constituents of milk solids, and defatted whole egg. Lactalbumin was tested for total amino acid content. The results of these experiments show the extent to which rice rations can benefit from addition of small amounts of milk solids, lactalbumin, and whole egg. This is of importance in those areas of the world where much rice is consumed and where these high protein foods are economically available.

IN AN ATTEMPT to improve rice diets further, studies were made with whole milk powder, nonfat dried milk (skimmilk), lactalbumin, and nonfat whole egg powder as supplements to whole rice and to white milled rice. An investigation was started on the effect of replacing whole rice proteins with those of milk solids and powdered whole egg; 1, 3, and 5% of these solids replaced equivalent amounts of proteins in milled and whole rice rations which were fed to albino rats for 70 days. Another series of experiments was made on the effect of adding small amounts of these solids to rations containing rice as the only source of protein. This paper also reports studies on the content of all members of the vitamin B complex, amino acids (including nonessentials), calcium, phosphorus, and iron, and on the growth value of the proteins of samples of commercial dry milk solids, lactalbumin, and whole egg powder.

Experimental Procedure and Results

Commercial samples of dry milk solids (spray-dried whole milk, nonfat dry milk solids, or skimmilk), lactalbumin, and fat-extracted whole egg powder were used for the determination of vitamins, minerals, amino acids, and supplementary and growth values of the proteins.

Supplementary values were determined in studies using albino rats as experimental animals fed milled white rice rations containing 5.5% protein and whole rice rations containing 6.38% protein. Milled and whole rice furnished the only source of protein in these rations and at levels to incorporate the necessary protein. The per cent of protein content of the white milled rice was 6.45, of whole brown rice 7.25, of dried whole milk 25.0, of fat-extracted whole egg 65.8, of lactalbumin 80.7, and of skimmilk 36.2. The composition of the rest of the rations was 4% of salt mixture No. 1 (*6*), 4% of hydrogenated vegetable shortening, 2% of cellu flour, 2% of cod liver oil, and 1% of wheat germ oil as sources of vitamins A, D, and E, and the rest as glucose (cerelose).

In the protein replacement experiments, the other solids were added at three levels—1, 3, and 5%; the proteins of these solids were added at the expense of the rice proteins, leaving the total protein in the ration the same. In the protein addition experiments, the solids were added also to the basal ration at these levels of 1, 3, and 5% at the expense of cerelose, and the protein content was slightly increased.

The following crystalline components of the vitamin B complex were administered daily to each animal separately from the rations with double doses on Saturday: 25 γ each of thiamine, riboflavin, pyridoxin, and niacin; 150 γ of calcium pantothenate; 3 mg. of *p*-aminobenzoic acid; 6 mg. of choline chloride; and 1 mg. of inositol. The animals, 28 to 32 days old when started on the experiment and weighing about 50 grams each, were divided in two groups, each having equal numbers of both sexes; they were fed for 70 days. Each animal was weighed weekly and accurate weekly records for food consumption were kept. From these data the protein efficiency ratios were calculated and expressed as gains in body weight per gram of protein intake.

In the study (*3*) of the supplementary value of the proteins of whole milk solids for those of white milled rice, an equivalent amount of the proteins of rice was replaced by the proteins of 1, 3, and 5% whole milk powder, leaving the protein level at 5.55. In experiments reported here, 1, 3, and 5% of whole milk were added, which increased the protein level to 5.86, 6.24, and 6.62%, respectively (Table I, *A*, ration 9 listed).

The protein content of the various foods was determined from nitrogen

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The protein content of the various foods was determined from nitrogen

Table I. Supplementary Relationship between Various Solids

(12 animals in each group. Average results per animal for a 10-week period)

	Ration	Protein in Ration, %	Gains in Body Weight, Grams	Gains in Body Weight, %	Protein Intake, Grams	Protein Efficiency Ratio[a], Grams	Protein Efficiency Ratio[a], %
	A. Proteins in Whole Milk Solids and Rice[b]						
1	Whole rice	6.38	82.3 ± 3.7	...	43.8	1.87 ± 0.03[c]	...
2	Whole rice + 1% whole milk	6.38	111.7 ± 5.5	35.7[d]	50.4	2.21 ± 0.06	18.1[a]
3	Whole milk solids	6.38	75.7 ± 4.5	...	44.6	1.69 ± 0.05	...
4	Whole rice + 1% whole milk	6.56	127.1 ± 3.4	54.3[d]	54.9	2.31 ± 0.13	23.8[d]
5	Whole milk solids	9.0	148.3 ± 4.0	...	72.5	2.04 ± 0.10	...
6	Whole rice + whole milk	9.0	175.8 ± 9.9	18.5[d]	86.8	2.02 ± 0.08	−1.0
7	Polished rice + whole milk	9.0	182.8 ± 13.0	23.2[d]	84.8	2.17 ± 0.10	6.3
8	Polished rice	5.55	63.6 ± 4.6	...	42.2	1.50 ± 0.04[c]	...
9	Polished rice + 1% whole milk	5.86	78.4 ± 5.0	23.1[d]	44.0	1.78 ± 0.07	18.6[d]
	B. Proteins in Dried Nonfat Milk Solids and Rice[b]						
10	Polished rice	5.55	63.6 ± 4.6	...	42.2	1.50 ± 0.04[c]	...
11	Polished rice + 1% N.F.M.	5.55	99 ± 3.3	55.6[d]	50.7	1.94 ± 0.04	29.3[d]
12	N.F.M.	6.38	124.4 ± 4.0	...	59.2	2.11 ± 0.06	40.6[d]
13	Polished rice + 1% N.F.M.	5.73	97.4 ± 6.0	53.1[d]	46.7	2.08 ± 0.08	38.6[d]
14	N.F.M.	5.68	97.7 ± 2.8	..	46.3	2.11 ± 0.05	40.6[d]
15	Whole rice	6.31	72.0 ± 4.4	...	41.5	1.73 ± 0.06	15.3[d]
16	Whole rice + 1% N.F.M.	6.31	87.0 ± 6.5	20.8[d]	43.6	1.98 ± 0.11	32.0[d]
17	N.F.M.	9.0	187.0 ± 11.8	160.0[d]	89.3	2.08 ± 0.13	32.0[d]
	C. Lactalbumin and Protein in Rice[b]						
18	Polished rice	5.42	71 ± 4.7	...	35.4	2.01 ± 0.07[c]	...
19	Polished rice + 1% lactalbumin	5.42	96 ± 5.2	35.2	39.8	2.41 ± 0.08	14.9[d]
20	Lactalbumin	5.42	20 ± 3.4	...	23.8	2.09 ± 0.19	...
21	Lactalbumin	9.0	179 ± 6.6	...	72.5	1.79 ± 0.08	...
22	Whole rice	6.31	72 ± 4.4	...	41.6	1.93 ± 0.06	...
23	Whole rice + 1% lactalbumin	6.31	93 ± 6.4	29.1[d]	41.3	2.23 ± 0.03	15.5[d]
	D. Proteins in Fat-Extracted Whole Eggs and Rice[b]						
24	Polished rice	5.55	73 ± 3.4	...	41.9	1.74 ± 0.03[c]	...
25	Polished rice + 1% whole egg	6.05	113.8 ± 8.8	55.9[d]	51.5	2.24 ± 0.04	28.7[d]
26	Whole egg	6.38	113.9 ± 8.8	...	51.5	2.21 ± 0.03	27.0[d]
27	Whole rice	6.38	82.3 ± 3.7	...	43.9	1.87 ± 0.03	...
28	Whole rice + 1% whole egg	6.38	143.3 ± 7.0	74.1[d]	58.2	2.46 ± 0.04	31.5[d]
29	Whole egg	9.00	142.2 ± 8.5	...	78.9	1.78 ± 0.08	...

[a] Gain in body weight per gram of protein intake.
[b] Milled white polished rice and whole brown rice.
[c] Standard error.
[d] Significant for $P = 0.05$.

Table II. Determination of Amino Acids

	In Whole Milk Powder, % 25.0% Protein		In Skimmilk Powder, % 36.2% Protein		In Fat-Free Whole Egg, % 65.8% Protein		In Lactalbumin, % 80.7% Protein		In White Milled Rice, % 6.01% Protein (4)	
	In dry matter	In protein	In dry matter	In protein	In dry matter	In protein	In dry matter	In protein	In dry matter	In protein
Alanine	0.80	3.20	1.30	3.58	2.10	3.19	4.24	5.29		
Arginine[a]	1.00	4.00	1.30	3.59	4.31	6.55	3.00	3.72	0.62	10.31
Aspartic acid	2.00	8.00	3.00	8.28	5.23	7.95	8.01	9.93	0.29	4.82
Cystine	0.25	1.00	0.40	1.10	1.54	2.34	3.10	3.84	0.09	1.48
Glutamic acid	5.20	20.8	6.90	19.1	8.93	13.57	12.0	14.80	0.68	11.31
Glycine	1.60	6.40	1.35	3.73	3.06	4.65	3.20	3.97	0.44	7.32
Histidine[a]	0.67	2.68	0.87	2.50	1.78	2.70	2.00	2.48	0.19	3.15
Isoleucine[a]	1.76	7.04	2.15	6.17	4.46	6.78	5.80	7.19	0.31	5.15
Leucine[a]	2.90	11.60	3.40	9.38	6.18	9.39	9.4	11.66	0.57	9.48
Lysine[a]	1.80	7.20	2.58	7.13	4.62	7.02	6.60	8.18	0.22	3.66
Methionine[a]	0.90	3.60	0.96	2.65	2.40	3.65	2.00	2.48	0.23	3.82
Phenylalanine[a]	1.25	5.00	1.76	4.86	3.70	5.62	4.50	5.58	0.29	4.82
Proline	2.20	8.80	2.60	7.46	3.08	4.68	4.00	4.96	0.28	4.65
Serine	1.32	5.20	1.50	4.14	4.00	6.08	3.60	4.46	0.30	4.99
Threonine[a]	1.16	4.64	1.60	4.42	3.70	5.62	4.32	5.36	0.31	5.15
Tryptophan[a]	0.40	1.60	0.50	1.38	0.92	1.40	2.00	2.48	0.05	0.83
Tyrosine	1.25	5.00	1.58	4.53	2.26	3.43	4.10	5.08	0.32	5.32
Valine[a]	1.60	6.40	2.23	6.15	4.87	7.40	5.55	6.85	0.46	7.65

[a] Nutritionally essential.

Table III. Vitamins and Other Constituents in Fat-Free Dry Whole Milk, Skimmilk, and Whole Egg Powder

	Dry Whole Milk	Skimmilk	Whole Egg
	Micrograms Per Gram		
Thiamine	2.8	3.0	4.3
Riboflavin	10.8	13.2	8.4
Nicotinic acid	15.0	14.0	4.0
Pantothenic acid			
Total	34	36.8	46.7
Free	15	23.2	39.0
Biotin	0.20	0.32	0.35
Folic acid			
Total	0.04	0.04	0.06
Free	Traces	Traces	Traces
Pyridoxine	12.0	9.3	7.6
Inositol	2300	1320	1200
Choline	1734	1750	2400
Aminobenzoic acid	0.64	0.85	0.70
	Per Cent		
Calcium	0.98	1.30	0.20
Phosphorus	0.71	0.91	0.83
Iron	0.0053	0.0060	0.0050
Nitrogen	3.97	5.67	10.28
Protein (nitrogen × factor)	25.3	36.2	65.8
Fat (in the original)	26.2	0.05	34.6
Moisture	2.3	5.6	2.5
Ash	5.3	7.2	2.3
Fiber	0	0	1.4

analyses using the factor 6.38 for the nonfat milk solids, 6.49 for lactalbumin, and 6.25 for whole egg. For conversion of total nitrogen to protein, the factor 5.95 was used for rice, as Jones (*2*) reported that the nitrogen content of oryzenin, 16.8%, fairly represents the nitrogen in the rice proteins as a whole.

In the study of the supplementary value of the proteins of whole milk solids for those of whole brown rice, the procedure followed was similar to that described for the white milled rice. In the replacement tests, the protein level was left at 6.38% (Table I, *A*, ration 2 listed), and in the addition tests, the protein level was increased from 6.38 to 6.56, 6.92, and 7.28%, respectively (Table I, *A*, ration 4 listed). The results of these supplementary studies are given in Table I. Only one level of each tested supplement is listed; similar results were obtained on higher levels.

The results, expressed as average gain per animal (Table I, *A*), indicate that the proteins of whole brown rice can also be improved by supplementation with whole milk solids like those of milled white rice, as previously demonstrated in ad libitum and paired feeding experiments (*3*).

The values are higher in the combination whole rice–whole milk than in each food separately and this constitutes a demonstration of a true supplementary relationship between two proteins (Table I, *A*, rations 1, 2, and 3). The results obtained from rations 5, 6, and 7 provide further proof of a supplementary relationship between proteins of milled and whole rice and of whole milk solids.

Table I, *B*, shows that the proteins of dried nonfat milk solids (skimmilk) have a supplementary effect for those of milled white rice and whole brown rice; Table I, *C*, shows that a supplementary relationship exists between lactalbumin and the proteins of milled white rice and of whole brown rice.

As previously shown (*3*) in ad libitum and paired feeding experiments, the proteins of whole egg supplemented those of white milled polished rice in rations where the protein level of 5.55 was kept constant and in whole rice rations fed rats at a constant level of 6.38% protein. Addition of small amounts (1, 3, and 5%) of whole egg to a white polished rice ration leads to large increases in growth and protein efficiency ratio (P.E.R.), although the protein levels of the rations are increased to a certain extent (ration 25, only one level listed, Table I, *D*). The proteins of whole brown rice are supplemented by those of whole egg as shown by the results obtained with ration 28. In the whole rice–whole egg ration 28, a higher value in gain and P.E.R. was obtained than found in either the whole rice ration alone or in the whole egg ration alone and this points to a true, real supplementation. Animals fed extracted whole egg at a 9% protein level of intake showed an average gain of 142 grams and a P.E.R. of 1.78 grams which is lower than the values found for whole milk, nonfat dry milk solids, and lactalbumin.

The results of these experiments indicate that the proteins of white milled polished rice and of whole brown rice can be further improved by supplementation with those of whole milk solids, nonfat dry milk solids, lactalbumin, and extracted whole egg. This is of importance in those areas where rice is consumed to a large extent and where milk solids, lactalbumin, and whole eggs are readily available at a reasonable cost.

A complete description of the experimental procedure for the determination of amino acids, vitamins, and the minerals calcium, phosphorus, and iron is presented in a previous publication (*5*).

Amino Acid Content. The results of the amino acid determinations in fat-free samples of whole milk solids, nonfat dry milk solids, lactalbumin, and whole egg, expressed as percentages, are given in Table II. These values are calculated on the air-dried samples and are also expressed as the percentage in the crude protein. The content of the amino acids (essential) agrees well with that reported elsewhere (*1*). Values for amino acids in rice have been reported previously (*4*) and are reproduced in Table II.

This table shows that the proteins of milk solids, whole egg, and lactalbumin have much higher amino acid content than rice proteins. Tryptophan, lysine, threonine, and the combination of cystine and methionine are much lower in rice proteins and it might be assumed that these amino acids are mainly responsible for the high supplementary effect observed in the growth experiments, especially as lysine is considered to be the main limiting amino acid in rice proteins.

A number of vitamins were determined by methods referred to elsewhere (*4*). The results of these tests are given in Table III, which also contains data on calcium, phosphorus, iron, and other constituents.

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Cystine Content of Proteins, Foods, and Feeds. Comparison of Chromatography on a Sulfonated Polystyrene Resin and Microbiological Methods of Determination

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The cystine contents of a number of proteins, foods, and feeds were determined by chromatography after oxidation to cysteic acid, and by three microbiological assay methods. The microbiological methods used a lanthionized casein medium after 30 minutes of hydrolysis or an oxidized peptone medium after 30 minutes or 6 hours of hydrolysis by autoclaving. The microbiological procedures using 30-minute hydrolysis gave higher values than the chromatographic method, but results were erratic. Most of the cystine values obtained by microbiological assay of the 6-hour digests were lower than the chromatographic values. One commercially isolated soybean protein preparation showed much more cystine by the chromatographic procedure than by microbiological assay, indicating a loss of cystine availability during preparation.

CYSTINE AND METHIONINE are among the amino acids most apt to be deficient in the common foods and feeds of vegetable origin (*11*). Cystine is not considered an essential amino acid, because it can be replaced in the diet by methionine. It is important, however, because it can supply part of the amino acid sulfur required and, thus, spare methionine.

Most values obtained for the cystine content of foods and feeds have been low, because some of the cystine is destroyed during acid hydrolysis in the presence of carbohydrates (*9*).

Recently, two new methods have been described for the determination of cystine in the presence of carbohydrates. Schram, Moore, and Bigwood (*13*) oxidized the protein-bound cystine to cysteic acid and determined the amount of cysteic acid by separation on ion exchange chromatographic columns of Dowex 2 after acid hydrolysis of the protein. Horn and Blum (*8*) determined cystine by microbiological assay, using "lanthionized" casein as a source of amino acids. They hydrolyzed their foodstuffs by refluxing for only 2 hours rather than the regular 24 hours.

The cystine contents of a number of biological materials were determined by chromatographic separation of cystine after conversion to cysteic acid and by three microbiological techniques. Cystine was determined in a number of different types of material, such as seeds, poultry feed ingredients, soybean products, and proteins. The effects of adding sucrose to the protein and autoclaving various proteins on the cystine content determined by four methods were also studied. Chromatographic determination of cystine as cysteic acid was used as the standard method, because cystine is quantitatively oxidized to cysteic acid, which is stable to acid hydrolysis even in the presence of large amounts of carbohydrates (*13*).

Experimental

Cystine was determined chromatographically essentially as described by Schram, Moore, and Bigwood (*13*), with modifications of Bandemer and Evans (*1*). The material to be analyzed was oxidized with performic acid to convert cystine and cysteine to cysteic acid. The oxidized protein was hydrolyzed by heating in the autoclave at 15 pounds' pressure for 6 hours with 20% hydrochloric acid. The hydrochloric acid was removed by evaporation on the steam bath. Water was added twice and evaporation repeated. The solution was made to volume and aliquots were chromatographed on a column of the chloroacetate form of 200- to 400-mesh Dowex 2X10 resin.

Two methods of microbiological assay were used for determination of cystine. *Leuconostoc mesenteroides* P-60 was the organism in both methods. The first method, the peptone medium method, used the medium of Lyman *et al.* (*10*), which contained oxidized peptone as the source of most of the amino acids. The second method, the lanthionized casein medium method, used the medium of Horn and Blum (*8*), which contained lanthionized casein hydrolyzate as the source of most of the amino acids.

Acid hydrolyzates were prepared by autoclaving 1.0 gram of the material with 20 ml. of 20% hydrochloric acid at 15 pounds' pressure for 30 minutes or 6 hours. The 6-hour hydrolysis has been used in this laboratory for some time in preparing hydrolyzates for microbiological assay of most of the amino acids. The 30-minute hydrolysis was chosen as a result of the following experiments.

One-gram samples of soybean oil meal were weighed into 125-ml. Erlenmeyer flasks, and 20 ml. of 20% hydrochloric acid was added. Duplicate samples were then autoclaved at 15 pounds' pressure for 15, 30, 45, or 60 minutes. The same soybean oil meal was used in a duplicate second experiment, in which samples were also autoclaved for 120 minutes. All samples were assayed for cystine, using both peptone and lanthionized casein media (Table I).

Sixty-one samples were analyzed by the chromatographic procedure. Cystine was also determined on most of these same samples by microbiological assay after 30 minutes of hydrolysis, using both the oxidized peptone and lanthionized casein media. Some samples were assayed microbiologically for cystine after 6 hours of hydrolysis, using the oxidized peptone medium.

Results and Discussion

The results of the hydrolysis studies were not conclusive because low values

were obtained with the peptone medium in the second experiment. Repeat assays gave consistently low values, showing the trouble to be in the digestion procedure (Table I). The 30-minute period was chosen for routine assays because it gave the highest values most often.

The question might be raised as to the reason for the differences in results obtained in experiments 1 and 2 of Table I. During hydrolysis of the soybean oil meal two reactions are taking place: the release of cystine, or of peptides that contain cystine in a form available for the assay organism; and reaction of cystine with carbohydrate to cause destruction of the cystine. The largest value is obtained where the curves of the two reactions intersect. Slight differences in time or conditions of hydrolysis will shift the point of intersection, or the highest point may be somewhere between two of the hydrolysis times used. The data in Table I indicate the possibility of erratic results from the use of short hydrolysis time for microbiological assay for cystine.

Nine different seeds, both raw and heated, were analyzed by the chromatographic and microbiological procedures, using a 30-minute hydrolysis and both oxidized peptone and lanthionized casein media (Table II). The results agree fairly well for the most part, except that the lanthionized casein medium gave slightly high results on some samples.

Several poultry feed ingredients and mashes were analyzed by all four methods (Table III). The chromatographic values were generally lower than the microbiological assays using 30-minute digests and higher than those using 6-hour digests.

Cystine was determined on several commercially available isolated soybean proteins as obtained, and after autoclaving alone or in a mixture of 80% protein and 20% sucrose (Table IV). The cystine contents of soybean oil meal and four of the five soybean proteins were nearly the same whether determined chromatographically or microbiologically after 30 minutes of hydrolysis using the peptone medium. The lanthionized casein medium gave higher values in most cases than either the peptone medium or the chromatographic method. The microbiological procedures consistently gave low values for one of the five isolated soybean protein preparations (Alpha protein) when compared with the chromatographic determination. The microbiological assay procedure gave values similar to or higher than those of the chromatographic method for the isolated milk protein, casein, and the isolated corn protein, zein (Table V).

Schram, Moore, and Bigwood (*13*) found that the chromatographic cystine determination is specific for cystine and cysteine because both are oxidized quantitatively to cysteic acid, and cysteic acid is not destroyed during acid hydrolysis even in the presence of large amounts of carbohydrates. Horn and Blum assumed that because the short hydrolysis time gave highest results for cystine, these are necessarily correct results. They considered the possibility of peptide stimulation, but dismissed it because peptides did not stimulate other amino acid assays. However, Evans and McGinnis (*6*) and Evans and St. John (*7*) observed that cystine peptides exerted a greater stimulating action on growth of *Lactobacillus arabinosus* on a cystine-free medium than did free cystine.

Table I. Effect of Time of Hydrolysis on Cystine Liberated from Soybean Oil Meal by Hydrochloric Acid

	Microbiological Assay, %			
	Peptone Medium		Lanthionized Casein Medium	
Time, Min.	Expt. 1	Expt. 2	Expt. 1	Expt. 2
15	1.04	0.71	0.93	0.90
30	0.98	0.74	0.95	1.01
45	0.96	0.81	0.87	0.95
60	0.88	0.76	0.80	0.88
120	...	0.63	...	0.78

Experiment 1 was conducted about a month before experiment 2, but portions of the same sample of soybean oil meal were used for each. The peptone and lanthionized casein medium assays were performed on aliquots of the same hydrolyzates.

Table II. Cystine Found in Seeds

(Calculated to basis of 16% nitrogen)

			30-Min. Hydrolysis, %	
Material[a]	% N	Chromatographic, %	Peptone medium	Lanthionized casein medium
Barley	1.53	2.3	2.1	2.7
Barley heated	1.60	1.9	2.0	2.5
Mung bean	3.89	0.6	0.7	0.6
Mung bean heated	3.99	0.5	0.6	0.6
Pea bean	3.84	1.1	0.6	1.2
Pea bean heated	3.84	1.1	0.5	1.2
Corn a[a]	1.73	2.0	1.8	2.4
Corn a heated	1.73	1.9	2.0	2.4
Millet	2.24	1.7	0.9	1.9
Millet heated	2.28	1.3	0.9	2.0
Rice	1.05	1.5	1.8	2.1
Rice heated	1.15	1.4	1.7	2.1
Sudan grass seed	1.65	1.4	1.5	1.4
Sudan grass seed heated	1.75	1.2	1.5	1.5
Sunflower seed	4.95	1.6	1.9	2.3
Sunflower seed heated	4.80	1.3	1.3	1.1
Wheat a	1.59	2.1	2.3	2.8
Wheat a heated	1.62	2.0	2.2	2.5

[a] Name of sample followed by a letter indicates that different samples were analyzed and data for each are given. Materials were obtained from various sources, and those obtained from any one source are grouped together.

Table III. Cystine Found in Poultry Feed Ingredients

(Calculated to basis of 16% nitrogen)

			30-Min. Hydrolysis, %		6-Hr. Hydrolysis, %,
Material[a]	% N	Chromatographic, %	Peptone medium	Lanthionized casein medium	Peptone Medium
Alfalfa meal	3.02	0.9	0.7	0.8	0.4
Dried buttermilk	4.92	0.7	1.0	1.5	0.8
Chick starter mash	3.55	1.3	1.6	1.9	0.9
Corn b	1.64	1.6	1.9	1.8	1.4
Corn fermentation solubles	4.11	2.3	...	...	...
Cottonseed meal	6.64	2.2	1.7	1.8	...
Fish meal	9.66	0.7	1.1	1.3	0.7
Fish meal (menhaden)	9.91	0.7	...	...	...
Oats	1.87	2.2	2.9	3.0	1.8
Poultry mash	3.18	1.3	1.7	2.0	1.0
Soybean oil meal a	8.34	1.2	1.5	1.7	0.9
Soybean oil meal b	7.90	1.2	1.3	1.4	...
Wheat b	1.88	1.9	2.1	2.2	1.4
Wheat bran	2.29	1.3	2.3	2.5	1.2
Wheat middlings	2.65	1.4	1.9	1.9	1.1
Whey	6.63	0.6	...	...	...

[a] See footnote, Table II.

Table IV. Cystine Found in Soybean Oil Meal and Some Isolated Soybean Proteins

(Calculated to basis of 16% nitrogen)

Material[a]	% N	Chromatographic, %	30-Min. Hydrolysis, % Peptone medium	30-Min. Hydrolysis, % Lanthionized casein medium	6-Hr. Hydrolysis, % Peptone Medium
Soybean oil meal c	7.90	1.54	1.60	2.07	1.14
Soybean oil meal c autoclaved	8.24	0.85	0.82	0.87	0.60
Alpha protein a[b]	14.39	0.69	0.29	0.31	0.24
Alpha protein a autoclaved	14.37	0.65	0.26	0.27	0.20
Alpha protein a + sucrose	11.63	0.59	0.30	0.18	0.17
Alpha protein a + sucrose autoclaved	12.20	0.59	0.26	0.20	0.17
Alpha protein b	14.39	0.66	0.34	0.33	...
Amisoy[b]	13.51	1.30	1.37	1.91	0.78
Amisoy acid washed	12.38	1.52	...	...	...
Buckeye protein a[b]	14.32	1.24	1.38	1.72	0.85
Buckeye protein a autoclaved	14.35	0.81	1.00	0.99	0.67
Buckeye a + sucrose	11.45	1.22	1.37	1.56	0.88
Buckeye protein a + sucrose autoclaved	12.12	0.95	0.82	1.03	0.73
Buckeye protein b	14.32	1.18	1.52	1.56	...
C-1 assay protein[b]	13.75	1.22	1.08	1.40	0.70
Protosoy[b]	13.64	1.14	1.10	1.46	0.70

[a] See footnote, Table II.

[b] Alpha, Buckeye, Protosoy, Amisoy, and C-1 assay are commercial isolated soybean proteins prepared by Glidden Co. (now Central Soya Co.), Buckeye Cellulose Corp., Mann Research Labs., Glidden Co., and Dracket Co. (now Archer-Daniels-Midland Co.), respectively.

Table V. Cystine Found in Proteins

(Calculated to basis of 16% nitrogen)

Material	% N	Chromatographic, %	30-Min. Hydrolysis, % Peptone medium	30-Min. Hydrolysis, % Lanthionized casein medium	6-Hr. Hydrolysis, % Peptone Medium
Casein	13.46	0.37	0.34	0.43	0.33
Casein autoclaved	13.34	0.31	...	...	0.28
Casein + sucrose	10.92	0.32	...	...	0.47
Casein + sucrose autoclaved	11.32	0.25	...	...	0.44
Zein	14.96	0.49	0.60	0.67	0.49
Zein autoclaved	14.96	0.42	...	...	0.38
Zein + sucrose	12.15	0.45	...	...	0.38
Zein + sucrose autoclaved	12.12	0.36	...	...	0.34
Dried whole egg (defatted)	11.64	2.34	...	...	2.89
Egg white	13.23	2.61	...	...	3.02
Egg yolk (defatted)	14.28	1.69	...	...	1.90

The higher values for cystine obtained by the microbiological procedures using 30-minute hydrolysis than by the chromatographic method appear to be better explained by a stimulating effect of cystine peptides on assay bacteria than by a loss of cystine in the chromatographic procedure.

Evans, Groschke, and Butts (*5*) observed a loss of 31% of the cystine from soybean oil meal that had been autoclaved for 4 hours at 15 pounds' pressure. None of the cystine of isolated soybean protein (Alpha protein) was destroyed when the protein was autoclaved either alone or as a mixture of 80% protein and 20% sucrose, glucose, dextrin, agar-agar, gum arabic, or soybean oil. Cystine was determined by assay with *L. mesenteroides* after hydrolysis by autoclaving with 20% hydrochloric acid for 6 hours. The data in Table IV confirm the results of Evans *et al.* (*5*), except that over 40% destruction of cystine occurred in autoclaved soybean oil meal. Contrary to the results with Alpha protein, autoclaving destroyed 27 to 42% of the cystine in Buckeye protein (depending upon method of determination). No further destruction took place when the protein was mixed with 20% of sucrose. Assuming that pure soybean protein contains 16% nitrogen, Buckeye protein contained 11% of impurities. These may be the cause of the cystine destruction when Buckeye protein was autoclaved.

Some destruction of cystine also occurred when zein or casein was autoclaved, and the addition of sucrose increased the destruction very little (Table V).

Alpha protein differed from the rest of the isolated soybean proteins in that it contained less cystine. The proteins (N × 6.25) of soybean oil meal contained 1.16 to 1.54% cystine by chromatographic analysis and 1.34 to 2.06% by microbiological assay after 30-minute hydrolysis. About half of the cystine was lost from the total soybean protein in preparation of Alpha protein according to chromatographic determination. When microbiological assay was used, at least 80% was lost or was inactivated so that it was not available for *L. mesenteroides* P-60. A cystine-rich portion of soybean proteins is, therefore, not included in the Alpha protein fraction. Van Etten *et al.* (*15*) found a higher concentration of cystine in heat-coagulated soybean protein than in the acid-precipitated protein.

Alpha and Buckeye proteins must be different fractions of the total soybean proteins. Buckeye protein contains the soybean trypsin inhibitor. Alpha protein does not. Buckeye protein contains about twice as much cystine as does Alpha protein determined chromatographically, and five times as much by microbiological assay after 30 minutes of hydrolysis.

Three possible explanations for the peculiar behavior of Alpha protein are apparent from the data. The first is that in the method of preparing Alpha protein, considerable cystine is oxidized to cysteic acid or converted to some form that is oxidized to cysteic acid by the method used in the chromatographic study. Cysteic acid or the other compound is not available to *L. mesenteroides* P-60. Another possible explanation is that the cystine in Alpha protein is combined with other amino acids in such a way that it is liberated from the protein more slowly than from the other soybean proteins. The amount of free cystine present in the hydrolyzate would then be small, because it would be destroyed almost as fast as it is liberated. The third explanation is also connected with the possibility of a different type of cystine binding in Alpha protein than in other soybean proteins. It is possible that some cystine peptides are more active for the microbiological assay than others and that the peptides of Alpha protein that are liberated by autoclaving with 20% hydrochloric acid for 30 minutes are not as active for the growth of *L. mesenteroides* as those liberated from soybean oil meal or Buckeye protein. The first explanation appears to the authors to be the most plausible, but there is evidence for the last, in that microbiological assay of the other soybean proteins using the lanthionized casein medium gave higher values than the chromatographic procedure, indicating stimulation of growth by something other than cystine.

The colorimetric method of Sullivan (*14*) has been the most specific chemical procedure for the determination of cystine, and most of the earlier data in the literature were obtained by this procedure. It, however, suffers from the limitation that the cystine has to be

first liberated from the proteins by acid hydrolysis. The colorimetric method was not used in the present study, because losses of cystine during prolonged acid hydrolysis of grains and other carbohydrate-containing materials would be expected (*9*). Sarkar, Luecke, Evans, and Duncan (*12*) compared the microbiological with the colorimetric method and obtained similar values by the two methods. They used aliquots of the same hydrolyzates for microbiological and chemical methods. They first precipitated cystine from the acid hydrolyzates as the cuprous mercaptide by the procedure of Csonka, Lichtenstein, and Denton (*2*) in the chemical procedure, and determined cystine by the colorimetric Sullivan reaction as modified by Evans (*4*). Values obtained by Sarkar *et al.* (*12*) are compared in Table VI with some of those from Tables II to V.

Evans (*3*) used a differential oxidation procedure for the determination of cystine and methionine in plant and animal materials. The method assumes that all of the organic sulfur oxidized to sulfate by concentrated nitric acid is cystine or cysteine sulfur. High results will be obtained if other easily oxidizable organic sulfur compounds are present. A comparison of some of the data obtained by the differential oxidation procedure (*3*) with those of Tables II to V is of interest (Table VI). The differential oxidation procedure gave slightly higher values for most of the materials listed except barley. Fish meal showed much more cystine by the differential oxidation procedure than by the other methods, but this was no doubt caused by differences in the fish meals studied. For the most part, the differential oxidation procedure gave values very close to the chromatographic and microbiological ones, even though analyses were not made on the same samples.

The data of Horn and Blum (*8*) agree well with those presented in this paper, except for wheat, zein, and whole egg, which are much higher. The values of Sarkar *et al.* (*12*) are low for cystine in zein.

Table VI. Comparison of Several Methods for the Determination of Cystine

(Per cent cystine. Calculated to a basis of 16% nitrogen)

Material	Chromatographic	Microbiological				Chemical (Sarkar) (12)	Differential Oxidation (3)
		30 min.	6 hr.	Horn (8)	Sarkar (12)		
Alfalfa	0.9	0.7	0.4	...	...	...	1.8
Barley	2.3	2.1	...	2.0	...	...	1.7
Casein	0.37	0.34	0.33	0.38	0.23	0.26	0.5
Corn	2.0	1.8	1.4	2.5	...	...	2.3
Cottonseed meal	2.2	1.7	...	1.7	...	...	2.4
Whole egg (defatted)	2.3	...	2.9	2.9	...	...	...
Fish meal	0.7	1.1	0.7	...	...	...	1.8
Oats	2.2	2.9	1.8	3.0	...	...	3.0
Rice	1.5	1.8	...	1.8	...	...	...
Soybean oil meal	1.3	1.5	1.0	1.9	...	...	2.0
Wheat	2.2	2.4	1.7	2.2	...	...	2.6
Wheat middlings	1.4	1.9	1.1	...	...	...	2.2
Whey	0.6	...	...	...	...	...	2.6
Zein	0.5	0.6	0.5	1.4	0.20	0.20	...

Summary

The cystine contents of a number of proteins, foods, and feeds were determined by chromatography and three microbiological procedures. Chromatography of cysteic acid gave the most reproducible results, was considered most satisfactory, and was used as the standard of comparison. Chromatography generally gave lower values than the two microbiological assay procedures using a 30-minute hydrolysis period, and the microbiological procedures gave erratic results. Most of the cystine values obtained by microbiological assay of the 6-hour digests were lower. The chromatographic procedure showed more cystine in one commercially isolated soybean protein preparation than microbiological assay, indicating a loss of cystine availability during preparation of the protein or presence of a bacterial growth inhibitor in the protein.

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Factors Affecting the Ascorbic Acid and Carotene Content of Broccoli

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The high ascorbic acid and carotene content of broccoli and the instability of these nutrients give significance to a study of factors affecting them. Investigations were made of the carotene content and of the oxidation and retention of ascorbic acid in broccoli as influenced by market form, length of storage, and cooking method. When cooked to optimum doneness, frozen and fresh broccoli retained about the same percentage of their original ascorbic acid, from 65 to 79% depending on the method of cooking used. Frozen broccoli contained only small amounts of dehydroascorbic and diketogulonic acids and no ascorbase enzyme. Frozen broccoli stored at 0° F. for 36 weeks showed only slight oxidation of ascorbic acid to dehydroascorbic and diketogulonic acids. No decrease in carotene content was found in frozen broccoli stored for 61 weeks at 0° F.

Work reported in previous publications describes the effect of cooking methods and other factors on the palatability and ascorbic acid and carotene content of fresh broccoli (*4*, *7*, *14*). Ascorbic acid was found to be rather evenly distributed between the head and the stem, but the smaller stems contained more ascorbic acid than those larger and more mature. Most of the carotene was found in the broccoli head, the content increasing with the maturity of the heads. The carotene present in the stems was concentrated chiefly in the outer portion.

In this paper are presented ascorbic acid retention values for fresh and frozen broccoli cooked by various methods and results of the effect of freezer storage and cooking of broccoli on the oxidation of ascorbic acid to dehydroascorbic acid and diketogulonic acid. Other factors investigated were the presence of reductones which interfere with the indophenol titration of ascorbic acid, and of ascorbase, an enzyme catalyzing the oxidation of ascorbic acid. The carotene content of fresh and frozen broccoli also was determined.

Experimental Methods

Materials. All broccoli was of the green sprouting type. The frozen broccoli was all of the same brand and was stored at 0° F. in a farm-type freezer. Most of it was obtained directly from the processor. That used for microwave cooking and for some of the carotene determinations was obtained at the retail market. The fresh broccoli was obtained at the retail market, iced, and stored overnight at 38° F.

Cooking Methods. A randomized block design [Cochran and Cox (*5*)] was followed for each cooking method. All cooking methods were replicated three times. Data from each exeriment were evaluated by analyses of variance. Differences between single means were examined for significance by applying the Duncan and Bonner test (*6*).

For determination of ascorbic acid retention, two 10-ounce packages of frozen broccoli were defrosted 1 hour at 72° F. Then 567-gram samples were weighed and cooked for various lengths of time by the following conventional methods:

Boiling in 236 grams of water heated to boiling before adding broccoli.

Boiling in 236 grams of water; broccoli added to cold water.

Boiling in 472 grams of water heated to boiling before adding broccoli.

Cooking by steaming (472 grams of water).

Cooking at a 15-pound steam pressure (118 grams of water).

In the study of the effect of cooking on reduced ascorbic acid, dehydroascorbic acid, and diketogulonic acid in frozen broccoli, a conventional method and a microwave method (*15*) were used. Two 10-ounce packages (567 grams) of frozen broccoli were cooked for various lengths of time by the two following methods: boiling in 236 grams of water; and microwave cooking, no water added.

Ascorbic Acid. In most of the analyses, reduced ascorbic acid was determined by the indophenol titration procedure (*2*). For investigation of the effect of cooking procedures and storage on reduced ascorbic, dehydroascorbic, and diketogulonic acids, the procedure of Roe *et al.* (*13*) was also used.

Reductones. Because any reductones present in the broccoli or produced by cooking would react with the indophenol dye used in titrating ascorbic acid, it was necessary to check for their presence. This was done by titrating in the presence of formaldehyde, as suggested by Robinson and Stotz (*12*). Results indicated that no reductones were present.

Ascorbase. Preliminary determinations of ascorbase were made by the procedure of McCombs (*9*) in which the unoxidized vitamin C is determined spectrophotometrically. This procedure was later modified by the substitution of a titration step for the photometric step, which made possible the use of larger samples for analysis.

Further work disclosed that the ascorbic acid was itself unstable in the citrate buffer used by McCombs. Accordingly, this buffer was replaced by a sodium oxalate phosphate buffer, pH 6, previously used by Ponting and Joslyn (*11*). The modified procedure used for ascorbase determination follows:

Twenty-five grams of broccoli were blended for 3 minutes with 100 ml. of cold 0.4*M* sodium chloride. The extract was diluted to 175 ml. with 0.4*M* sodium chloride, the suspension held for 2 hours at 0° C., and then centrifuged for 20 minutes at 3000 r.p.m. The supernatant liquid was decanted and kept in an ice bath until used.

The reaction mixture consisted of the following: 5 ml. of standard ascorbic acid solution containing 0.625 mg. of ascorbic acid per ml. in oxalate phosphate buffer, 35 ml. of buffer solution, and 10 ml. of centrifuged plant extract. The control mixture used was identical with the reaction mixture except for the substitution of 10 ml. of 0.4*M* sodium chloride for

10 ml. of the plant extract. The mixtures were held at 30° C. for 15 minutes. Five-milliliter aliquots were then withdrawn, diluted with 5 ml. of 1*M* oxalic acid, and titrated with 2,6-dichlorophenolindophenol in the usual manner.

Carotene. Carotene determinations were made by the procedure of Wiseman, Irvin, and Moore (*16*). So that larger samples might be tested, the following modification was used: Fifty-gram portions each of broccoli heads and stems were blended for 2 minutes at high speed with 100 grams of 95% ethyl alcohol. A 40-gram portion of the resulting slurry was then extracted as described in the Wiseman procedure. A 10-ml. aliquot was chromatographed and the eluate made to 25 ml.

Storage Study. Ascorbic acid and carotene determinations were made on frozen broccoli obtained directly from the processor and stored in a farm-type freezer at 0° F. for various periods of time. The storage time of the broccoli used in the ascorbic acid determinations ranged from approximately 2 to 36 weeks and that for carotene from 17 to 61 weeks.

Results and Discussion

Effect of Cooking Method on Ascorbic Acid. In Figure 1, *A*, are shown ascorbic acid retention values for 567-gram samples of frozen broccoli cooked by five methods. Best retention of ascorbic acid was found in methods using 236 grams of water or less. In Figure 1, *B*, are shown comparable values for ascorbic acid contained in the liquids, expressed as percentage of ascorbic acid originally in the broccoli. Retention values obtained on fresh broccoli cooked by four methods (*14*) are shown in Figure 1, *C*, for purposes of comparison. The amount of fresh broccoli used in each case was 1 pound (454 grams).

There were marked differences in the behavior of fresh and frozen broccoli with respect to retention of ascorbic acid. With frozen broccoli, the percentage of ascorbic acid retained in the solids cooked 5 minutes ranged from 66 to 77% (Figure 1, *A*). Losses of ascorbic acid in frozen broccoli cooked for periods longer than 5 minutes were not statistically significant. With fresh broccoli cooked 5 minutes in no more than 300 ml. of water, the percentage retained in the broccoli solids ranged from 80 to 91% (Figure 1, *C*). Further cooking, however, caused significant decreases in ascorbic acid content.

The relatively porous structure of the frozen broccoli resulting from the blanching and freezing seems to be responsible for the more rapid transfer of ascorbic acid in the early stages of cooking. Despite this difference in transfer rate, however, fresh and frozen broccoli, when cooked until the stems, as evaluated by panel (*7*), were tender yet firm (2 minutes by pressure and 10 minutes by the other methods), did not differ greatly in ascorbic acid retention. The range in mean values for ascorbic acid retention in broccoli was 65 to 79% for the frozen broccoli and 72 to 75% for the fresh. However, the actual mean ascorbic acid content of 39 samples of fresh broccoli ranged from 85 to 129 mg. per 100 grams with a mean of 106 mg., whereas the ascorbic acid content of 15 samples of frozen broccoli used in this portion of study ranged from 81 to 86 mg. per 100 grams with a mean of 84 mg. Therefore, the fresh broccoli was actually higher in ascorbic acid after cooking than was the frozen broccoli.

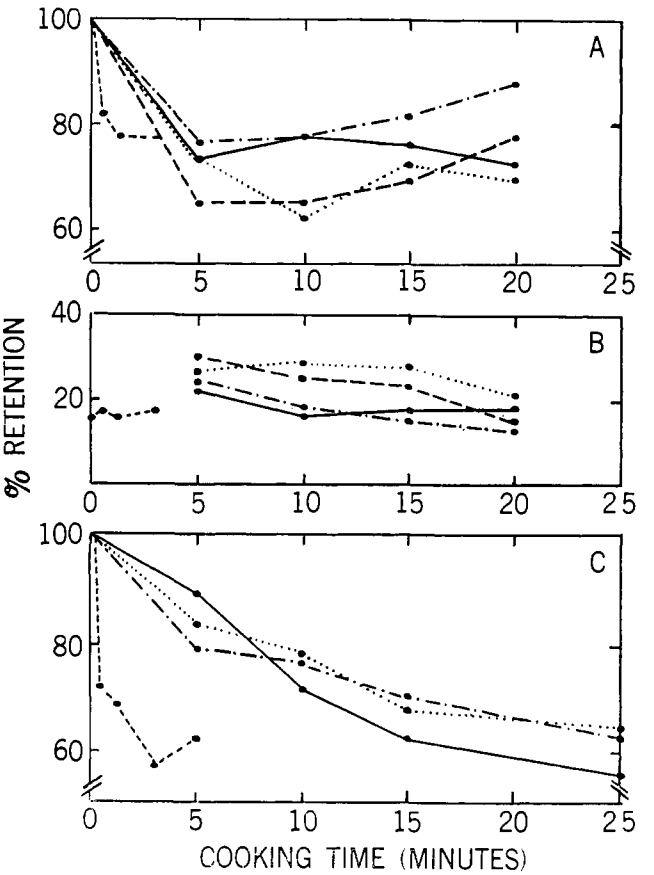

Figure 1. Retention of ascorbic acid

A. In frozen broccoli
B. In cooking liquid of frozen broccoli
C. In fresh broccoli

—— A and B, boiled in 236 grams of water (boiling start); C, 300 grams
– – A and B, boiled in 236 grams of water (cold start); C, 300 grams
— — A and B, boiled in 472 grams of water (boiling start)
– – – A, B, and C, cooked at 15-pound pressure (118 grams of water)
. . . A and B, steamed (472 grams of water); C, 600 grams

Effect of Cooking Methods on Reduced Ascorbic Acid, Dehydroascorbic Acid, and Diketogulonic Acid Content of Frozen Broccoli. Results of quantitative differentiation of reduced ascorbic acid, dehydroascorbic acid, and diketogulonic acid in frozen broccoli cooked by two methods for various periods of time are given in Table I. The values obtained for dehydroascorbic acid tended to be lower for frozen broccoli than those reported previously for fresh broccoli (*14*). Cooking, in general, caused decreases of all three compounds in both fresh and frozen broccoli. However, in some samples of fresh broccoli in which the dehydroascorbic acid content was very high there appeared to be some conversion of dehydroascorbic acid to reduced ascorbic acid on cooking, with a consequent increase in the reduced ascorbic acid content (*14*). In the frozen broccoli, the initial dehydroascorbic acid content was never great enough to cause a measurable increase in the reduced ascorbic acid content of the cooked broccoli.

Ascorbic Acid Oxidase. Ascorbic acid oxidase is shown to catalyze the oxidation from reduced to dehydroascorbic acid (*11*). Several studies have been conducted which show that maceration of plant tissue increases the dehydroascorbic acid content (*3, 9, 10*). As certain of the cooking methods investigated involved placing the fresh broccoli in cold water, it was believed advisable

Table I. Effect of Cooking Method on Content of Reduced Ascorbic Acid, Dehydroascorbic Acid, and Diketogulonic Acid in Frozen Broccoli

Sample and Cooking Method	Cooking Time, Min.	Reduced Ascorbic Acid, Mg./100 G.	Dehydroascorbic Acid, Mg./100 G.	Diketogulonic Acid, Mg./100 G.
Boiled in 236 g. water[a]				
Lot 1	0	30.8	5.2	6.2
	5	25.0	4.2	2.1
	20	22.7	6.2	2.3
Lot 2	0	79.8	5.3	8.8
	5	63.4	8.3	1.9
	10	60.6	7.9	3.8
	15	64.0	8.8	2.5
	20	56.9	7.3	4.2
Cooked by microwave, no water added				
Lot 1	0	61.9	9.1	8.5
	15	46.5	6.1	4.7
Lot 2	0	52.8	6.0	8.8
	15	37.8	5.9	4.9

[a] Water heated to boiling before adding broccoli.

Table II. Changes in Ascorbic Acid Content of Commercially Processed Frozen Broccoli Stored at 0° F. for Various Lengths of Time

Type of Sample	Storage Time at 0° F., Weeks	Reduced Ascorbic Acid, Mg./100 G.	Dehydroascorbic Acid, Mg./100 G.	Diketogulonic Acid, Mg./100 G.
Heads and stems	2	93.8	4.7	1.1
	13	79.8	5.3	8.8
	15	92.3	5.9	8.0
	17	81.0	10.9	6.0
	25	78.2	12.9	7.1
Heads	36	80.9	19.1	8.0
Stems	36	93.8	11.4	4.5

Table III. Carotene Content of Frozen and Fresh Broccoli

Description	Storage Conditions	Sample No.		Carotene Content, γ/100 G.
Frozen				
From processor	0° F., 17 weeks	1		1020
		2		1030
		3		1100
		4		1355
	0° F., 61 weeks	1		1020
		2		1070
		3		1110
		4		1150
			Mean	1107
From retail market	Overnight at 0° F.	1		640
		2		780
		3		780
		4		830
		5		840
		6		1040
		7		1060
		8		1180
			Mean	894
Fresh				
From retail market	Iced and stored overnight at 38° F.	1		620
		2		680
		3		680
		4		710
		5		740
		6		770
		7		770
		8		870
		9		890
		10		1170
		11		1180
		12		1280
			Mean	863

to determine the content of ascorbic acid oxidase so that its effect on reduced ascorbic acid might be evaluated.

Four samples of fresh broccoli tested contained sufficient ascorbase to oxidize 2.1 to 2.2 mg. of ascorbic acid. Tests on two samples of frozen broccoli showed no measurable amount of ascorbase. The absence of ascorbase in the frozen broccoli indicated that this enzyme had been destroyed by the blanching prior to freezing. The presence of ascorbase in fresh broccoli did not significantly affect ascorbic acid retention in broccoli cooked by the cold start method (Figure 1, *C*).

Stability of Ascorbic Acid and Carotene in Frozen Broccoli. As vegetables, when stored, are known to undergo changes in diketogulonic acid and in reduced and dehydroascorbic acid content, determinations of these substances were made on freshly processed frozen broccoli and on samples stored at 0° F. and withdrawn at various time intervals. The results are summarized in Table II. There appeared to be an increase in dehydroascorbic acid in the interval between 15 and 17 weeks of storage. There was an increase in diketogulonic acid between 2 and 13 weeks and a gradual decrease in reduced ascorbic acid with increased length of storage time.

As both dehydroascorbic acid and reduced ascorbic acid are biologically available, the decrease in reduced ascorbic acid is partially compensated for by the increase in dehydroascorbic acid, and the results of this study would indicate only slight decreases in ascorbic acid value after 36 weeks storage at 0° F. Determinations made separately on heads and stems after 36 weeks of storage indicated that the dehydroascorbic acid and diketogulonic acid content of the heads was higher and the reduced ascorbic acid lower in the heads than in the stems.

In Table III are shown results of carotene determinations made on frozen and fresh broccoli. Determinations on 4 samples of frozen broccoli obtained directly from the processor and stored for 17 weeks at 0° F. and 4 samples stored 61 weeks showed a range of 1020 to 1355 γ of carotene per 100 grams with a mean of 1107 and no apparent decrease in the period between 17 and 61 weeks. For eight samples obtained at the retail market the range was from 640 to 1180 γ per 100 grams with a mean of 894. The carotene content of 12 samples of fresh broccoli procured at the retail market ranged from 620 to 1280 γ per 100 grams with a mean of 863 γ.

Carotene is water insoluble and fairly stable to heat—almost 100% was retained in broccoli cooked by the methods studied (*14*). However, it is sensitive to light, auto-oxidation, and atmospheric oxygen (*1*, *8*). The lower carotene values of fresh and frozen broccoli obtained on the retail market demonstrate

this instability and indicate that carotene determinations are of importance in quality evaluation.

Acknowledgment

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β-Apo-8′-carotenal in oil solution was assayed for vitamin A activity in a series of U.S.P. vitamin A curative rat-growth assays. Combining the data from two assays yielded an average potency of 1,200,000 U.S.P. units of vitamin A per gram of all-*trans* β-apo-8′-carotenal with a range of 1,070,000 to 1,330,000 units at $P = 0.05$ or 72 ± 8% of the activity of all-*trans* β-carotene on a weight basis. Dry stabilized beadlets of β-apo-8′-carotenal were also assayed in three separate tests. Within the limits of the assay procedure no significant difference was seen in vitamin A potency. The possibility is discussed that β-apo-8′-carotenal is an intermediate product in the biological conversion of β-carotene to vitamin A.

Euler, Karrer, and Solmssen (*4*) reported β-apo-8′-carotenal to be the main product resulting from the partial oxidation of β-carotene with calcium permanganate. Daily supplements of 5 γ fed to vitamin A–deficient rats stimulated growth. Glover and Redfearn (*6*) prepared β-apo-8′-, β-apo-10′-, and β-apo-12′-carotenal and fed them to vitamin A–deficient rats. All were transformed into vitamin A. It was noted that β-apo-10′-carotenal undergoes β-oxidation to β-apo-12′-carotenal. The structural relationship of vitamin A, β-carotene, and β-apo-8′-carotenal is shown on page 391. The commonly accepted vitamin A potency of β-carotene is 1,667,000 I.U. per gram as compared to 3,333,000 for vitamin A alcohol. On the basis of the formulas of the two molecules, conversion of β-carotene to vitamin A has been postulated to proceed via hydrolytic cleavage at the central double bond. Theoretically this would be expected to yield two molecules of vitamin A from one of β-carotene. However, attempts to carry out this unusual reaction chemically have generally been very inefficient. Conversion in the animal body under most conditions yields only one rather than two molecules of vitamin A as indicated by the relative potencies given above, which are based on numerous bioassays. Under certain conditions, however, a much more efficient conversion, approaching closely the theoretically possible two molecules of vitamin A, has been reported (*3, 7, 11*).

The stepwise oxidation of β-carotene from one end, yielding successively β-apo-8′-carotenal, β-apo-10′-carotenal, β-apo-12′-carotenal, and subsequently vitamin A aldehyde, which is reduced to vitamin A, has been postulated by Glover and Redfearn (*6*) as a possible mode of its conversion to vitamin A. In support of this they observed that the hitherto unidentified substances related to the carotenoids found by Festenstein (*5*) in horse intestine had spectroscopic and chromatographic properties identical to those of the β-apo-10′- and β-apo-12′-carotenals. Winterstein (*16*) isolated β-apo-8′-carotenal from oranges, tangerines, and spinach and demonstrated the identity of the naturally occurring substance with synthetic β-apo-8′-carotenal.

Isler *et al.* (*8*) synthesized β-apo-8′-carotenal as part of an intensive program to produce synthetically many of the naturally occurring carotenoids. This carotenoid has very desirable properties for coloring foods and beverages. Also, as a feed ingredient, it has been reported by Steinegger, Streiff, and Zeller (*14*), Steinegger and Zanetti (*15*), and Marusich, Kadin, and Bauernfeind (*13*) to be an efficient pigmenter for coloring egg yolks uniformly with a pleasing yellow equal to that obtained with high quality ingredients rich in natural xanthophylls.

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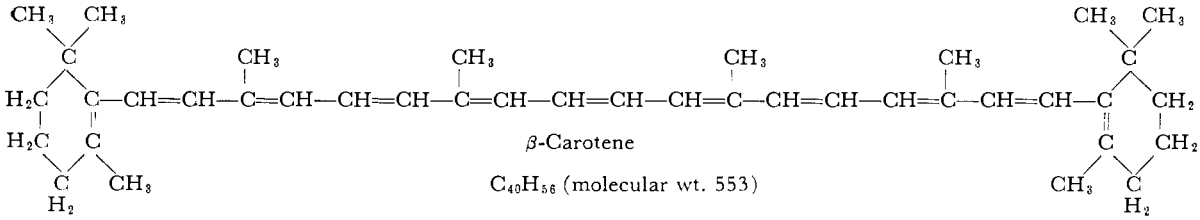
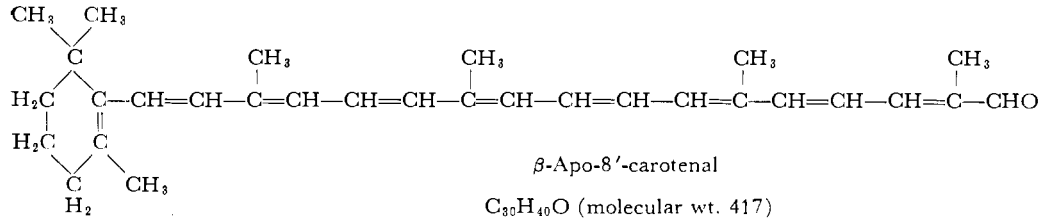
Structural Formulas



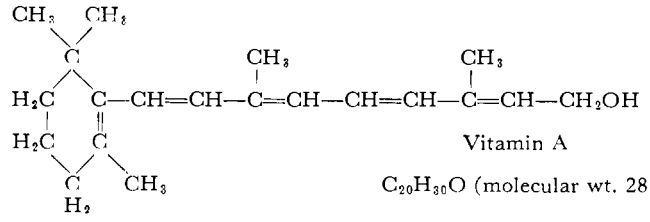
β-Carotene

$C_{40}H_{56}$ (molecular wt. 553)



β-Apo-8′-carotenal

$C_{30}H_{40}O$ (molecular wt. 417)



Vitamin A

$C_{20}H_{30}O$ (molecular wt. 286)

In view of such potential applications of β-apo-8′-carotenal in foods and feeds, its vitamin A activity has been determined quantitatively by means of the U.S.P. rat curative growth test. Because this carotenoid resembles β-carotene and many other carotenoid pigments in being readily destroyed by oxidative processes, it has been found desirable to stabilize it by incorporation into gelatin-sugar beadlets similar to the vitamin A and carotene beadlets which have gained wide acceptance in the food and feed fields. Therefore dry beadlets containing β-apo-8′-carotenal were included in the present rat bioassay in addition to oil solutions of the pure crystalline material synthesized by the method of Isler *et al.* (*8*).

Chemical Assay of β-Apo-8′-carotenal

Crystalline all-*trans* β-apo-8′-carotenal or oil solutions thereof are assayed by dissolving in petroleum ether (Skellysolve B), making the appropriate dilutions, and reading in a Beckman DU spectrophotometer at the maximum at 454 to 455 mμ. An $E^{1\%}_{1cm.}$ of 2640 is used in calculating the potency of the sample.

In the assay of mixtures of cis and trans stereoisomers of β-apo-8′-carotenal, such as are present in the dry beadlet preparations used in this study, a somewhat different procedure is used. The method of assay utilizes the technique described by Bunnell, Driscoll, and Bauernfeind (*2*) for β-carotene preparations, which involves isomerization to an equilibrium mixture. The procedure for assay is the same as reported in this paper, except that the sample is read in the Beckman spectrophotometer in petroleum ether (Skellysolve B) at the maximum at 454 to 455 mμ and potency is calculated using an $E^{1\%}_{1cm.}$ of 2530.

The latter extinction is the value for the stereoisomeric mixture determined experimentally by iodine isomerization. This value gives the best approximation of the total β-apo-8′-carotenal content that can be made without the elaborate and time-consuming separation and individual determination of all the stereoisomers.

Biological Assay of β-Apo-8′-carotenal

An oil solution of β-apo-8′-carotenal and stabilized gelatin-sugar beadlets containing 2.53% β-apo-8′-carotenal were compared to the U.S.P. vitamin A reference oil in two successive assays. The same beadlet preparation plus a second containing 7.18% β-apo-8′-carotenal was tested in a third assay using both the U.S.P. vitamin A reference oil and a dry stabilized vitamin A beadlet as the standard. For all assays, the oil solutions of β-apo-8′-carotenal and vitamin A standard were dosed orally. Each rat was given the appropriate dosage in 0.1 ml. of cottonseed oil every fourth day for 4 weeks using a 1-ml. tuberculin syringe with a blunt-tipped needle. The β-apo-8′-carotenal beadlets were fed in the diet in all assays, as were the vitamin A beadlets in the third assay. An amount of both substances equivalent to that dosed orally in 0.1 ml. was added to 40 grams of the U.S.P. vitamin A–free ration. The rats were given access to feed *ad libitum* and feed intake was recorded. The levels fed, number of rats per group, growth responses, and calculated potencies of β-apo-8′-carotenal are shown in Table I for the three assays.

Discussion of Results

The potencies obtained for β-apo-8′-carotenal in the three assays are in good agreement. To determine whether overall average potencies and error limits could be calculated, the data for the three assays of the beadlets and those for the two assays of oil solution were tested for homogeneity by the X^2 test (*1*). As the potencies obtained in the individual assays were found to be homogeneous at the 5% level, the data were combined. The composite vitamin A value obtained for the stabilized beadlets is 1,070,000 U.S.P. units per gram of β-apo-8′-carotenal with a range of 950,000 to 1,190,000 units at $P = 0.05$. In oil solution, 1 gram of β-apo-8′-carotenal has vitamin A activity equal to 1,200,000 U.S.P. units with a range of 1,070,000 to 1,330,000 units.

It is of interest to compare these values with the vitamin A activity of all-*trans* β-carotene, which has yielded an average potency of 1 I.U. per 0.58 γ in repeated rat-growth assays in this laboratory under the conditions used in the present

Table I. Vitamin A Activity of β-Apo-8′-carotenal in Oil Solution and Stabilized Beadlets

Supplement	Mode of Administration	No. of Rats Surviving	Supplement per Rat per 4 Days	Weight Gain in 28 Days, G.	Calcd. Vitamin A Activity, U.S.P. Units per Gram β-Apo-8′-carotenal: Potency	Range of potency at P = 0.05
Assay 1			*U.S.P. Units*			
U.S.P. vitamin A reference oil	Oral	6	3.2	25		
		10	4.7	37		
		13	6.9	51		
			Micrograms[a]			
Oil solution of β-apo-8′-carotenal	Oral	10	3.8	31	1,150,000	960,000–1,400,000
		12	5.6	44		
		14	8.3	84		
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	8	3.1[b]	33		
		12	5.0	52	1,000,000	700,000–1,400,000
		14	8.4	75		
Assay 2			*U.S.P. Units*			
U.S.P. vitamin A reference oil	Oral	34	4.1	34		
		27	6.0	42		
		34	8.9	65		
			Micrograms			
Oil solution of β-apo-8′-carotenal	Oral	30	4.9	44		
		31	7.2	66	1,200,000	1,080,000–1,340,000
		35	10.7	85		
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	12	4.3	34		
		16	6.6	49	1,060,000	940,000–1,190,000
		32	11.5	85		
Assay 3			*U.S.P. Units*			
Stabilized vitamin A beadlet	In diet	14	4.9	26		
		17	7.4	42		
		21	12.4	68		
U.S.P. vitamin A reference oil	Oral	15	4.2	14		
		20	6.1	39	107[c]	91–125%
		11	9.0	58		
			Micrograms			
Stabilized beadlets β-apo-8′-carotenal (2.53%)	In diet	8	5.1	25		
		10	7.8	44	940,000	760,000–1,160,000
		9	12.4	63		
Stabilized beadlets β-apo-8′-carotenal (7.18%)	In diet	10	5.1	19		
		11	8.0	40	930,000	830,000–1,110,000
		12	13.1	74		

[a] Weight of β-apo-8′-carotenal in supplement.
[b] Av. per 4 days calculated from total feed intake over 28-day assay period for all in-diet feeding.
[c] % of in-diet standard response.

assays (*12*). This value is very close to the International Standard value of 1 I.U. per 0.6 γ. In oil solution all-trans β-apo-8′-carotenal has 72% (range at $P = 0.05$: 64 to 80%) of the activity of all-trans β-carotene. The beadlets in which the β-apo-8′-carotenal is partially stereoisomerized are 64% (range at $P = 0.05$: 57 to 71%) as active as all-trans β-carotene. While the differences between the oil solution and beadlet form are not statistically significant, the somewhat lower activity of the beadlets could be due to the presence of less active stereoisomers formed in manufacturing. This difference may also be influenced by the fact that the accuracy and precision of the assay procedure are less for the beadlets than for oil solutions of the pure trans isomer.

The conversion of β-carotene to vitamin A via β-apo-8′-carotenal would require cleavage of the β-ionone ring from one end of the molecule. This has been achieved chemically by Karrer and his coworkers (*9, 10*) by permanganate treatment. The fact that β-apo-8′-carotenal has considerable vitamin A activity has led to speculation (*6*) that conversion of β-carotene to vitamin A in vivo may proceed by such cleavage of one ionone ring followed by β-oxidation to vitamin A aldehyde, which is reduced to vitamin A. If β-apo-8′-carotenal were totally converted to vitamin A, it would have a potency of 2,280,000 I.U. per gram. The actual vitamin A potency in the rat as determined in the present assays is only 53% of this theoretical value. The fact that all-trans β-apo-8′-carotenal has less vitamin A activity than all-trans β-carotene even on a weight basis suggests the existence of an important route or routes of conversion of β-carotene to vitamin A other than that postulated above. The formation under certain conditions (*3, 7, 11*) of more than one molecule of vitamin A from one molecule of carotene would require an alternate metabolic pathway.

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ENZYME INHIBITION

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KENDALL (*3*) reported that in vitro hydrolysis of casein by trypsin is inhibited by aqueous extracts of the fresh forage of alfalfa, ladino clover, and soybeans. Beauchene and Mitchell (*1*) detected a similar inhibition of trypsin by extracts of commercial dehydrated alfalfa, indicating that the inhibitory substance withstands the heat of the dehydration process.

A trypsin inhibitor in alfalfa meal conceivably could be partially responsible for the growth depression which has been observed when alfalfa meal is added to broiler rations at levels of 10% or more. Investigation of this possibility will require isolation of the inhibitor in sufficient quantity for in vivo studies. This report is concerned with a method of isolating the trypsin inhibitor and attempts to determine its chemical nature.

Experimental

The inhibitory activity of extracts of alfalfa meal, and of the fractions obtained from them, was determined by measuring the decrease in the amount of amino acids and peptides released during the in vitro digestion of casein by trypsin when the extracts or fractions were present. Amino acids and peptides were determined by a modification of the method of Spies and Chambers (*6*), which depends on the reaction of these substances with cupric ions under proper conditions to produce complexes having a blue color.

Measurement of Inhibition. A modification of the procedure of Beauchene and Mitchell (*1*) was used.

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Beaker	Mixture
1	Casein, trypsin
2	Casein, inactivated trypsin
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4	Casein, inactivated trypsin, inhibitor

The beakers were covered with small watch glasses and placed in a water bath maintained at 37° C. After 4 hours, 5 ml. of the solutions were placed in test tubes and the undigested casein was precipitated by adding 5 ml. of a 10% trichloroacetic acid solution. The samples were filtered and the filtrates were adjusted to pH 7 with 40% sodium hydroxide. Five milliliters of each filtrate were placed in 15-ml. graduated centrifuge tubes and 3 ml. of a suspension of copper phosphate in pH 9.1 borate buffer were added. The contents of the tubes were mixed, allowed to stand 5 minutes, and centrifuged at 2000 r.p.m. to sediment the excess copper phosphate. The intensity of the blue color of the supernatant solutions was measured at 620 mμ with a Beckman DU spectrophotometer. A standard curve was prepared by carrying known quantities of alanine through the procedure.

Isolation of Trypsin Inhibitor. Preliminary extraction studies were performed by extracting 25-gram portions of commercial dehydrated alfalfa meal in a Waring Blendor with 200 ml. of various solvents. After blending for 10 minutes, the samples were filtered through a cloth held in a Büchner funnel. When organic solvents were used, the solvent was removed by evaporation on a steam plate under a current of air from an electric fan, and the residue was extracted with water. The final volume in each case was adjusted to 200 ml. and the solutions were centrifuged.

Dialyzability of the inhibitor was determined by placing a water extract in a cellophane dialysis bag and dialyzing against distilled water for several days. The water was changed frequently, and the dialyzates so obtained were combined and concentrated to the same volume as the original extract.

The inhibitory activities of the various extracts are shown in Table I, from which it is apparent that the inhibitor was soluble in water but was not extracted by acetone or 95% ethyl alcohol. However, aqueous ethyl alcohol extracted the substance, with a progressive increase in inhibition as the alcohol concentration was decreased. The dialyzed extract was as inhibitory as the undialyzed extract, while the dialyzate was noninhibitory. These properties were utilized in the procedure which was adopted for isolation of the inhibitor.

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Table I. Effect of Extracting Solvent, Alcohol Precipitation, and Dialysis on Inhibitory Activity as Measured by Inhibition of in Vitro Hydrolysis of Casein by Trypsin

Preparation Added to Casein Solution	Amino Nitrogen, Mg. per Tube after Incubation	Inhibition, %
None	1.09	0
Acetone extract	1.07	2
Ethyl alcohol extract		
95%	1.08	2
60%	0.71	35
40%	0.68	38
Water extract	0.62	43
Dialyzed water extract	0.63	42
Dialyzate	1.06	2
Supernatant after alcohol precipitation of water extract	1.08	2

five grams of meal and 200 ml. of water were placed in the cup of a Waring Blendor and the Blendor was operated at half speed for 10 minutes. The resulting slurry was filtered through a cloth supported in a Büchner funnel, and the residue was washed several times with water. When low temperature meal was used, the filtrate was heated almost to boiling and the coagulated protein was removed by centrifugation. This step was not necessary when commercial meal was used, as it does not contain water-soluble protein (*2*). However, extracts of the latter usually were turbid, and filtration under vacuum through Whatman No. 50 paper covered with a layer of Supercel was used to clarify them. The protein-free amber colored filtrate was concentrated to a small volume under reduced pressure at 40° C. The concentrate was placed in a cellophane dialysis sack and was dialyzed against continuously replenished tap water for 12 hours. A portion of the amber color was eliminated in this manner. A slight turbidity which developed during dialysis was eliminated by centrifuging at 14,000 r.p.m. for 30 minutes.

The clear amber solution again was concentrated to about 30 ml. and 250 ml. of ethyl alcohol were added. After standing in a refrigerator for a few hours, the precipitate which formed was collected by centrifugation. On drying, the precipitate was amber and had a glassy appearance. About 0.6 gram was obtained per 100 grams of alfalfa meal. It was readily soluble in water and could be purified further by repeating the centrifugation, dialysis, and alcohol precipitation. The amber color was never completely eliminated, however.

The material which was obtained by this procedure strongly inhibited the in vitro digestion of casein by trypsin.

Table II. Activity of Trypsin Inhibitor of Alfalfa

Substances Added to Casein Solution	Absorbance at 620 Mμ	Amino Nitrogen, Mg. per Tube after Incubation	Net	Inhibition, %
Trypsin	0.215	1.10	1.09	0
Boiled trypsin	0.003	0.01	...	..
Trypsin, 2 ml. of extract[a]	0.190	0.9	0.62	43
Boiled trypsin, 2 ml. of extract[a]	0.070	0.34	...	..
Trypsin, 4 mg. of isolated inhibitor	0.114	0.58	0.56	49
Boiled trypsin, 4 mg. of isolated inhibitor	0.005	0.02	...	..

[a] 25 grams of meal per 200 ml. of water.

The activity of a typical preparation is shown in Table II.

Heat Stability. Although extracts of commercial dehydrated alfalfa meal are inhibitory (*1*), it should not be assumed that the inhibitor is heat stable. It is possible that the duration of heating during dehydration is too short to have an appreciable effect on the inhibitor.

Heat stability of the isolated inhibitor was studied by dissolving it in water and heating the solution at 98° C. on a steam plate. Equal aliquots were removed after 0, 0.5, 1, 3, and 7 hours of heating, and were tested for inhibitory power. The results (Table III) show that inhibitory activity had decreased markedly after 0.5 hour, and was almost completely absent after 3 hours. It was concluded that, although the substance is heat labile, the time required for drying alfalfa commercially is too short for inactivation to occur.

Electrophoretic Behavior. One hundred milligrams of the inhibitor were dissolved in pH 8.6 borate buffer, and the solution was subjected to a current of 20 ma. for 30 minutes in an Aminco portable electrophoresis apparatus. Two components were detected under these conditions, indicating a lack of homogeneity of the inhibitor as isolated by the procedure employed.

Nature of Inhibition. The nature of the inhibition was studied by measuring the effect of a given concentration of inhibitor with varying concentrations of substrate. A 5% casein solution was prepared by dissolving 15 grams of casein in 300 ml. of pH 8.4 phosphate buffer and adjusting the pH to 8.4 with a few drops of 40% sodium hydroxide solution. Aliquots of this solution were diluted with buffer to yield solutions of 0.5, 1, 2, 3, and 4% casein. A trypsin solution was prepared by dissolving 30 mg. of the enzyme in 100 ml. of water. The inhibitor was dissolved in pH 8.4 buffer so that 5 ml. of the solution contained 25 mg. of the inhibitor. Three reaction mixtures were prepared in 100-ml. flasks for each substrate concentration:

Flask A. 20 ml. of casein, 5 ml. of buffer, 2 ml. of trypsin

Table III. Effect of Heating at 98° C. on Activity of Inhibitor

Time of Heating, Hr.	Inhibition, %
0	35
0.5	13
1	11
3	2
7	0

Flask B. 20 ml. of casein, 5 ml. of inhibitor, 2 ml. of trypsin

Flask C. 20 ml. of casein, 5 ml. of inhibitor, 2 ml. of boiled trypsin

The reaction flasks were placed in a water bath maintained at 37° C. The enzyme solution was added at zero time and a 5-ml. aliquot was withdrawn and added to 5 ml. of 10% trichloroacetic acid to stop the reaction. Additional aliquots were treated similarly after 2 hours. Each sample so obtained was allowed to stand 15 minutes and was filtered through dry filter paper. The pH was adjusted to 7, and 5 ml. of the resulting filtrate were used for amino acid determination. Flask C was used as a blank to correct for small quantities of free amino acids in the casein and enzyme.

The analytical data are presented in Table IV. From these values a plot of $1/S$ and $1/V$ was prepared (Figure 1) according to the double reciprocal method of Lineweaver and Burk (*4*). Failure of the two lines to intersect

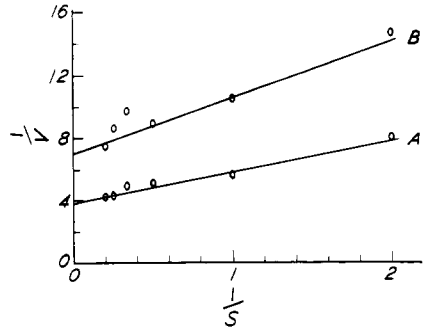

Figure 1. Double reciprocal plot of the data of Table IV

A. Inhibitor absent B. Inhibitor present

Table IV. Effect of Inhibitor and Substrate Concentration on Hydrolysis of Casein by Trypsin

	A. Inhibitor Absent						B. Inhibitor Present					
Casein concentration, S, %	5	4	3	2	1	0.5	5	4	3	2	1	0.5
Absorbance at 620 mμ after 2 hours at 37° C., V	0.238	0.237	0.201	0.196	0.178	0.123	0.133	0.115	0.102	0.112	0.095	0.068
$1/S$	0.20	0.25	0.33	0.5	1	2	0.20	0.25	0.33	0.5	1	2
$1/V$	4.2	4.2	5.0	5.1	5.6	8.1	7.5	8.7	9.8	9.0	10.5	14.7

indicates that the inhibition was noncompetitive.

Chemical Nature of Inhibitor. Because the active principle was precipitated from water by dilution with a relatively large volume of ethyl alcohol, it was thought that it might be inorganic in nature. However, this was shown not to be true when a portion of the isolated substance was ashed and the residue was found to be noninhibitory.

The inhibitor probably is not a heat-coagulable protein, as coagulable material was eliminated during isolation. However, it could be a polypeptide with a sufficiently low molecular weight not to coagulate with heat. This possibility was investigated by refluxing 5 mg. of the isolated material with 6N hydrochloric acid overnight. The hydrochloric acid was removed by repeated distillation at reduced pressure at 40° C. The final residue was dissolved in 1 ml. of water and 25 μl. were chromatographed on Whatman No. 1 filter paper, using 8 to 2 phenol-water as the developing solvent.

When the paper was sprayed with ninhydrin, several sharply defined spots were observed. A similar chromatogram of unhydrolyzed inhibitor gave no spots with ninhydrin. Hence, the spots obtained with the hydrolyzate were due to amino acids released during hydrolysis.

Although the data suggest the inhibitor is a polypeptide, it is recognized that the amino acids might have arisen from hydrolysis of a polypeptide impurity. Further investigation of its purity will be needed before it can be characterized unequivocally.

Discussion

It is necessary to include adequate blanks when assaying various preparations for inhibitory activity by the method employed in this work. Water extracts of alfalfa contain free amino acids,and a correction for them must be made when enzymic hydrolysis of casein in the presence of an extract is used to measure the degree of inhibition. Furthermore, extracts of dehydrated plant tissue are amber in color. Often the intensity of this color is sufficient to cause appreciable light absorption at the wave length used to measure the blue color of the amino acid–copper complex.

Inspection of Table II will indicate the variability of blanks. Both casein and trypsin are essentially devoid of free amino acids, and a very low absorbance was obtained in the boiled trypsin experiment. When the plant extract was added, a marked increase in absorbance of the blank resulted. The addition of 4 mg. of the isolated inhibitor had essentially no effect on absorbance. Hence, dialysis and ethyl alcohol precipitation eliminated the free amino acids and much of the substances responsible for the amber color.

Isolation of the trypsin inhibitor will permit in vivo studies to determine whether it is involved in chick growth depression. If the inhibitor were altered while in the crop or gizzard, it might no longer inhibit trypsin in the intestinal tract. Crude extracts are not suitable for such tests, because other constituents of alfalfa are known to inhibit growth (*5*).

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MEASUREMENT OF URINARY ANDROGENS

The Effect of Interfering Chromogens in Spectrophotometric Determination of Ruminant Urinary Androgens

W. S. RULIFFSON, A. M. GAWIENOWSKI, and J. S. HUGHES

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As has been demonstrated, androgenic 17-ketosteroids (17-KS) frequently appear in the urine, originating in most mammals as steroid metabolites of either the testes or adrenal glands (*3*). Of these compounds, androsterone [androstane-3 (α)-ol-17-one] and dehydroepiandrosterone [Δ^5-androstene-3(β)-ol-17-one], or androstenolone, as recently suggested (*4*), are not only produced in the greatest amounts, but are also among those which are the most active biologically.

The quantitative determination by various spectrophotometric methods of these and related compounds, all of which are frequently referred to as "urinary androgens," has classically been interpreted as a measure of testicular and/or adrenal cortical function.

Among the spectrophotometric methods are the Zimmerman (*5*), Pincus (*9*), and Allen (*1*) reactions, each of which produces chromogens having characteristic absorption maxima in the visible wave length range. These reactions have been employed by many workers to study the 17-KS content of urines isolated from a variety of subjects (*3*). However, certain nonsteroid ketones as well as non-17-KS present in biological materials probably confound the interpretation of results obtained as a consequence of using the chemical methods of 17-KS assay.

Only relatively recently has the probable nature of at least one group of these compounds been recognized (*6*) as ionone derivatives. In addition, cow urine has been shown to contain a group of chromogens (*7*) which very probably are ionone derivatives arising from dietary carotenoids. These compounds are ex-

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Absorbance at 620 mμ after 2 hours at 37° C., V	0.238	0.237	0.201	0.196	0.178	0.123	0.133	0.115	0.102	0.112	0.095	0.068
$1/S$	0.20	0.25	0.33	0.5	1	2	0.20	0.25	0.33	0.5	1	2
$1/V$	4.2	4.2	5.0	5.1	5.6	8.1	7.5	8.7	9.8	9.0	10.5	14.7

indicates that the inhibition was noncompetitive.

Chemical Nature of Inhibitor. Because the active principle was precipitated from water by dilution with a relatively large volume of ethyl alcohol, it was thought that it might be inorganic in nature. However, this was shown not to be true when a portion of the isolated substance was ashed and the residue was found to be noninhibitory.

The inhibitor probably is not a heat-coagulable protein, as coagulable material was eliminated during isolation. However, it could be a polypeptide with a sufficiently low molecular weight not to coagulate with heat. This possibility was investigated by refluxing 5 mg. of the isolated material with 6N hydrochloric acid overnight. The hydrochloric acid was removed by repeated distillation at reduced pressure at 40° C. The final residue was dissolved in 1 ml. of water and 25 μl. were chromatographed on Whatman No. 1 filter paper, using 8 to 2 phenol-water as the developing solvent.

When the paper was sprayed with ninhydrin, several sharply defined spots were observed. A similar chromatogram of unhydrolyzed inhibitor gave no spots with ninhydrin. Hence, the spots obtained with the hydrolyzate were due to amino acids released during hydrolysis.

Although the data suggest the inhibitor is a polypeptide, it is recognized that the amino acids might have arisen from hydrolysis of a polypeptide impurity. Further investigation of its purity will be needed before it can be characterized unequivocally.

Discussion

It is necessary to include adequate blanks when assaying various preparations for inhibitory activity by the method employed in this work. Water extracts of alfalfa contain free amino acids,and a correction for them must be made when enzymic hydrolysis of casein in the presence of an extract is used to measure the degree of inhibition. Furthermore, extracts of dehydrated plant tissue are amber in color. Often the intensity of this color is sufficient to cause appreciable light absorption at the wave length used to measure the blue color of the amino acid–copper complex.

Inspection of Table II will indicate the variability of blanks. Both casein and trypsin are essentially devoid of free amino acids, and a very low absorbance was obtained in the boiled trypsin experiment. When the plant extract was added, a marked increase in absorbance of the blank resulted. The addition of 4 mg. of the isolated inhibitor had essentially no effect on absorbance. Hence, dialysis and ethyl alcohol precipitation eliminated the free amino acids and much of the substances responsible for the amber color.

Isolation of the trypsin inhibitor will permit in vivo studies to determine whether it is involved in chick growth depression. If the inhibitor were altered while in the crop or gizzard, it might no longer inhibit trypsin in the intestinal tract. Crude extracts are not suitable for such tests, because other constituents of alfalfa are known to inhibit growth (*5*).

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MEASUREMENT OF URINARY ANDROGENS

The Effect of Interfering Chromogens in Spectrophotometric Determination of Ruminant Urinary Androgens

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As has been demonstrated, androgenic 17-ketosteroids (17-KS) frequently appear in the urine, originating in most mammals as steroid metabolites of either the testes or adrenal glands (*3*). Of these compounds, androsterone [androstane-3 (α)-ol-17-one] and dehydroepiandrosterone [Δ^5-androstene-3(β)-ol-17-one], or androstenolone, as recently suggested (*4*), are not only produced in the greatest amounts, but are also among those which are the most active biologically.

The quantitative determination by various spectrophotometric methods of these and related compounds, all of which are frequently referred to as "urinary androgens," has classically been interpreted as a measure of testicular and/or adrenal cortical function.

Among the spectrophotometric methods are the Zimmerman (*5*), Pincus (*9*), and Allen (*1*) reactions, each of which produces chromogens having characteristic absorption maxima in the visible wave length range. These reactions have been employed by many workers to study the 17-KS content of urines isolated from a variety of subjects (*3*). However, certain nonsteroid ketones as well as non-17-KS present in biological materials probably confound the interpretation of results obtained as a consequence of using the chemical methods of 17-KS assay.

Only relatively recently has the probable nature of at least one group of these compounds been recognized (*6*) as ionone derivatives. In addition, cow urine has been shown to contain a group of chromogens (*7*) which very probably are ionone derivatives arising from dietary carotenoids. These compounds are ex-

The presence of nonsteroidal ketones and non–17-ketosteroid chromogens in biological materials, particularly urine of ruminants, has been recognized for some time. The possible extent of interference of these materials in the spectrophotometric determination of 17-ketosteroids remains to be defined completely. This investigation indicates that: The effect of these urinary chromogens is to displace toward the shorter wave lengths the absorption maxima of the chromogens produced by treating known 17-ketosteroids according to the Allen, Pincus, and Zimmerman reactions; the effect of the urinary chromogens in each of these reactions is to produce an intensity of color equal to or greater than that produced by similar treatment of known 17-ketosteroid alone; and the absorption maxima produced by urinary chromogens in the presence of known 17-ketosteroid are due to an apparent additive effect and in every case are higher than that produced by an equal amount of known ketosteroid alone.

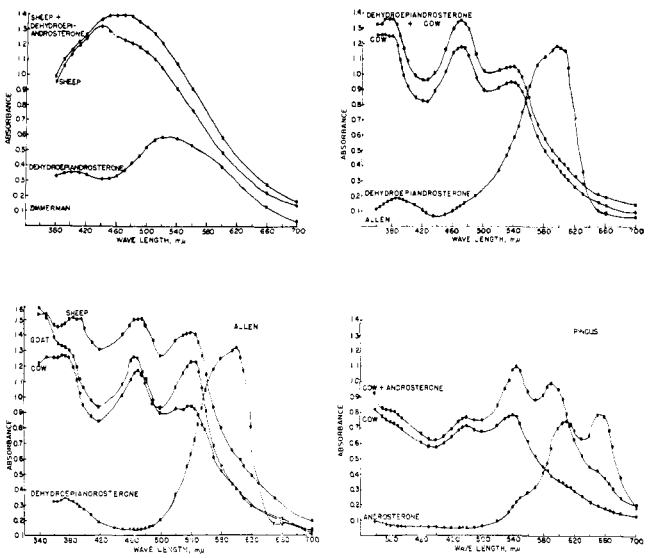

Figure 1. Distinctive and reproducible curves, each containing characteristic maxima, result when each of the three types of reactions (Allen, Pincus, and Zimmerman) is conducted in the presence of neutral extracts of urine plus known amounts of pure 17-KS

tractable by the same procedures as those used for the isolation of urinary androgens, but give different spectra when subjected to the conditions of the Allen, Pincus, and Zimmerman reactions.

This paper presents data which indicate that non–17-KS chromogens not only confound spectrophotometric determinations of urinary androgens, but their presence may actually enhance the response of the very small amounts which may be inherently present, or which are intentionally added.

The experimental procedures and/or results described herein are not claimed to be amenable to practical application. However, the results do have an important bearing on the general problem of recognizing that even standard and well-accepted bench procedures may have certain pitfalls which need to be recognized by workers in a variety of fields.

The mechanism of the effect of crystalline steroid hormones and of certain hormonally active synthetic compounds on endogenously produced hormones and on tissue anabolism needs considerable clarification. The natural products whose level in biological media the methods described in this paper are presumed to measure, play an important role not only in the maintenance of most mammals, including livestock, but also in the production of certain edible products derived from them.

Methods

Neutral extracts of urine were prepared according to the acid hydrolysis method of Mixner (*7*), employing 15% hydrochloric acid (v./v.) and a 10-minute boiling period.

The color-developing reactions employed in this study were those of Zimmerman, as modified by Holtorff and Koch (*5*), Pincus (*9*), and Allen (*1*). Five milliliters of urine equivalent were used in the Zimmerman reaction, 10 ml. in the Pincus reaction, and 5 ml. in the Allen reaction. A Beckman DU spectrophotometer was used for all measurements.

Table I. Chick Comb Bioassay of Androgens

Steroid Treatment, γ	*Comb Weight, Mg. / Body Weight, G.*
Control	0.61
Androsterone, 28	1.17
Androsterone, 56	1.49
Androsterone, 84	1.58
Unknown I	0.60
Unknown II	0.55

Biological assays to determine the androgenic potency of the neutral extracts of urine were performed by employing the chick comb test as modified by Munson (*8*). Reference standards and unknowns were dissolved in absolute alcohol. Three different levels, 4, 8, and 12 γ of the standard (androsterone), were applied daily for a 7-day period. The unknowns were neutral extracts of two different wether urines applied in amounts equivalent to 10 ml. of urine. Absolute ethyl alcohol was used as the control. Each group of day-old chicks contained 10 individuals of approximately equal size.

Ovine urine collection was accomplished by modifying the apparatus of Dick and Mules (*2*). Instead of using a specially manufactured rubber collection bag, a hemisphere made of an ordinary plastic beach ball was employed. Bovine urine collection was accomplished by conventional methods.

Results

The absorption spectrum produced by the Zimmerman reaction (Figure 1, upper left) treatment of dehydroepiandrosterone is characterized by a maximum at approximately 520 mμ. In the presence of neutral extracts of ovine urine, the maximum becomes broadened and undergoes a shift to 440 mμ. When the reaction is conducted in the presence of neutral extracts of urine to which known amounts of pure 17-K S(65

γ) have been added, the maximum is fairly broad and still displaced to 440 to 470 mμ.

The absorption spectrum of the Allen reaction (Figure 1, upper right; lower left) in the presence of dehydroepiandrosterone (65 γ) demonstrates a remarkably distinct and prominent maximum at 600 mμ and a much less prominent absorption at 380 mμ. In the presence of neutral extracts of bovine urine, this reaction demonstrates absorption maximum at 370, 465, and 540 mμ, respectively (Figure 1, upper right, lower left). These same effects are noted when neutral extracts of wether and goat urine are employed (Figure 1, lower left). When the reaction is performed in the presence of a neutral extract of bovine urine to which has been added 50 γ of dehydroepiandrosterone (Figure 1, upper right), the urinary chromogens completely obscure the maxima characteristic of the steroid alone, but are still consistent with the urinary, neutral extract spectra.

The absorption spectrum of the Pincus reaction (Figure 1, lower right) in the presence of androsterone (150 γ) demonstrates a prominent double peak, the first wave of which is at 610 mμ and the second, a slightly higher wave, at 660 mμ. In addition, there is a slight, although definite, indication of absorption occurring at 545 mμ. When this reaction is performed in the presence of a neutral extract of bovine urine, two obvious maxima located at 470 and 540 mμ are discernible. When a mixture of a neutral extract of bovine urine and known 17-KS is subjected to this reaction, three distinct maxima at 470, 545, and 590 mμ, respectively, become obvious. In addition, there is a faint, but again a definite, peak at about 650 mμ.

Biological assays (Table I), employing the chick comb test indicate that neutral extracts of wether urine contain few, if any, androgenic ketones, despite the characteristic, albeit displaced, maxima demonstrable by spectrophotometric methods.

Discussion

In the case of the Zimmerman reaction, interfering materials in the neutral extracts of urine largely displace the absorption maximum characteristic of a known 17-KS (dehydroepiandrosterone) in the presence of this reaction. In the case of the Allen reaction, the prominent maximum occurring at 600 mμ, when pure steroid (dehydroepiandrosterone) is subjected to the reaction conditions, completely disappears when the neutral urine extract containing steroid is treated likewise. In the case of the Pincus reaction, not only is there apparent displacement of the maxima which are characteristic of the pure steroid (androsterone) in the presence of this reaction, but also virtually complete disappearance of the second prominent wave at 660 mμ.

These data indicate that the characteristic absorption spectra may be obtained employing the Allen, Pincus, and Zimmerman reactions in the presence of pure 17-KS, neutral extracts of urine, and neutral extracts of urine to which have been added known amounts of pure 17-KS. In each case, however, the absorption spectra, although characteristic, are different. Moreover, unidentified compounds, normally present in bovine, ovine, and goat urine, not only may be responsible for the shifts in maxima which have been demonstrated, but also may serve to enhance or otherwise confound the effect of small amounts of 17-KS present either normally or as a consequence of intentional addition. Thus, it would appear that small amounts of endogenous 17-KS would not be detectable in neutral extracts of ruminant urine which also contain these interfering substances, when subjected to the reaction conditions of any of these methods.

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FUNGICIDE EVALUATION

Fungicidal Activity of Some New Amino Alcohols Synthesized from Citrus (+)-Limonene

ROGER PATRICK and W. F. NEWHALL

College of Agriculture, Agricultural Experiment Stations, University of Florida, Gainesville, Fla.

The synthesis of several new amino alcohols from (+)-limonene has been reported (*2*). Partial hydrogenation involving the exocyclic double bond of (+)-limonene followed by oxidation of the endocyclic double bond with peracetic acid afforded *p*-menthane-1,2-epoxide. The epoxide ring was readily cleaved by ammonia to give the mixed trans isomers of 2-amino-1-*p*-menthanol (I). Cleavage of the epoxide by methylamine and dimethylamine afforded the mixed trans isomers of 2-methylamino-1-*p*-menthanol (II) and 2-dimethylamino-1-*p*-menthanol (III), respectively. Since this work was reported, an additional new amino alcohol, 2-dimethylamino-$\Delta^{8(10)}$-*p*-menthen-1-ol (IV), has been prepared by cleavage of $\Delta^{8(10)}$-*p*-menthene-1,-2-epoxide by dimethylamine. This series of amino alcohols was originally prepared for animal testing with the hope that some of the compounds would show physiological activity. No such activity was observed for any member of this series.

A preliminary investigation of these compounds as fungicides, for sanitizing of food processing plants, was investigated after becoming familiar with the properties of another derivative of (+)-limonene, tetrahydrocarvone (*1, 3*). It bore a menthollike odor and, in dilution, the odor resembled other compounds used for sanitizing purposes. The amino alcohols—I, II, III, and IV—although not as pleasant in odor, were also investigated as possible fungicides.

Experimental Procedure

Fungicidal Tests on Gauze Pads. Emulsions of the compounds to be tested were prepared in a 1 to 10 dilution with 2 drops of Tween 80 to ensure

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Several derivatives of 2-amino-1-*p*-menthanol synthesized from (+)-limonene have been found to be fungicidal when tested as antimildew agents on gauze pads and as chemicals for the control of decay in Valencia oranges.

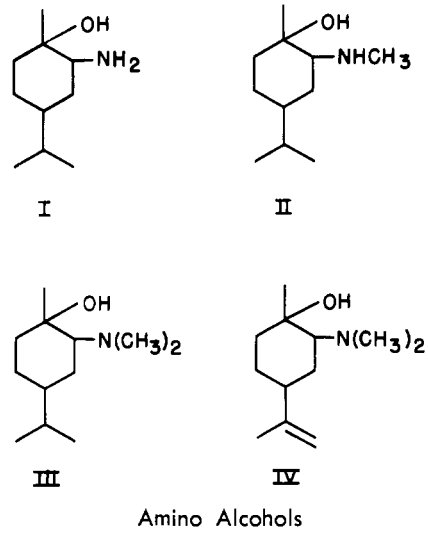
complete dispersion. The total volume in all cases was 100 ml. Further serial dilutions of 1 to 100, 1 to 1000, and 1 to 10,000 were then made. Gauze was cut in strips about 3 inches wide and folded into squares six layers thick. These were dipped into the emulsions and thoroughly wetted. Six pads were treated with each dilution of each compound tested, beginning with the greatest dilution. The pads were wrung free of excess emulsion by hand and inoculated by pressing one surface on the moldy wall of a storage room. They were then folded with the inoculated surface inside, placed in Petri dishes, and incubated in a dark space at room temperature. Control pads were treated individually with water, water and Tween 80, and salicylanilide (put into solution using anhydrous sodium carbonate) at dilutions of 1 to 1000, 1 to 10,000, 1 to 100,000, and 1 to 1,000,000. These pads were inoculated and incubated with the pads treated with the test compounds. All the pads were kept moist by adding, when needed, a mildew test solution of nutrient salts comprising 3 grams of sodium nitrate, 1 gram of dipotassium phosphate, 0.25 gram of magnesium sulfate, 0.25 gram of potassium chloride, and sufficient water to give a total volume of 1000 ml.

Fungicidal Test on Fresh Fruit. Freshly picked Valencia oranges were randomized into six lots of 50 each. The oranges were not washed prior to treatment and one untreated lot was used as a check. Aqueous emulsions containing 0.0001, 0.001, 0.1, and 1.0% of 2-dimethylamino-1-*p*-menthanol (III) were prepared using a few drops of Tween 80 as the emulsifying agent. Each emulsion was applied by a small brush to the stem-end portion of the oranges in one 50-fruit lot. The stem end was treated because most of the decay organisms are at this location. The treated fruit was stored with the untreated check lot at 21° and 85 to 90% relative humidity for 4 weeks. Each lot was examined at weekly intervals for decay. Decay in citrus fruits is caused by the molds, *Penicillium digitatum* Sacc., *Penicillium italicum* Wehmer, and the rot-inducing *Diplodia natalensis* Pole-Evans and *Phomopsis citri* Fawc.

The food-plant "cleanup" tests were made using specimens and rinse water from a citrus processing pilot plant. The sanitizing tests employed inoculations of raw sewage, specimens collected from the facilities and tile floors of the men's wash room, and strains of unidentified enteric streptococci. Beef yeast–extract agar was melted, cooled, and inoculated with the above-mentioned materials. After the inoculum was blended with the liquid agar, plates were poured and permitted to solidify. The chemicals to be tested were spotted on the surfaces of the agar with a standard loop inoculation needle. The plates were incubated at 30° C. Salicylanilide was not used in the sanitizing tests.

Table I. Fungicidal Effect of (+)-Limonene Derivatives Tested Using Inoculated Gauze Pads

Chemicals	Incubation Period, Days	Highest Dilution Showing No Growth	Results for Next Higher Dilution
Tetrahydrocarvone	57	1:100	1:1000 slightly musty, scant growth on all pads
2-Amino-1-*p*-menthanol (I)	53	1:1000	1:10,000 slightly musty, spotted. Less than 50% were damaged
2-Methylamino-1-*p*-methanol (II)	43	1:100	1:1000 inconsistent, occasionally musty, moldy, and spotted
2-Dimethylamino-1-*p*-menthanol (III)	43	1:1000	1:10,000 musty, moldy, and spotted
2-Dimethylamino-$\Delta^{8(10)}$-*p*-menthen-1-ol (IV)	31	1:1000	1:10,000 moldy and spotted
Salicylanilide control	43	1:10,000	1:100,000 musty and spotted



Amino Alcohols

Results and Discussion

The preliminary investigation of compounds I, III, and IV as fungicides is encouraging (Table I). Only tetrahydrocarvone and compound II do not justify further testing as fungicides. The gauze pads treated with tetrahydrocarvone showed no inhibition after 57 days of incubation. At a dilution of 1 to 1000, all pads were musty and showed growth of mold. After repeated tests, compound II was inconsistent in preventing growth at dilutions of 1 to 1000. When inhibition occurred at 1 to 1000, the next higher dilution permitted slight mustiness, moldiness, and spotting in a number of pads.

The greatest fungicidal activity was noted for the three remaining compounds —I, III, and IV. In tests on I growth was completely inhibited at a dilution of 1 to 1000. At the next higher dilution there was no visible growth of mold and only a slight mustiness and spots on less than half of the pads. The other two amino alcohols, III and IV, were about equal in their mold-inhibiting properties. Dilutions of 1 to 1000 completely prevented growth, while at the next higher dilution a very few pads remained undamaged.

Salicylanilide was used as a fungicide control. It prevented mold growth on gauze pads at a dilution of 1 to 10,000; the next higher dilution, 1 to 100,000, prevented growth in more than half of the pads.

The nutrient solution added to the inoculated gauze pads supplied adequate growth conditions for the microorganisms; therefore it is felt that these amino alcohols have been given a severe test.

The amino alcohols were not effective for cleanup and sanitizing purposes at any dilution, as determined by inoculated beef yeast–extract agar plates. Neither did any dilution prevent the growth of enteric streptococci on the surface of the same medium.

The fungicidal tests on Valencia oranges using various concentrations of III are summarized in Table II. Compound III was selected because the first tests on gauze pads indicated that it might be more fungicidal than the other amino alcohols prepared. These results show that some reduction of decay

Table II. Valencia Oranges Given Stem-End Treatment of 2-Dimethylamino-1-p-menthanol (III)

III, Concn., %	Decay, %			
	1 wk.	2 wk.	3 wk.	4 wk.
0.0 (check)	4	8	10	14
0.0001	4	10	10	16
0.001	2	4	6	14
0.01	0	6	8	16
0.1	0	4	4	8
1.0	0	0	2	6

is provided after 3 weeks' storage by the emulsion containing only 0.001% of III. The emulsion containing 1.0% of III provides very effective decay control (80%) after 3 weeks' storage at 21° and moderate control (57%) after 4 weeks' storage. Decay control refers to per cent reduction of decay based on the untreated check. This decay control is of the same order of magnitude as that provided by citrus fruit fungicides such as Dowicide A. However, results comparing the effectiveness of compounds III with Dowicide A are not complete. It is likely that compounds I and IV will show a similar degree of decay control, but they have not yet been tested on citrus fruits.

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PESTICIDE RESIDUE ANALYSIS

Microcoulometric Gas Chromatography of Pesticides

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A new rapid screening method of pesticide residue analysis has been developed. Quantitative analysis for several pesticides, such as γ-BHC, aldrin, dieldrin, DDT, chlordan, endrin, toxaphene, and other chlorinated organic pesticides, can be made in a single determination requiring only 1 hour. The method, based on gas chromatography and coulometric detection, can also be used for thiophosphates.

THE PROBLEM of identifying and quantitatively measuring pesticide residues on agricultural products has become increasingly difficult owing to the wide variety of chemicals used for controlling pests. The number of chemicals in use increases annually, which continually increases the number of combinations of pesticides and plant materials. In order to make the task of the analyst somewhat less difficult, a program of development of rapid screening tests for pesticide residues on fresh vegetables was undertaken. The main objective was to develop a rapid, yet sensitive, technique that would be useful for a wide variety of plant materials. In addition to good sensitivity, the method must be reasonably simple to perform, and yet it should give quantitative information concerning a wide variety of materials. At the beginning of this study, a complete survey of available equipment and methods was made, and it was decided that a new and fresh approach to the problem was needed. Such techniques as paper chromatography were considered, along with column chromatography and various classical colorimetric methods. At that time there was no published work on gas chromatography as a tool for rapid screening pesticide residue analyses. After a cursory laboratory evaluation of the potentiality of gas chromatography, it was decided that this technique, if properly developed, showed great promise (*3*). Commercially available equipment was surveyed and found to be lacking in certain necessary features for this particular problem. Therefore, a method and instrument development program was undertaken. This resulted in development of new rapid screening procedures for pesticide residues on leafy vegetables and other food products.

In order to accomplish a rapid screening procedure for a variety of pesticides, it was necessary to develop new equipment. This new equipment had to be consistent with the concept that a single catchall type of extraction would be used, followed by a rapid step in which the extract would be concentrated to a small volume, with or without additional cleanup procedures. If a cleanup procedure was necessary, it could be relatively crude.

Equipment

Figure 1 shows a block diagram demonstrating the principles on which the new instrument operates. The instrument consists of three major modules and a titration cell. The first module contains a gas chromatographic column and microcombustion furnace. The second, not shown in Figure 1, is a power supply for the gas chromatograph. The third module is the coulometer.

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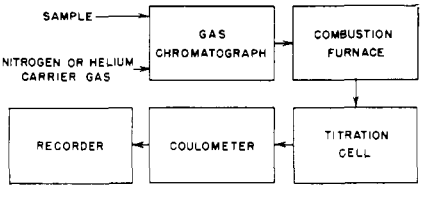

Figure 1. Block diagram of pesticide analyzer

Table II. Valencia Oranges Given Stem-End Treatment of 2-Dimethylamino-1-p-menthanol (III)

III, Concn., %	Decay, %			
	1 wk.	2 wk.	3 wk.	4 wk.
0.0 (check)	4	8	10	14
0.0001	4	10	10	16
0.001	2	4	6	14
0.01	0	6	8	16
0.1	0	4	4	8
1.0	0	0	2	6

is provided after 3 weeks' storage by the emulsion containing only 0.001% of III. The emulsion containing 1.0% of III provides very effective decay control (80%) after 3 weeks' storage at 21° and moderate control (57%) after 4 weeks' storage. Decay control refers to per cent reduction of decay based on the untreated check. This decay control is of the same order of magnitude as that provided by citrus fruit fungicides such as Dowicide A. However, results comparing the effectiveness of compounds III with Dowicide A are not complete. It is likely that compounds I and IV will show a similar degree of decay control, but they have not yet been tested on citrus fruits.

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PESTICIDE RESIDUE ANALYSIS

Microcoulometric Gas Chromatography of Pesticides

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A new rapid screening method of pesticide residue analysis has been developed. Quantitative analysis for several pesticides, such as γ-BHC, aldrin, dieldrin, DDT, chlordan, endrin, toxaphene, and other chlorinated organic pesticides, can be made in a single determination requiring only 1 hour. The method, based on gas chromatography and coulometric detection, can also be used for thiophosphates.

THE PROBLEM of identifying and quantitatively measuring pesticide residues on agricultural products has become increasingly difficult owing to the wide variety of chemicals used for controlling pests. The number of chemicals in use increases annually, which continually increases the number of combinations of pesticides and plant materials. In order to make the task of the analyst somewhat less difficult, a program of development of rapid screening tests for pesticide residues on fresh vegetables was undertaken. The main objective was to develop a rapid, yet sensitive, technique that would be useful for a wide variety of plant materials. In addition to good sensitivity, the method must be reasonably simple to perform, and yet it should give quantitative information concerning a wide variety of materials. At the beginning of this study, a complete survey of available equipment and methods was made, and it was decided that a new and fresh approach to the problem was needed. Such techniques as paper chromatography were considered, along with column chromatography and various classical colorimetric methods. At that time there was no published work on gas chromatography as a tool for rapid screening pesticide residue analyses. After a cursory laboratory evaluation of the potentiality of gas chromatography, it was decided that this technique, if properly developed, showed great promise (*3*). Commercially available equipment was surveyed and found to be lacking in certain necessary features for this particular problem. Therefore, a method and instrument development program was undertaken. This resulted in development of new rapid screening procedures for pesticide residues on leafy vegetables and other food products.

In order to accomplish a rapid screening procedure for a variety of pesticides, it was necessary to develop new equipment. This new equipment had to be consistent with the concept that a single catchall type of extraction would be used, followed by a rapid step in which the extract would be concentrated to a small volume, with or without additional cleanup procedures. If a cleanup procedure was necessary, it could be relatively crude.

Equipment

Figure 1 shows a block diagram demonstrating the principles on which the new instrument operates. The instrument consists of three major modules and a titration cell. The first module contains a gas chromatographic column and microcombustion furnace. The second, not shown in Figure 1, is a power supply for the gas chromatograph. The third module is the coulometer.

It was found that such chlorinated pesticides as lindane (γ-BHC), aldrin, dieldrin, endrin, DDT, toxaphene, chlordan, gamma-chlordan, heptachlor, heptachlor epoxide, ronnel, and DD could be successfully gas chromatographed, only if materials of construction were carefully selected. After considerable research it was found that a $^1/_4$-inch in outside diameter by 6-feet long aluminum column packed with 30-to-60-mesh Chromosorb (Johns-Manville Co.) coated with 15 to 30% by weight of Dow-Corning high vacuum silicone grease is a satisfactory gas chromatographic column. In order to avoid chemical effects it was necessary to acid-wash the Chromosorb with hot 6*N* hydrochloric acid, followed by a thorough

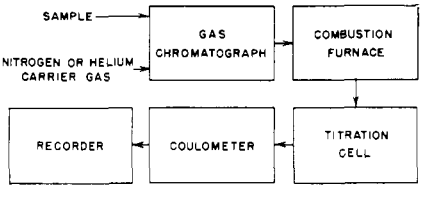

Figure 1. Block diagram of pesticide analyzer

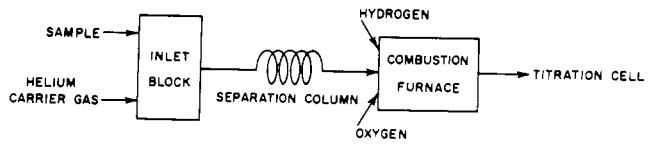

Figure 2. Block diagram of gas chromatograph

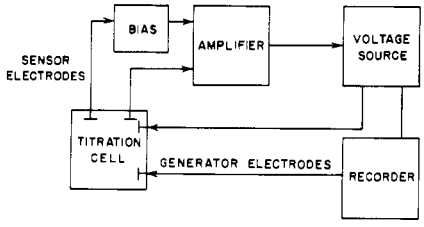

Figure 3. Block diagram of coulometer

washing with distilled water. This solid packing material is relatively inert to various chlorinated hydrocarbons, and participates in gas chromatography primarily as a solid support medium for the liquid phase. However, there is some evidence of adsorption by Chromosorb itself.

The stationary liquid phase was prepared by extracting Dow-Corning high vacuum silicone grease with ethyl acetate to remove some of the more volatile components. Complete solvent removal at this state is not necessary since solvent removal occurs during the final drying of the column packing. Some of the other liquid phases that were tested, and found to be inferior to high vacuum silicone grease, were polyethylene, several silanes, several Apiezon greases, and paraffin oil. The sample introduction block and separation column, in the column oven, and the combustion furnace (Figure 2) are combined into one module.

A coulometric detection system is used to follow the elution of the pesticides from the separation column. This system has been described by Coulson and Cavanagh (*2*). It is based on the continuous titration of chloride and sulfide with silver ions that are electrically generated in the titration cell. The success of this coulometric method of analysis was dependent on the design of a suitable titration cell. A four-electrode system is used, including two sensor electrodes and two generator electrodes. The gases eluted from the separation column are mixed with oxygen in the furnace and burned to form carbon dioxide, water, hydrogen chloride, and other inorganic substances. The technique of burning organic chlorinated pesticides in a tube furnace was reported by Agazzi, Peters, and Brooks (*1*) and by Peters, Rounds, and Agazzi (*5*). A similar combustion technique is used for the decomposition of the pesticides in the combustion furnace. These gases are then bubbled into the titration cell. Under these conditions the coulometric detector is specific for chloride (bromide and iodide would also be detected). If the combustion is followed by catalytic hydrogenation in the high temperature furnace, sulfur is converted to hydrogen sulfide and both chloride and sulfide are detected. Thus the sulfur-containing constituents may be determined by the difference between samples thermally degraded oxidatively and reductively. This makes possible the detection of both chlorine- and sulfur-containing materials by means of the coulometric system.

The coulometer is based on a continuously balancing system, in which the titrating agent present in the titration cell is kept at a fixed concentration throughout the titration. Thus, the environment within the titration cell remains constant even during intervals when sample constituents are entering the titration cell. Figure 3 is a block diagram of the coulometer portion of the pesticide analyzer. The silver ion concentration may be varied as a result of changing the bias voltage in the sensor electrode circuit. It was found that a bias voltage of +250 mv. *vs.* a saturated silver acetate electrode gives maximum sensitivity with an electrolyte in the titration cell composed of 15% water in glacial acetic acid. Under these conditions, the titration curve, a current-time curve on the strip chart recorder, follows the elution of pesticides from the column very precisely.

The electrical current used to maintain a constant silver ion concentration in the titration cell is recorded on a strip chart recorder as a function of time. The type of pesticide present may be determined by the elution time from the gas chromatograph. Calibrations may be made by measuring elution times for known pesticides. The quantity of the pesticide represented by an elution peak is determined by a theoretical calculation based on the area under the titration curve and the per cent of chlorine in the molecule. The area under the titration curve is proportional to the amount of silver ion generated. The relation between the area under an elution peak and the amount of a chlorinated organic pesticide is given by Equation 1.

The power supply module permits the temperature in the gas chromatograph to be varied over a broad range. Column temperatures may be varied from room temperature to as high as 400° C. For the common pesticides, a column temperature of 220° to 250° C. has been found satisfactory. The combustion furnace, containing a platinum gauze catalyst, is normally maintained at 800° C., but may be operated at temperatures as high as 1000° C. The power supply module also houses pyrometers for continuous indication of temperature at various critical points in the gas chromatograph and combustion furnace.

Results and Discussion

A gas chromatogram on a mixture of γ-BHC, aldrin, dieldrin, and DDT is given in Figure 4. This was based on 2 mg. each of the first three compounds and 4 mg. of DDT, using a thermal conductivity type detector. It was recognized from the outset that thermal conductivity detection would probably not be sensitive enough for the detection of pesticide residues. Consequently, research was undertaken to develop a much more sensitive and selective type detector for use in a pesticide residue gas chromatograph. For this purpose an electrochemical technique was developed that is sensitive only to chloride or chloride and sulfide, depending on the procedure of operation.

In this equipment, the gas chromatographic column operates in the conventional manner. The effluent gases from it are oxidatively decomposed and bubbled through the solution in a coulometric titration cell. The resulting inorganic chloride is coulometrically titrated, quantitatively with an automatic titrator. The coulometric method requires no empirical calibration factors for quantitative work. It is, however, necessary to determine empirically the time required for each of the pesticides to pass through the gas chromatographic separation column in order to identify the pesticide present.

Figure 5 shows a microcoulometric gas chromatographic determination of a mixture of γ-BHC, aldrin, dieldrin, and DDT. In this case, the sample consisted of 10 μl. of a xylene solution that contained 0.02% of γ-BHC, 0.04% of aldrin and of dieldrin, and 0.08% of DDT. These quantities are smaller by a factor of 1000 than necessary for the ordinary gas chromatographic procedure. The peaks are considerably sharper than those in Figure 4, indicating that with smaller

$$\text{Micrograms} = \frac{\left(\frac{\text{peak}}{\text{area}}\right) \times \left(\frac{\text{recorder}}{\text{sensitivity}}\right) \times 35.5\ \frac{\text{grams}}{\text{equivalent}} \times 60\ \frac{\text{second}}{\text{minute}} \times 10^{6}\ \frac{\gamma}{\text{grams}} \times 10^{-3}\ \frac{\text{volts}}{\text{mv.}} \times 10^{2}}{\left(\frac{\text{recorder input}}{\text{resistance, ohms}}\right) \times (\%\ \text{chlorine in pesticide}) \times 96{,}500\ \frac{\text{coulombs}}{\text{equivalent}}} \quad (1)$$

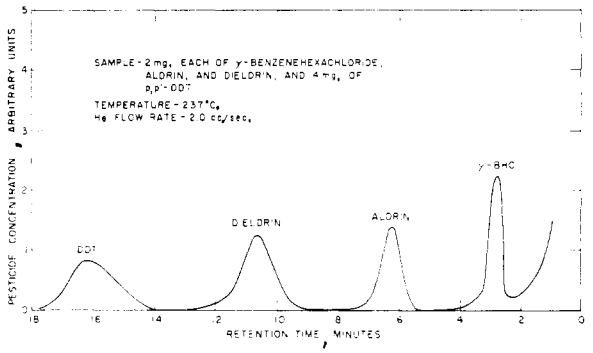

Figure 4. Gas chromatogram on a mixture of chlorinated hydrocarbons

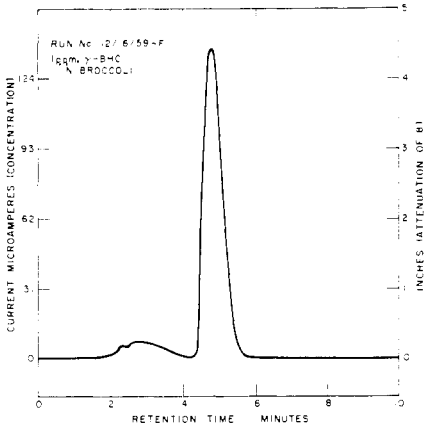

Figure 6. Gas chromatogram on broccoli extract containing γ-BHC

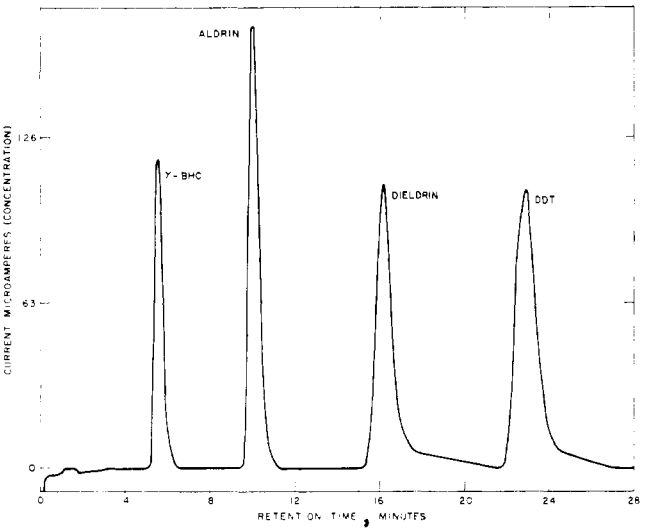

Figure 5. Microcoulometric gas chromatogram on a pesticide mixture

Temperature: 252° C.
Helium flow rate: 1 ml./second
Column: 6 feet × ¼ inch in outside diameter, 20% silicone grease on 40/60-mesh Chromosorb
Recorder: 10 mv. ordinate, 0.5 inch/minute; abscissa attenuation factor of 8

quantities of pesticide the gas chromatographic behavior improved.

Figure 6 is a gas chromatographic determination on broccoli fortified with γ-BHC. In this case, the sample introduced into the gas chromatograph represented 1 gram of broccoli and the peak shown on the figure represents 1 γ of γ-BHC. The quantity of γ-BHC represented by this peak can be calculated from theoretical factors, including Faraday's constant and the per cent of chlorine in γ-BHC. Quantitative recovery was obtained in this case. The only factor that is empirical in this analysis is the retention time. Under the conditions of operation of the gas chromatograph used for this experiment, aldrin would be eluted in 10 minutes, dieldrin in 16 minutes, and DDT in approximately 23 minutes.

Earlier laboratory models of the equipment gave somewhat less than quantitative recoveries, oftentimes 50% or lower. As the equipment was improved and the problems became somewhat better understood, recoveries were improved to the point where it is now possible to get virtually quantitative recoveries for the common chlorinated hydrocarbon-type pesticides.

Table I gives results for γ-BHC, aldrin, and dieldrin standards. In each case the pesticide concentration was 0.10% in xylene. Two types of problems resulted in losses during preliminary experiments not reported here. One cause of low results was the complexity of the gas chromatographic system which contained a number of components coupled together mechanically. This problem was overcome by designing a complete unit with the gas chromatographic oven combined with the combustion furnace. A second cause for losses was decomposition of certain pesticides in the gas chromatographic system. Aldrin has little tendency to decompose during gas chromatography under the conditions used in these experiments. The most serious decomposition problems were observed with DDT, while γ-BHC gave some difficulty. In these cases, it was necessary to precondition the gas chromatographic system with preliminary samples before good recoveries could be obtained. This problem was solved in part by developing an improved column packing material.

Table II contains typical results for aldrin residues in lettuce and broccoli extracts. In every case, these extractions were made, after the addition of the pesticide directly to the chopped material, with either a tetrahydrofuran-hexane or an acetonitrile-hexane mixture. These results, therefore, do not represent studies of extraction efficiency, but they can be used to demonstrate the usefulness of the gas chromatographic, microcoulometric technique on crude extracts of vegetables without previous cleanup procedures. A complete determination may be completed within 1 to 2 hours from the time the experiment is undertaken until the final result is calculated. The procedure involves chopping the vegetable, followed by a Waring Blendor-type extraction, removal of 95 to 99% of the solvent, followed by gas chromatographic, microcoulometric analysis of the concentrate. These extracts were dark green and contained large quantities of plant pigments. Most residue methods require rather elaborate cleanup procedures, prior to the measurement step. In the case of microcoulometric gas chromatography, the cleanup and measurement steps have been combined. The gas chromatographic column performs the cleanup and the titration cell and coulometer measure the amount of each pesticide. For the purposes of detecting the presence of any one of several unknown pesticides on a plant material, it is desirable to minimize the amount of cleanup preceding the measurement step in order to avoid partial or complete losses of pesticides during the cleanup. If, after having established the presence of a particular pesticide, it is desirable to make a more sensitive measurement, it then would be appropriate to perform a suitable cleanup procedure prior to subjecting the sample to microcoulometric gas chromatography. In this case, an even greater degree of sensitivity can be reached due to additional sample concentration that can be effected.

To date this research has involved samples of uncontaminated vegetables to which known quantities of pesticides were added. It is now felt that the techniques have been developed to the point where practical problems can be successfully undertaken. This has been done to a limited extent. The gas

chromatographic, microcoulometric procedure was employed on several samples of potatoes grown under controlled conditions in which dieldrin (Shell Development Co.) had been applied in known quantities to the soil. In one sample, to which dieldrin had been applied at the 0.1 p.p.m. level in the soil before the potatoes were planted, approximately 0.06 p.p.m. of dieldrin was found when the potatoes were harvested 109 days after planting. A somewhat smaller quantity of dieldrin was found to be present in potatoes harvested 116 days after planting. It was convenient to extract the potatoes with acetonitrile, hexane, and water, with the pesticides partitioning into the hexane phase. Sucrose was used to prevent stable emulsions. The hexane phase was then evaporated to a small volume in a Kuderna-Danish evaporator. An aliquot of the concentrate, representing approximately 20 grams of potatoes, was injected into the gas chromatograph.

The new microcoulometric, gas chromatographic procedure was also employed in the analysis of several hay and silage samples. The results of the gas chromatographic analyses on Skellysolve B extracts are given in Table III.

Samples number 2 and 8 were pea hay. Samples number 10 through 13 were alfalfa hay, and the others were pea vine silage. As a check on the gas chromatographic, microcoulometric procedure samples number 2 and 6 were analyzed by the classical nitration, colorimetric method (*6*). The results were 122 and 9 p.p.m., respectively. This excellent check with the gas chromatographic procedure indicates that the rapid gas chromatographic microcoulometric procedure is capable of yielding quantitative results for DDT on hay and silage.

In these experiments, the total elapsed time from beginning to end can be as little as 1 hour. The instrument time per sample may be from 5 to 20 minutes, depending on the time required for the pesticide present to go through the gas chromatographic column. As soon as one sample is completed, a second sample may be injected into the gas chromatograph without changing the solution in the titration cell or making any other modifications of the equipment. In the case of excessively oily plant materials, and meat and dairy products, it may be necessary to carry out a separation procedure prior to subjecting the sample to microcoulometric gas chromatography. Certain chlorinated organic pesticides can be separated almost quantitatively from butter by a simple liquid-liquid partition procedure using acetonitrile and hexane as the partitioning solvents. This procedure is commonly used in the preparation of butter samples for paper chromatography (*4*).

It is also possible to measure sulfur-containing materials such as thiophosphates by the same method, with a minor modification in operating procedure. In this case, after gas chromatographic separation, the pesticides are decomposed under reducing conditions; this results in the formation of hydrogen sulfide which is then titrated in the coulometric titration detector. Malathion, parathion, and Systox have been successfully determined by this procedure. Under these conditions, both classes of pesticides, the chlorinated organic compounds and the thiophosphates, are detected and quantitatively determined.

Although most of the research to date has been on pesticides, the new coulometric, gas chromatographic apparatus will certainly find broad application in the fields of food additives, flavor constituents, petroleum chemistry, health research, and other problems dealing with volatile organic materials.

Research on specific detection, in gas chromatography, of elements other than sulfur and chlorine is in progress. It now appears that specific and theoretically quantitative detection of hydrogen and carbon is possible. With the coulometric method the specific detection of functional groups such as amines and aldehydes is also possible.

Table I. Pesticide Standards by Gas Chromatography

No.	γ Taken	γ Found	Recovery, %
	Lindane		
95	10	0.6	6
96	10	3.0	30
98	10	5.7	57
100	10	7.1	71
12-3-59C[a]	5	5.2	104
12-15-59H[a]	2	1.9	95
12-16-59B[a]	1	1.1	110
	Aldrin		
170	10	9.4	94
37	5	4.2	84
81	5	5.5	110
61	2.5	2.1	84
12-15-59M[a]	4	4.2	105
12-15-59N[a]	3	3.8	93
	Dieldrin		
45	20	13.8	69
88	10	5.3	53
62	5	3.8	76
12-15-59J[a]	8	7.4	93
12-29-59A[a]	4	4.1	102
12-29-59B[a]	4	4.1	102

[a] Results obtained with prototype equipment.

Table II. Aldrin in Vegetable Extracts

No.	P.P.M. Taken	P.P.M. Found	Recovery, %	Vegetable
74	5.0	6.4	128	Lettuce
108	1.0	0.94	94	Lettuce
159	0.25	0.36	144	Lettuce
13[a]	10	10.0	100	Broccoli
14[a]	5.0	4.8	96	Broccoli

[a] Results obtained with prototype equipment.

Table III. Gas Chromatographic Results for Hay and Silage

No.	DDT, P.P.M. Gas Chromatography	DDT, P.P.M. Schechter-Haller (6)
1	90	...
2	130	122
3	0	...
4	3	...
5	6	...
6	10	9
7	1	...
8	0.5	...
9	5	...
10	0.3	...
11	0.6	...
12	0	...
13	0	...

Acknowledgment

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Residue Analysis of a Chlorinated Insecticide (Thiodan) by Combination of Gas Chromatography and Infrared Spectrophotometry

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A combination gas chromatography–infrared spectrophotometry technique is used to analyze quantitatively small traces of a chlorinated insecticide (Thiodan) extracted from plant materials. The advantages of this method are the relative simplicity, the possible application to other compounds, and the simultaneous qualitative and quantitative analyses of the compound under investigation.

Quantitative analyses of trace amounts of chlorinated insecticides, as may be required for residue analyses in crop materials, have been performed by one of the following techniques: analysis by total chloride (*4*); bioassay using different test organisms (*10*); and specific chemical analyses (*9*). The first two methods suffer from their nonspecificity and the interference of other chlorine-containing or toxic compounds other than the material under investigation. The disadvantages of a specific method of analysis are the cumbersomeness of purification, and in some cases, the unavailability of a truly specific chemical method, especially for newly developed compounds.

Therefore, it was felt desirable to develop a general method of trace analysis for a chlorinated pesticide which might be applicable to a number of such compounds and, at the same time, retain the advantage of great specificity. This objective has now been accomplished by the combination of gas chromatography and subsequent quantitative analysis by infrared spectrophotometry. The use of infrared spectrophotometry also afforded the qualitative identification of the material being analyzed.

As a model compound, the newly developed insecticide Thiodan (6,7,8,9,-10,10-hexachloro-1,5,5*a*,6,9,9*a*-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) was chosen.

Present methods of trace analysis for Thiodan are by total chloride or by the colorimetric determination of sulfur dioxide after hydrolysis of the parent compound (*7*). The quantitative analysis of Thiodan in formulations by gas chromatography has been reported recently (*12*). The method reported here is an extension of this work.

Equipment and Materials

Gas Chromatography. Aerograph Model A-100-C and Burrell Model K-3, equipped with a Honeywell-Brown recorder, 1-mv., 1-second response. Six-foot ($^1/_4$ inch in outside diameter) stainless steel, spiral columns were packed with Dow Corning No. 11 high-vacuum silicone grease on Chromosorb 35/80 (30% w./w.). All other conditions for gas chromatography have been described (*12*).

Infrared Spectrophotometry. Model 221 Perkin-Elmer infrared spectrophotometer, equipped with scale expansion. Liquid microcells with sodium chloride windows and a light path of 3.0 mm. were used. A variable thickness liquid absorption cell was used for the reference beam.

Reagents. Mixed hexane and carbon disulfide (analytical reagent grade) were used without further purification. Thiodan standards and a formulation (24.00%) in xylene (Niagara Chemical Division, Food Machinery and Chemical Corp.) were used.

Experimental Methods

Standard Curve. Five microliters of Thiodan standard, dissolved in toluene and containing 4.8 to 120 γ of Thiodan, were injected into the chromatographic column with a 10.0-μl. Hamilton syringe-type pipet. The column temperature was kept at 225° C. and the gas flow (helium) had an effluent rate of 167 ml. per minute. Under these experimental conditions, the two isomers of Thiodan (*6*) had a retention time between 4.0 and 7.2 minutes. During this period the effluent stream was collected by a tapered 1.0 × 14 cm. glass tube, fitted with a 7/15 standard taper at one end. A small wad of glass wool soaked in carbon disulfide was inserted into the exit end of the tube just prior to the time of collection. At high concentrations of Thiodan, the material was deposited as a white film on the walls of the collector near the collection point.

A slight difference in design between two commercial gas chromatographic apparatus might be mentioned. The Aerograph has a rubber O-ring gasket at the collection port, into which the tapered end of the collection tube is inserted. The Burrell fraction collector consists of a stainless steel male standard tapered joint to which the open end of the collection tube is attached. Just before the removal of the collection tube from the Burrell, the temperature of the collector must be decreased because of different expansion coefficients of the glass and the metal.

After the sample had been collected, the inside of the glass tube and the outside of the restricted end were washed thoroughly with generous amounts of carbon disulfide (about 25 ml.). The washings were evaporated at room temperature in a stream of warm air, and the dry residue was redissolved in carbon disulfide (0.20 to 5.0 ml.). This solution was then transferred to a liquid microcell for infrared spectrophotometry, and the spectrum was scanned between 8.0 and 8.5 microns using pure carbon disulfide in the reference beam. The setting of the infrared instrument was as follows: slit

program 9.80; scanning time 16; suppression 10; and scale expansion 5 ×. The absolute absorbance at 8.37 microns was measured and calculated, using the base-line method (*1*).

It was important to flush the microcell after each analysis with pure carbon disulfide and to evacuate the empty cell with a vacuum pump for several seconds. Failure to observe this procedure resulted in a spurious absorption band at 8.25 microns. The origin of this band may be due to a volatile reaction product from carbon disulfide and Koroseal, because Koroseal bulbs were employed to empty the microcell. A typical scan in the range of 8.0 and 8.5 microns for a Thiodan standard solution is shown in Figure 1. Figure 1 also shows the base line used for calculating the absorbance values. Absorbance was plotted *vs.* concentration (γ/ml.) and a straight line was obtained, following Beer's law. For confirmatory work the absorbance at 10.16 microns could also be employed, but because of the lower sensitivity at this wave length, the reading at 8.37 microns was preferred (Figure 1).

Trace Analysis of Thiodan from Crop Material. In order to validate the method of residue analysis a typical agricultural crop was chosen. Winter-Nellis pears with an unknown spray history were surface-extracted with mixed hexane (1000 grams of fruit per 1000 ml. of solvent) using the roller-stripping technique (*4*). Prior to stripping, 5 p.p.m. of insecticide solution in toluene were added. After 1 hour of stripping, the mixture was dried over anhydrous sodium sulfate and then filtered through Whatman No. 1 paper. One hundred milliliters of this solution, equivalent to 100 grams of crop, were evaporated to complete dryness in a stream of warm air. A small amount of a waxy material remained as a residue. Then 100 μl. of toluene were added, the solution was warmed slightly to melt the waxes, and the total volume of this solution was measured, usually between 112 and 118 μl. Fifty microliters of this solution were injected into the gas chromatographic column with a 100-μl. Hamilton syringe-type pipet, and the procedure for analysis was followed exactly as described above under standard curve.

Other crop materials analyzed for Thiodan by this procedure without further modifications were alfalfa and peaches.

Results and Discussion

Reproducibility of Method. In order to test all possible experimental errors due to pipetting, loss during collection from the chromatographic column, and errors due to infrared instrumentation, 11 samples, containing 120 γ of Thiodan, were analyzed by this procedure.

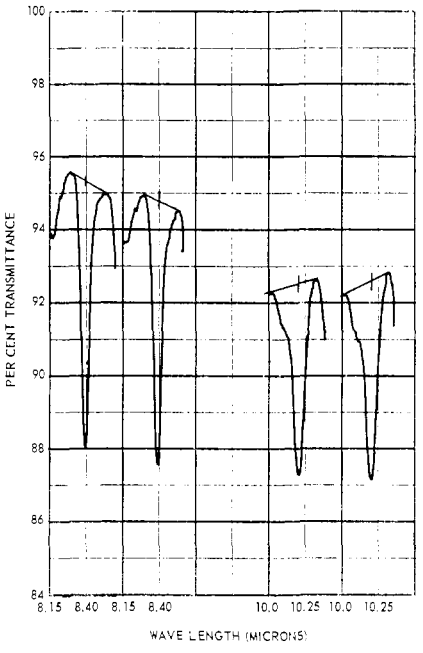

Figure 1. Infrared scan 8.0 to 8.5 and 10.0 to 10.5 microns of Thiodan standard

Note base line is drawn in

Table I lists the absolute absorbance of each sample as well as the duplicate readings of the spectrophotometer. The calculated standard deviation was 0.002 or ±10.2% and included all sources of error enumerated above. When 12 other standard solutions were analyzed directly by infrared spectrophotometry, without prior gas chromatography, an average value of 148.62 γ was obtained, using the standard curve. This indicated a loss of about 23% due to the gas chromatography step. However, because standard curves were obtained by processing the standards by the two-step process (gas chromatography and infrared spectrophotometry), these experimental errors canceled out in the analysis for an unknown amount of Thiodan.

The sensitivity of the method is 20 γ per ml. and a total volume of 0.2 ml. is required to fill the microcell, so that the absolute sensitivity is 4 γ. One difficulty in obtaining even greater sensitivity was the slow bleeding of the stationary phase (Silicone Dow 11) when using a comparatively new column. A column which had been in continuous use over a 6-month period resulted in readable peaks at a concentration of 4.8 γ per ml. Furthermore, a scale expansion of "20 ×" by the infrared spectrophotometer would increase the sensitivity by a factor of 4. However, in the present work only the scale expansion factor of "5 ×" was employed. If other scale expansions are used, new standard curves must be constructed for each because the scale expansion factors are not exactly linear (*8*).

Table I. Calculation of Standard Deviation for Analysis of Thiodan

(All samples contained 60 γ of Thiodan per ml. of carbon disulfide solution)

Absorbance at 8.37 Microns		
0.018	0.022	0.020
0.017	0.024	0.021
0.027	0.024	0.020
0.022	0.022	0.022
0.022	0.021	0.020
0.021	0.021	0.021
0.023	0.019	0.021
0.024		

$m = 0.21$
$\Sigma x = 0.473$
$n = 22$
$\sigma = \pm 0.002 \pm 10.2\%$

Table II. Recovery Study of Thiodan Added to Crop Material

Sample	*P.P.M. Added*	*P.P.M. Found*	*Recovery, %*[a]
Pear AR 1[b]	5.00	4.32	86.4
AR 2	5.00	4.93	98.6
ARR 1	5.00	4.30	86.0
ARR 2	5.00	4.33	86.6
ARR 165[c]	5.00	5.75	115.0
ARR 165	5.00	5.34	106.8
Peach ARR 168[d]	5.00	4.90	98.0
ARR 168	0.96	0.88	91.7

AR = addition of Thiodan before processing of crop.
ARR = sample to which known amounts of Thiodan were added after extraction and concentration just prior to gas chromatography.

[a] 43.47 grams of plant material; value corrected for an average wax concentration of 15% in final concentrate.
[b] Winter-Nellis pear.
[c] Bartlett pears.
[d] Lovell peaches.

Residue Analysis from Crop Materials. Gas chromatography by itself may be used for trace analyses of insecticides by measuring the area of the retention-time curves. However, peak areas corresponding to less than 25 γ of Thiodan have been shown to be unreliable, using the hot-wire Katharometer detector (*12*). Direct analysis by gas chromatography alone may not be possible if there are interfering substances present in the plant material that have similar retention times as Thiodan. This was observed in the case of several untreated pear samples which exhibited at least two components within the retention time period of 4.0 to 7.2 minutes. These components were collected and did not show the characteristic infrared absorption peak of Thiodan at 8.37 microns. However, when the effluent stream from a treated sample was collected during the predetermined retention-time period, an uncontaminated infrared absorption peak due to Thiodan could be measured at 8.37 microns.

Table II lists the recovery studies of

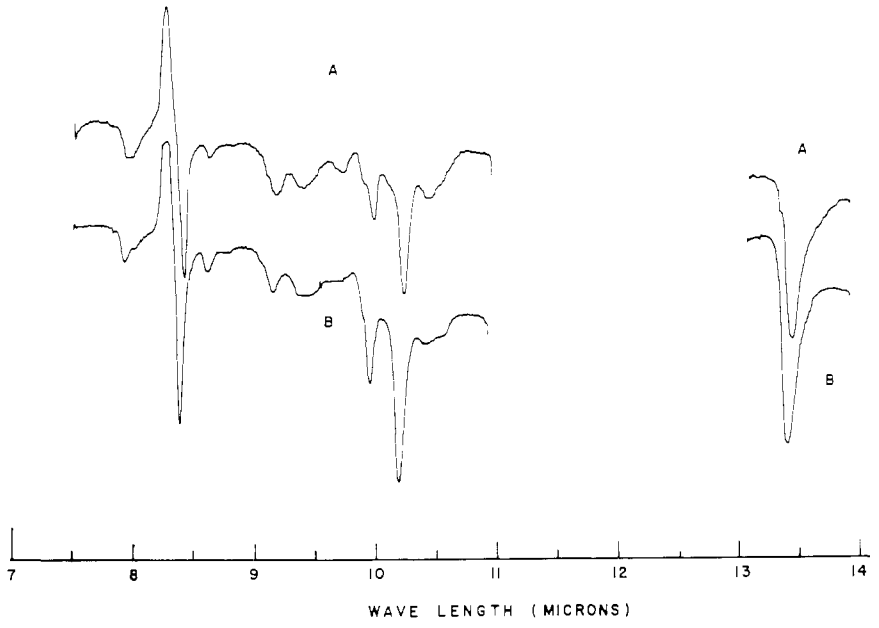

Figure 2. Infrared absorption curves of Thiodan

A. Isolated from Bartlett pear
B. Analytical standard in carbon disulfide solutions

several fortified pear and peach samples. The recoveries ranged consistently from 86 to 115%.

A further advantage of the combination of gas chromatography and infrared spectrophotometry is illustrated in Figure 2. The infrared spectrum depicted in this figure shows the identification of Thiodan from a pear sample to which Thiodan had been added. This method thus provided simultaneously a quantitative and qualitative analysis of the material under investigation. The chromatographic column should be properly aged, as bleeding of the stationary phase would interfere in qualitative identification. Silicone Dow 11 exhibits strong absorption bands in the regions of 7.9, 8.6 to 9.9, and 11 to 12 microns. These bands are especially cumbersome when scale expansion is applied.

Choice of Solvents for Infrared Spectrophotometry. A number of solvents were investigated for suitability in the 8.0- to 8.5-micron region. Some of the solvents which show some transmittance in this region are acetonitrile, carbon tetrachloride, chloroform, cyclohexane dichloromethane, methylcyclohexane, and carbon disulfide. It was possible to obtain a satisfactory 100% transmittance line using scale expansion only with carbon disulfide. Furthermore, it was possible to use carbon disulfide (analytical reagent grade) without any further purification. Gunther and Blinn have also used carbon disulfide for the determination of Tedion by infrared spectrophotometry (*5*).

Comparison with Other Methods. The combination gas chromatography-infrared method was compared with two other methods available for trace analysis of Thiodan (*7*). For this purpose several alfalfa samples were analyzed in the authors' laboratories by the method described above. Table III summarizes these results and those obtained by the sulfur dioxide and total chloride methods in another laboratory (*2*). In all cases, except one (sample 221), the results were in good agreement with the sulfur dioxide method. Total chloride seemed to give consistently higher results than either of the other two methods.

Future Research. Preliminary research at this and other laboratories (*3, 11*) has indicated the feasibility of the separation of a number of insecticides by gas chromatography. It might be possible, therefore, to combine gas chromatography and infrared spectrophotometry for the analysis of each, and possibly a number of insecticides simultaneously. The prerequisites are that these compounds be separated by gas chromatography and that they possess specific infrared absorption bands for quantitative analysis. If two compounds were not resolved chromatographically, it might even be possible to analyze them by infrared as a multicomponent mixture (*1*).

Table III. Comparison of Thiodan Analyses by Three Methods

(Alfalfa samples)

	Parts Per Million		
Sample No.	Total chloride[a]	Sulfur dioxide	Gas chromatography-infrared
221	0	0	0.11
213	1.1	0.03	0.06
214	2.5	0.07	0[b]
217	3.3	0.38	0.44
219	5.4	2.06	2.86
221	11.0	6.21	10.11

[a] Analyses (*7*) made by Niagara Chemical Division, Food Machinery and Chemical Corp., Richmond, Calif.
[b] Less than 0.05 p.p.m.

Acknowledgment

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Solubility of Carbon-14 DDT in Water

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A knowledge of the solubility of DDT in water was required to study the effect of dissolved and undissolved toxicant on bioassays with mosquito larvae. In the present study the DDT was analyzed radiometrically, undissolved particles (>41 A.) were removed by an average ultracentrifugal force of 84,150 G with the amount of impurity held to a minimum by avoiding the use of a large excess of solute. The solubility of DDT in water was found to be 1.2 p.p.b. or less at 25° C. Data on sizes of undissolved DDT particles and on the recovery of DDT after ultracentrifugation at 1 G are presented.

During investigations on the physical behavior of DDT in aqueous suspensions (*2*), it became necessary to know the solubility of DDT in water in order to evaluate the effect of dissolved and undissolved toxicant on mosquito larvae bioassays. Several solubility values are recorded in the literature. Neal *et al.* (*5*) reported 1000 p.p.b. at room temperature. Richards and Cutkomp (*6*) obtained 0.2 to 1.0 p.p.b. after limited ultracentrifugation and bioassay with mosquito larvae at 15° C. Roeder and Weiant (*7*) reported 10 to 100 p.p.b. at room temperature based upon symptoms of DDT poisoning in the ventral nerve cord of roaches while Gauvadan and Poussel (*3*) approximated 100 p.p.b. at 18° C. by nephelometry. Babers (*1*) obtained 37.4 p.p.b. at 25° C. from radiometric analyses. The determination of the solubility of DDT was undertaken to resolve these conflicting values.

The extraordinarily low solubility of DDT made the determination of its precise value an unusually difficult task, and sources of error not associated with the solubility study of a more soluble compound had to be eliminated. Minute, undissolved particles of DDT formed during the preparation of saturated aqueous solutions do not aggregate readily, so that the use of conventional methods, such as filtration or mild centrifugation, fails to remove all the particles and gives high results. Ultracentrifugation was therefore used in this study.

To permit the estimation of a few parts per billion, in the limited volume of sample available, through the use of prolonged ultracentrifugation, DDT was determined radiometrically by using a sample of high specific activity. In using this procedure it was found that a small percentage of radiolabeled impurity in the DDT markedly influenced the determination. This impurity was kept below the experimental error by avoiding the use of a large excess of solute. The purity of the carbon-14 DDT was established by paper chromatographic and radiometric analyses.

Another complicating factor was the tendency of DDT to accumulate at the interfaces of aqueous suspensions, with a consequent decrease in its concentration in the liquid (*2*). It was necessary to determine the loss of DDT from the filtrate at 1 G—no centrifugation—for several intervals of time, in order to evaluate the results of ultracentrifugation for the corresponding periods.

The solubility of DDT in water at 25° was not more than 1.2 p.p.b., a value that differs markedly from all but one of those reported.

Experimental

A Spinco Model L preparative ultracentrifuge equipped with a 26° No. 40 rotor was used. The samples were centrifuged at 25° ± 0.5° C. in stainless steel tubes fitted with aluminum caps. The loss of DDT due to codistillation (*2*) was prevented by using closed systems.

The diameter, d (cm.), of an undissolved particle of DDT that can be removed by ultracentrifugation from water at 25° C. may be calculated as follows:

$$d = \sqrt{\frac{370\ \eta}{\pi^2 T_s P_i (\sigma - \rho)}} \qquad (1)$$

where η is the viscosity of the medium (0.00894 poise), T_s is the precipitation time (minutes), σ and ρ are the specific gravities of DDT (1.556 grams per ml.) and of water (0.997 gram per ml.), and P_i is the performance index of the ultracentrifuge rotor,

$$P_i = \frac{(\text{r.p.m.})^2}{\log_e R_{max} - \log_e R_{min}} \qquad (2)$$

where r.p.m. is the revolutions of the rotor per minute, and R_{max} (5.9 cm.) and R_{min} (3.8 cm.) are the radial distances to the bottom of the top half and to the meniscus of the fluid.

Chromatography of DDT. About 20 γ of the *p,p'*-DDT-4-C^{14} (*3*)—specific activity 2.1 μc. per mg.—was chromatographed with dimethylformamide and heptane by the procedure described by Mitchell (*4*). The developed chromatogram was sprayed by the procedure of Winteringham (*8*) with potassium permanganate–benzidine reagent. The sprayed chromatogram was dried, sectioned, and counted in the proportional counter.

Preparation of Dispersion. The aqueous solution of the carbon-14 DDT was prepared in a glass-stoppered flask by removing the solvent at 30 to 40 mm. of mercury pressure from an acetone solution containing the appropriate quantity of carbon-14 DDT, and then sufficient water, redistilled from glass, was added to the residue. The mixture (15 p.p.b.) was heated and shaken for 1 hour in a water bath at 90° to 100° C. Then it was mechanically shaken at approximately 25° C. for 1 week and filtered through a fritted glass funnel (porosity 4.5 to 5 microns), and the filtrate was stored in a glass-stoppered flask at this temperature.

Methods of Sampling and Analysis. The flask with the filtrate was shaken to ensure a homogeneous dispersion of the small particles immediately before each pipetful was withdrawn either for analysis or for addition to the centrifuge tubes.

At the end of each experiment, the top half of the liquid was removed from all the ultracentrifuge tubes and combined. This was accomplished by means of a

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syringe equipped with a 40.5-mm., 18 gage stainless steel cannula that was inserted through the cap after the setscrew was removed. The syringe and cannula were finally rinsed with isooctane, which was added to the combined aqueous samples. The mixture was extracted with isooctane and the extract was analyzed radiometrically in the manner described by Bowman *et al.* (*2*). The top half of the liquid was analyzed because the particles removed from the top half are smaller than those removed from the entire liquid and the error caused by stirring back is minimized.

Distribution of Carbon-14 DDT at 1 G and after Ultracentrifugation. The decrease in concentration of carbon-14 DDT in the filtrate was determined after it had stood for various periods in the stainless steel, ultracentrifuge tubes at 1 *G*. Five groups of six tubes, each tube filled with 12.8 ml. of the filtrate, were capped. The liquid was sampled and analyzed immediately or after 1, 3, 6, and 12 hours.

The change in concentration of carbon-14 DDT at various centrifugal forces *vs.* time was also determined. Groups of six tubes were prepared as described above and centrifuged either at 5000 r.p.m. for 1 hour or at 39,400 r.p.m. for 3, 6, or 12 hours. The contents of the tubes were then sampled and analyzed.

Particle Size of Carbon-14 DDT in Filtrate. The diameters of the particles that could be precipitated by ultracentrifugation, as described above, were calculated by substituting the appropriate values of P_i and T_s in Equation 1. Particles larger than 1100 A. were removed during 1 hour of centrifugation at 5000 r.p.m. and those larger than 83, 59, and 41 A. during 3, 6, and 12 hours at 39,400 r.p.m. These calculated values were then related to the quantities of carbon-14 DDT that were lost from the filtrate during the various periods of centrifugation. The periods of acceleration and deceleration are short in the Spinco instrument and were disregarded in calculations because their influence is negligible. At 5000 and 39,400 r.p.m., average centrifugal forces of 1356 and 84,150 G were applied to the top half of the liquid.

Results and Discussion

The carbon-14 DDT used in this investigation was found by paper chromatography to contain a zone of impurity (more polar than DDT) that remained at the origin and reduced permanganate. This zone (57 counts per minute) and that of DDT (9757 counts per minute) at R_f 0.85 were the only zones that were detected radiometrically on the chromatogram. Mitchell (*4*) reported R_f 0.83 for DDT. The zone of impurity contained 0.58% of the total radioactivity detected on the chromatogram.

Preliminary experiments were made with filtrates from 1000 p.p.b. dispersions to determine the appropriate type of centrifuge tube, the effect of stirring back, and the approximate solubility of DDT in water. Adsorption of DDT from the filtrate by stainless steel tubes was found to be considerably less than by cellulose nitrate or polypropylene tubes, quantitative recovery of the insecticide being obtained only from the steel tubes. The fact that concentrations of DDT in the filtrate from the top and bottom halves of the steel tubes after prolonged ultracentrifugation were almost identical indicated that stir back was negligible. After prolonged centrifugation, the 1000 p.p.b. filtrate was found to contain radioactivity equivalent to 6.5 p.p.b. of DDT. However, as much as 5.8 p.p.b. of this could be due to the 0.58% radiolabeled impurity, depending on its water solubility.

In subsequent experiments, the initial concentration of DDT in the dispersion was reduced to 15 p.p.b. Only 0.09 p.p.b. (0.58% of 15 p.p.b.) could be impurity. The filtrate from this preparation contained 7.4 p.p.b. of carbon-14 DDT (corrected for impurity) and included undissolved particles less than 5 microns in diameter that the filter permitted to pass. The concentrations in the filtrate after standing in stainless steel tubes at 1 G for the intervals used in the centrifugation experiments are shown in Table I. When the filtrate was placed in the tubes and withdrawn immediately, the concentration decreased sharply, and then finally decreased during prolonged standing to about 42% of its original value. It is recognized that this affinity of the DDT for the walls of the tubes might have had some influence on the values for the solubility and particle sizes reported in this paper. However, since the change in concentration was less than that incurred by the precipitation of particles >1100 A. and the change during the 6- to 12-hour interval was small, no adjustment of the data was made for this effect.

The concentrations of DDT in the filtrate after various periods of ultracentrifugation are also shown in Table I. These results demonstrate the presence of undissolved particles of carbon-14 DDT in the submicron range, since a change in concentration was effected after particles larger than 1100 A. had been precipitated. After the 6-hour ultracentrifugation period, the DDT concentration remained substantially unaffected, changing only within experimental error (±0.12 p.p.b.) during the subsequent 6-hour centrifugation interval. Based upon the concentration of DDT remaining after removal of particles larger than 41 A., the solubility of DDT in water at 25° C. was approximated at 1.2 p.p.b., which must be considered as a maximum value. In another experiment using the filtrate from a dispersion initially 50 p.p.b., this solubility was confirmed when the value 1.0 p.p.b. (corrected for impurity) was obtained. Even the preliminary experiment with the 1000-p.p.b. suspension falls nicely into line, if the apparent DDT solubility value is corrected for 0.58% impurity assuming complete solubility of impurity: 6.5 (found) − 5.8 (impurity) = 0.7 p.p.b. This value is surprisingly close to the 1.2 p.p.b. found, considering that most of the radioactivity was due to impurity.

The impurity that caused the high apparent solubility value in the preliminary experiment may have been largely responsible for the high value reported by Babers (*1*) who also determined DDT's solubility radiometrically. It is surprising that the solubility reported by Richards and Cutkomp (*6*) approaches the value reported herein, since they used a much less sensitive method of analysis and apparently did

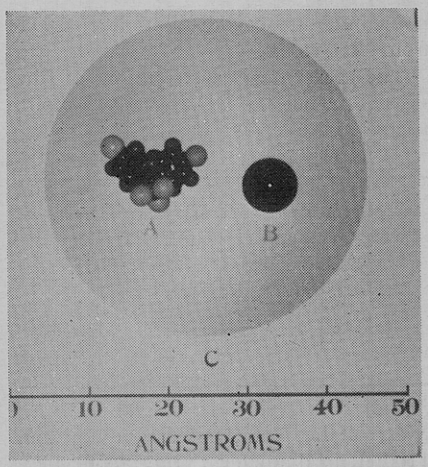

Figure 1. Relative sizes of the DDT molecule *A*, a sphere *B* equal in volume to *A*, and the smallest particle of DDT C that can be precipitated by ultracentrifugation under the experimental conditions employed

Table I. Concentration of Carbon-14 DDT in Filtrate, Initially 7.4 P.P.B., after Various Periods of Time in Stainless Steel Tubes

Hours	Carbon-14 DDT[a], P.P.B. After standing at 1 G	Carbon-14 DDT[a], P.P.B. After centrifugation
0	5.3	...
1	5.0	2.8[b]
3	4.1	1.8[c]
6	3.4	1.4[c]
12	3.1	1.2[c]

[a] Corrected for 0.09 p.p.b. impurity.
[b] At 5000 r.p.m.
[c] At 39,400 r.p.m.

not consider the codistillation of DDT from test systems.

The percentage distribution of the sizes of particles of DDT in the 15-p.p.b. preparation is shown below:

Diameter, A.	Per Cent
>50,000	50.7
1100–50,000	30.7
83–1100	6.6
59–83	2.7
41–59	1.3
7–41	8.0

Most of the particles were larger than 1100 A. in diameter. Only about 10% were in the range of 41 to 1100 A., but this quantity, if it were not removed from the filtrate, is sufficient to cause serious error in the solubility value.

Figure 1 shows a model of the DDT molecule—constructed from Fisher-Hirshfelder-Taylor atomic models—as compared with a sphere 7.1 A. in diameter that has a volume equivalent to that of the DDT molecule, and with the smallest particle, 41 A. in diameter, that could have been removed from the filtrate in the centrifugation experiments. The shape of the molecule is close enough to a sphere that Stokes' law may be used for the determination of particle size without appreciable correction. A centrifugation time in the No. 40 rotor of about 17.5 days at 39,400 r.p.m. is required to remove undissolved amicron particles of DDT larger than molecular size from the top half of the filtrate. Fortunately, stability to ultracentrifugal force was approached within 6 hours and it was not necessary to centrifuge for such an impractical period.

Acknowledgment

Several helpful suggestions were received from Herbert E. Hellwege, Rollins College, Winter Park, Fla., and Morton Beroza of the Entomology Research Division, U. S. Department of Agriculture. P. A. Dahm, Iowa State College, Ames, Iowa, kindly supplied the labeled DDT.

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INSECTICIDE RESIDUES IN MILK

Effects of Feeding Low Levels of Heptachlor Epoxide to Dairy Cows on Residues and Off-Flavors in Milk

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Heptachlor epoxide was fed to dairy cows at 0.5 and 1.0 p.p.m. of roughage intake. Analysis of the butter fat indicated residues reached a level of 0.38 and 1.94 p.p.m. for the two levels, respectively. These feeding rates simulate the residue of heptachlor epoxide shown to be present on alfalfa after practical application of heptachlor.

HEPTACHLOR is an effective insecticide for controlling many insects. It is recommended, particularly for the control of the meadow spittlebug and alfalfa weevil in the Northeast, at dosages ranging from 0.25 to 1 pound per acre, (Gyrisco *et al.*, *2*). Gannon and Decker (*1*) reported that heptachlor was converted to its epoxide on alfalfa and resulted in a residue of 2.42 p.p.m. when as little as 1 pound per acre of heptachlor was applied; 0.17 p.p.m. of the epoxide was still present after 21 days.

Heptachlor epoxide is more persistent and toxic than heptachlor. An experiment was conducted, therefore, to determine if heptachlor epoxide is excreted in the milk of cows fed levels of it, duplicating what comes from forage as a result of practical applications of heptachlor for insect control. Technical heptachlor epoxide was fed to dairy cows at levels of 0.5 and 1.0 p.p.m. of their average total daily roughage for a period of 2 weeks.

Materials and Methods

Three Holstein cows were assigned to each level of technical heptachlor epoxide and five cows were to serve as controls. The cattle were housed at random, in stables so constructed that no animal could steal food from her neighbor. Each cow was fed 40 pounds of hay, 50 pounds of silage, and grain of a good general herd mix at a rate roughly approximating 1 pound of grain for each 4 pounds of milk produced. All feed was carefully weighed out and portions not eaten were weighed and recorded. Thus, an accurate record of food intake was available for each animal over the 2-week standardization or pretest period and the following 2-week test period.

Each week the actual dosage of technical heptachlor epoxide fed to each cow was based on the dry weight of the previous week's intake of roughage. The amount of technical epoxide to be fed each cow to obtain the desired 0.5 or 1.0 p.p.m. dosage was weighed out carefully on a microbalance for each day, divided in half, and added to 1-pound lots of grain. Just prior to grain feeding for the herd each morning and evening, the pound of treated grain was fed in metal containers to each cow. Untreated grain was fed in a similar manner to the five control cows. Samples of milk consisting of 1 quart of fresh, well-mixed, raw milk were taken at the regular morning and evening milkings at −1, 1, 2, 3, 4, 5, 7, and 14 days, while the heptachlor epoxide was being fed, and again at 16, 18, 21, and 28 days after the initiation of the experiment.

Prior to feeding of the heptachlor epoxide and once a week thereafter, additional samples of milk were taken for butterfat, flavor, and odor tests.

The analytical procedure of Meyer, Malina, and Polen (*3*) was used, with several modifications, to determine hep-

not consider the codistillation of DDT from test systems.

The percentage distribution of the sizes of particles of DDT in the 15-p.p.b. preparation is shown below:

Diameter, A.	Per Cent
>50,000	50.7
1100–50,000	30.7
83–1100	6.6
59–83	2.7
41–59	1.3
7–41	8.0

Most of the particles were larger than 1100 A. in diameter. Only about 10% were in the range of 41 to 1100 A., but this quantity, if it were not removed from the filtrate, is sufficient to cause serious error in the solubility value.

Figure 1 shows a model of the DDT molecule—constructed from Fisher-Hirshfelder-Taylor atomic models—as compared with a sphere 7.1 A. in diameter that has a volume equivalent to that of the DDT molecule, and with the smallest particle, 41 A. in diameter, that could have been removed from the filtrate in the centrifugation experiments. The shape of the molecule is close enough to a sphere that Stokes' law may be used for the determination of particle size without appreciable correction. A centrifugation time in the No. 40 rotor of about 17.5 days at 39,400 r.p.m. is required to remove undissolved amicron particles of DDT larger than molecular size from the top half of the filtrate. Fortunately, stability to ultracentrifugal force was approached within 6 hours and it was not necessary to centrifuge for such an impractical period.

Acknowledgment

Several helpful suggestions were received from Herbert E. Hellwege, Rollins College, Winter Park, Fla., and Morton Beroza of the Entomology Research Division, U. S. Department of Agriculture. P. A. Dahm, Iowa State College, Ames, Iowa, kindly supplied the labeled DDT.

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INSECTICIDE RESIDUES IN MILK

Effects of Feeding Low Levels of Heptachlor Epoxide to Dairy Cows on Residues and Off-Flavors in Milk

C. A. BACHE, GEORGE G. GYRISCO, S. N. FERTIG, E. W. HUDDLESTON, D. J. LISK, F. H. FOX, G. W. TRIMBERGER, and R. F. HOLLAND

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Heptachlor epoxide was fed to dairy cows at 0.5 and 1.0 p.p.m. of roughage intake. Analysis of the butter fat indicated residues reached a level of 0.38 and 1.94 p.p.m. for the two levels, respectively. These feeding rates simulate the residue of heptachlor epoxide shown to be present on alfalfa after practical application of heptachlor.

HEPTACHLOR is an effective insecticide for controlling many insects. It is recommended, particularly for the control of the meadow spittlebug and alfalfa weevil in the Northeast, at dosages ranging from 0.25 to 1 pound per acre, (Gyrisco *et al.*, *2*). Gannon and Decker (*1*) reported that heptachlor was converted to its epoxide on alfalfa and resulted in a residue of 2.42 p.p.m. when as little as 1 pound per acre of heptachlor was applied; 0.17 p.p.m. of the epoxide was still present after 21 days.

Heptachlor epoxide is more persistent and toxic than heptachlor. An experiment was conducted, therefore, to determine if heptachlor epoxide is excreted in the milk of cows fed levels of it, duplicating what comes from forage as a result of practical applications of heptachlor for insect control. Technical heptachlor epoxide was fed to dairy cows at levels of 0.5 and 1.0 p.p.m. of their average total daily roughage for a period of 2 weeks.

Materials and Methods

Three Holstein cows were assigned to each level of technical heptachlor epoxide and five cows were to serve as controls. The cattle were housed at random, in stables so constructed that no animal could steal food from her neighbor. Each cow was fed 40 pounds of hay, 50 pounds of silage, and grain of a good general herd mix at a rate roughly approximating 1 pound of grain for each 4 pounds of milk produced. All feed was carefully weighed out and portions not eaten were weighed and recorded. Thus, an accurate record of food intake was available for each animal over the 2-week standardization or pretest period and the following 2-week test period.

Each week the actual dosage of technical heptachlor epoxide fed to each cow was based on the dry weight of the previous week's intake of roughage. The amount of technical epoxide to be fed each cow to obtain the desired 0.5 or 1.0 p.p.m. dosage was weighed out carefully on a microbalance for each day, divided in half, and added to 1-pound lots of grain. Just prior to grain feeding for the herd each morning and evening, the pound of treated grain was fed in metal containers to each cow. Untreated grain was fed in a similar manner to the five control cows. Samples of milk consisting of 1 quart of fresh, well-mixed, raw milk were taken at the regular morning and evening milkings at −1, 1, 2, 3, 4, 5, 7, and 14 days, while the heptachlor epoxide was being fed, and again at 16, 18, 21, and 28 days after the initiation of the experiment.

Prior to feeding of the heptachlor epoxide and once a week thereafter, additional samples of milk were taken for butterfat, flavor, and odor tests.

The analytical procedure of Meyer, Malina, and Polen (*3*) was used, with several modifications, to determine hep-

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Days after First Feeding	Heptachlor Epoxide in Butterfat, P.P.M.[a]	Absorbance	
		Sample	Check
Cow No. 22 fed at the 0.5 p.p.m. level[b]			
−1[c]	0.01		
1[d]	0.13	0.093	0.062
2	0.21	0.124	0.068
3	0.25	0.139	0.068
4	0.36	0.182	0.074
5	0.38	0.191	0.074
7	0.35	0.169	0.068
14	0.29	0.153	0.068
16	0.30	0.188	0.082
18	0.19	0.147	0.082
28	0.24	0.162	0.098
Cow No. 30 fed at the 1.0 p.p.m. level[e]			
1	0.05	0.082	0.062
3	1.34	0.444	0.078
4	1.04	0.303	0.078
7	1.71	0.460	0.057
14	1.94	0.460	0.057
16	1.20	0.431	0.062
21	0.72	0.287	0.103
28	0.52	0.230	0.103

[a] Uncorrected for check and recovery.
[b] $t = 9.25$. $t(0.01) = 3.25$.
[c] 1 day before feeding heptachlor epoxide was begun.
[d] First day of feeding heptachlor epoxide.
[e] $t = 5.12$. $t(0.01) = 3.49$.

tachlor epoxide. The cream was separated from the milk using a mechanical separator. The butter oil was extracted from the cream as in the procedure of Westlake (*5*). About 50 grams of the cream were tumbled with 300 ml. of pentane for 15 minutes. One hundred fifty grams of sodium sulfate were added and the pentane extract was decanted. One hundred milliliters of pentane were added, tumbled for 30 minutes, and the pentane was decanted off. One hundred milliliters more of pentane were added, tumbled for 45 minutes, and the remaining pentane was decanted off. The pentane extracts were combined and the pentane was distilled off through a Snyder column, on a hot water bath. Fifteen grams of butter oil were taken for analyses. Activated Florex XXS was used in the chromatographic step and 6% of diethyl ether was employed to elute heptachlor epoxide from this column. A new standard recovery curve was prepared with each pair of samples analyzed. The curve followed Beer's law. Recovery of heptachlor epoxide added to cream averaged 113.5%. Time did not permit analyses of the milk from all of the cows in the experiment.

Results and Discussion

Table I shows the residues of heptachlor epoxide found in milk. All standard curves prepared were recovery curves; hence, the check values become 0 γ. Because milk from epoxide-fed cows and control cows was taken at every sampling date, it was believed desirable to analyze the milk for heptachlor epoxide together with the check taken on the same day, so the values might be compared as paired values. These values could then be tested by the Student's t test for significance (*4*).

Heptachlor epoxide was present in the milk when either level of epoxide was fed to the dairy cattle (Table I). The amounts present at any sampling data were real and greater than that in the controls at the 1% level.

Prior to the feeding of the heptachlor epoxide, at the middle of the test period, and again at the termination, a trained veterinarian examined each cow for any signs of poisoning by checking its weight, temperature, pulse, respiration, nature of feces, etc. No flavors or odors that could be attributed to the feeding of heptachlor epoxide were found present in the milk. None of the cows exhibited any signs of poisoning or showed any weight loss. All test animals remained normal and healthy during the course of the experiment.

Conclusion

These data suggest that if heptachlor epoxide does appear in the field as residues from normal applications of heptachlor for insect control, the feeding of such residues will result in the secretion of heptachlor epoxide in milk.

Acknowledgment

The authors thank the Velsicol Chemical Corp. for financing, in part, the work reported here, for technical assistance, and for supplying the materials used. The Northeast Regional Project NE-36 also contributed financially. Thanks are also rendered to the many individuals in the Departments of Entomology and Agronomy for their help.

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INSECTICIDE RESIDUES IN MILK

The Effects of Feeding High Levels of Sevin on Residue, Flavor, and Odor of the Milk of Dairy Cattle

GEORGE G. GYRISCO, D. J. LISK, S. N. FERTIG, E. W. HUDDLESTON, F. H. FOX, R. F. HOLLAND, and G. W. TRIMBERGER

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SEVIN (1-naphthyl-*N*-methyl carbamate), a new insecticide with relatively low mammalian toxicity and a wide range of effectiveness on many insects, was particularly effective on the gypsy moth caterpillar, and hence was chosen as a possible replacement for DDT for 1959 in the U. S. Department of Agriculture gypsy moth eradication program. Because forage as pasture, hay, grain, and silage would be contaminated by the blanket coverage needed in the eradication program, it was the purpose of this experiment to determine if the feeding of high levels of Sevin would affect the health of dairy cattle, cause residues to appear in the milk, or result in off-flavors or odors.

Earlier tests by the U. S. Department of Agriculture at Kerrville (*2*) had indicated that Sevin was not detected in milk of dairy cows fed levels of Sevin varying from 2.5 to 50 p.p.m. Therefore, it was arbitrarily decided to feed technical Sevin for a period of 2 weeks at levels of 50, 150, and 450 p.p.m. of the average total daily roughage (hay and silage) intake of the dairy cattle.

Five cows of the breeds Brown Swiss, Holstein, Jersey, and Ayrshire were as-

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Earlier tests by the U. S. Department of Agriculture at Kerrville (*2*) had indicated that Sevin was not detected in milk of dairy cows fed levels of Sevin varying from 2.5 to 50 p.p.m. Therefore, it was arbitrarily decided to feed technical Sevin for a period of 2 weeks at levels of 50, 150, and 450 p.p.m. of the average total daily roughage (hay and silage) intake of the dairy cattle.

Five cows of the breeds Brown Swiss, Holstein, Jersey, and Ayrshire were as-

Technical Sevin was fed to dairy cows of the Brown Swiss, Jersey, Holstein, and Ayrshire breeds at 50, 150, and 450 p.p.m. of their average total daily roughage intake (dry weight) for a period of 2 weeks. Samples of milk were taken at regular intervals and the cream was analyzed for Sevin by means of the p-nitrobenzenediazonium fluoborate coupling method (Union Carbide). The concentration of Sevin, if present, was below the sensitivity of the analytical method, 0.01 p.p.m.

signed at random to each of the three insecticide levels fed and to a control. Each treatment had, as far as possible, at least one animal of each breed.

Experimental Design and Methods

The cows were housed in a completely randomized order in stalls so constructed that no animal could steal from her neighbor.

Jerseys were fed 25 pounds of hay and 30 pounds of good corn silage daily, while Ayrshires received 30 pounds of hay and 40 pounds of silage. Brown Swiss and Holsteins each were given 40 pounds of hay and 50 pounds of silage. Grain of a good general herd mix was fed on a formula which took into account the amount and quality of roughage fed, milk and butterfat produced, etc., but which in general amounted to about 1 pound of grain for each 4 pounds of milk produced. All feed and roughage for each cow were weighed carefully into the mangers at each feeding and weighbacks of portions not eaten were recorded. Thus, an accurate record of food intake was available for each animal over the test period and a 2-week standardization, or pretest period.

The moisture content of each roughage was determined and computed as a mean of several samples, and the dosage of Sevin fed was then based on the mean dry weight of the daily roughage intake averaged over a week's feeding period. Each week the dosage of insecticide fed to each cow was based on the previous week's intake of roughage and was adjusted accordingly for each cow. The level of technical Sevin to be fed each cow daily was weighed out separately on a Mettler H-5 balance, divided in half and added to two 1-pound lots of grain. Just prior to grain feeding each morning and afternoon, the pound of treated grain was fed to each cow in metal baskets. At the same time, nontreated 1-pound lots were fed to the 5 control cows. The treated grain was quickly consumed as the cows soon learned that more grain was forthcoming, once the treated grain was consumed. None of the treated grain was ever rejected by any of the cows even at the 450 p.p.m. level of Sevin.

The experiment was initiated on January 5, and on January 18, the first Sevin-treated grain was fed. Feeding of treated grain continued until February 1. Samples of milk for residue analysis were taken at −1, 0, 1, 2, 3, 4, 5, 7, and 14 days, while the Sevin was being fed, and again at 16, 18, 21, and 28 days in the third week after the insecticide had been stopped.

Milk samples were taken twice daily at the regular morning and afternoon milking times and consisted of 1 quart of raw, well-mixed milk from each cow.

Just prior to the start of the feeding of Sevin, and once weekly thereafter, samples of milk were taken for butterfat, flavor, and odor tests.

Prior to the feeding of Sevin, at the middle of the experimental period, and again at its conclusion, an experienced veterinarian checked the weight, temperature, pulse, respiration, and type of feces of each animal, observing them for any symptoms of insecticide poisoning.

All feed (hay, silage, and grain) was of excellent quality and was from a single source in sufficient quantity to last for the entire experimental period. Analysis of all cattle feed was made to ascertain that no contaminants were present.

Methods of Chemical Analysis

Mechanically separated cream was extracted with hexane-ether and partition extracted with hexane-acetonitrile according to the unpublished method of Westlake (*3*) as follows:

The cream was extracted by mechanical separation from 400 grams of milk, three times in a separatory funnel with 150-ml. portions of a mixture of equal volumes of *n*-hexane and ether. The extracts were combined, filtered through dry cotton, and evaporated to a 25-ml. volume. The evaporated solvent mixture was transferred to a separatory funnel and extracted three times with 15-ml. portions of acetonitrile. The acetonitrile solutions were combined in a separatory funnel, 200 ml. of water were added and the mixture was extracted three times with 50-ml. portions of diethyl ether. The combined ether extracts were filtered through dry cotton and the filtrate was evaporated to dryness. Spectrophotometric determination of Sevin then followed using the *p*-nitrobenzenediazonium fluoborate coupling method (*1*). Recovery of Sevin added to cream was usually about 84%. The sensitivity of the analytical method was about 0.01 p.p.m.

Discussion and Results

Seventy-eight samples of milk including those of the checks were analyzed for Sevin. The net residues in the milk ranged from (−)0.0062 to 0.0087 p.p.m. Therefore, the concentration of Sevin, if present in the milk, was below the sensitivity of the analytical method. All of the milk samples were not analyzed for residues because milk from the highest levels of Sevin fed failed to disclose measurable Sevin residues. However, some samples of milk from cows fed lower levels of Sevin were selected at random and analyzed. The readings from these samples did not differ from the milk of cows fed 450 p.p.m. of Sevin.

No difference in the reaction of cattle to Sevin was noted among the four breeds.

Unsuccessful attempts were made to recover 1-naphthol, a hydrolysis product of Sevin, added to cream. It was thought that 1-naphthol or esters of it which are known to be formed in other animals might exist in cow's milk. Saponification of milk, followed by analysis for 1-naphthol, was unsatisfactory; hence this work was not continued.

No off-flavors or odors that could be attributed to Sevin were found in any of the milk samples.

None of the cows showed any abnormal changes in weight or exhibited any symptoms of poisoning. Food intake and excretion were normal.

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The authors thank H. Y. Forsythe, Jr., C. S. Koehler, S. W. Jacklin, G. O. Poinar, Jr., R. L. Ridgway, and H. H. Shorey for parts of the work reported here.

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Calcium Sulfate Transitions in Superphosphate

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Thermodynamic equilibrium curves are given for the system calcium sulfate–phosphoric acid–water. Applicability to the liquid phase in superphosphate is discussed. Based on this the calcium sulfate transitions in superphosphate are explained in agreement with several observations.

THE OLD LITERATURE on calcium sulfate and its crystal forms is highly confusing. To explain existing data, several varieties of dihydrate, semihydrate, and anhydrite were postulated. The excellent work by Kelley, Southard, and Anderson (*3*) has, however, made things clear. Obscurities in nomenclature are elucidated by using the system suggested by Kruis and Späth (*4*).

In the calcium sulfate–phosphoric acid–water system, only three crystal forms of calcium sulfate can exist: dihydrate, α-semihydrate,* and anhydrite II. The asterisk indicates an over-stoichiometric crystal water content. Kelley, Southard, and Anderson (*3*) have given equations for the temperature dependence of the free energy for all real and hypothetical reactions in the calcium sulfate–water system. When the activity of water differs from unity, as in electrolyte solutions, an additional term, $nRT \times \ln a$, must be inserted, where:

n = moles of water in the reaction formula
R = general gas constant
T = temperature, °K.
a = activity of water in the solution

For zero free energy, equilibrium conditions between the two modifications of calcium sulfate in the reaction formula are obtained, the transition temperature being a function of water activity. By combining this finding with water vapor pressure data of phosphoric acid solutions (*1*), the thermodynamic equilibrium curves for the transitions dihydrate–anhydrite II and dihydrate–α-semihydrate* as a function of acid concentration have been obtained (Figure 1). For the metastable equilibrium dihydrate–α-semihydrate,* curves are also obtained from solubility measurements by Taperova and Shul'gina (*7*, *8*) and from direct observations in wet process phosphoric acid by Hultbom and Lehrecke (*5*). Agreement is good except for low concentrations, where Hultbom and Lehrecke extrapolated their curve to a transition temperature of 107°C. in pure water, and which today is known to be incorrect. The other thermodynamic curve shows the stable equilibrium dihydrate–anhydrite II.

The transitions of calcium sulfate that occur in superphosphate are of particular interest, as they explain several observation data and, partly, the caking of granulated material during storage. A superphosphate consists mainly of monocalcium phosphate and calcium sulfate as solid phases and a rather high concentration of phosphoric acid as the liquid phase.

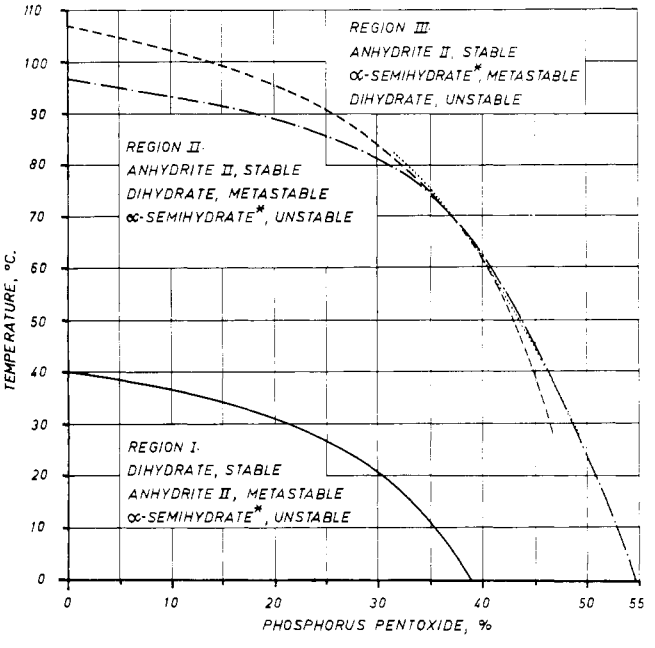

Figure 1. The transitions dihydrate-α-semihydrate and dihydrate-anhydrite II in phosphoric acid solutions

When calculating their thermodynamic relations, Kelley, Southard, and Anderson (*3*) neglected the influence of dissolved calcium sulfate on water vapor pressure because of its low solubility. The solubility measurements by Taperova and Shul'gina (*7*, *8*) show that the solubility of calcium sulfates increases up to about 15 to 20% phosphorus pentoxide and then decreases. At 40 to 45% phosphorus pentoxide it will be about the same as in pure water. The influence of dissolved calcium sulfate on the water vapor pressure at phosphorus pentoxide concentrations in the liquid phase of a normal superphosphate might be neglected. The solubility diagram of the calcium oxide–water–phosphorus pentoxide system (*9*) shows that for a normal concentration of phosphorus pentoxide in the liquid phase of superphosphate and at the corresponding temperature range for the equilibrium dihydrate–α-semihydrate,* the liquid phase may not contain more than 4% calcium oxide. A comparison between vapor pressure data of the previously mentioned system (*2*) at 25°C. and of the pure phosphoric acid solution (*1*) with the same phosphorus pentoxide content gives a corresponding decrease in water activity of about 0.015 to 0.020. Information at higher temperatures is not available. Furthermore, the presence of sulfate will be influential. However, the decrease in the equilibrium temperature caused by monocalcium phosphate is estimated to be 4° to 5°C., within the actual range.

In the liquid phase of a superphosphate other ions from impurities are to be considered. There is no information on their influence on water vapor pressure. However, the equilibrium diagram given here is applicable to a qualitative discussion of the transitions in superphosphate. It will be applicable more quantitatively to wet process acids, where impurities have less influence.

The first part of the acidulation of a phosphate rock takes place within region III, Figure 1. Some formation of α-semihydrate* simultaneously as anhydrite II is therefore possible during the re-

action in the den and the first part of the maturing period. Later, during maturing in the piles the temperature decreases, and the system is moving into region II, where the semihydrate can convert into dihydrate, if the supersaturation is high enough for dihydrate nucleation. Within those two regions there is a trend of semihydrate and dihydrate conversion into anhydrite II, but without high temperatures, as in the first part of the reaction in the den, this is known as an extremely slow process.

A matured and bagged superphosphate with a normal moisture content has a temperature and acid concentration in the liquid phase that correspond to region I, where anhydrite may go into solution and crystallize as dihydrate. This can proceed until so much water has been removed from the liquid phase that, because of the corresponding increase in acid concentration, the equilibrium curve is reached.

With extra hard drying all semihydrate probably converts into anhydrite II. Therefore in region II nothing can happen and the acid concentration in the liquid phase of the superphosphate will be so high that region I may not be reached. The above is in good agreement with several observations reported by Scheel (*6*) and the remedies recommended to prevent caking of the superphosphate.

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FERTILIZER QUALITY CONTROL

Fertilizer Sampling to Protect Consumers and Producers

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Methods are given for devising fertilizer sampling plans which manufacturers can use for assessing the quality of their products with confidence, or on which government regulations can be based. The sampling plans use defined consumer's and producer's risks to avoid the false information that single snap samples usually give. The methods can be applied to other chemicals which require costly chemical analyses.

The South African Department of Agricultural Technical Services is revising its regulations which control the qualities of fertilizers and fertilizer mixtures that may be sold to consumers. The regulations will be based on an acceptance sampling plan which recognizes that fertilizers cannot be produced without some unavoidable variation in quality. Hence, whenever a fertilizer is sampled by a government inspector, a number of samples instead of one snap sample will be taken. By this means the true quality of the material sampled will be realistically estimated. However, there remains the possibility that a bad estimate may be obtained by chance. Thus producers run a risk of being penalized although their fertilizers are of good quality and consumers run a risk of accepting poor quality material. The sampling plan envisaged in South Africa is based on defined small producer's and consumer's risks.

This paper discusses not the particular plan adopted in South Africa, but general sampling plans for fertilizers. With the formulas provided it is possible to devise plans to suit particular needs and purposes.

The first part of the discussion introduces the concept of defined risks. This may be tediously obvious to many, but it has been included because a sampling plan cannot efficiently be used without fully understanding its principles and operation. The second part shows how to devise a sampling plan for accepting or rejecting a particular batch of fertilizer. The third part shows how to devise a sampling plant to judge the performances of fertilizer producers over a period of time.

The Concept of Defined Risks

Variation in Fertilizer Quality. Consider the chemical analyses of single samples taken from consecutive batches of superphosphate produced by a hypothetical manufacturer. If the phosphate content is plotted against the order in which the batches were made, a diagram such as Figure 1 will be obtained.

Figure 1 shows that variation has occurred, and it must be admitted that a certain amount of variation is unavoidable. In fact, to produce absolutely uniform material would require absolute control of all variables, including the quality of the raw materials used. Such perfect control is impossible.

In the absence of perfect control, some variation must be accepted. The degree of variation can be reduced with better quality control of manufacture, but an irreducible amount of variation remains.

Representation of Variation by Distribution Curves. Figure 1 helps to view as a whole the variation in quality of the superphosphate. It shows that although the average phosphate content is 19.0% P_2O_5, about half the points fall below this value.

The data can be summarized in a more useful way. If the graph in Figure 1 is turned on end, the % P_2O_5 scale can be divided into classes, or intervals, and the number of points falling into each class can be counted. The percentage frequency with which the points fall into each class can be calculated and the histogram shown in Figure 2 can be constructed.

This histogram is only an approximate representation of the true variation in the superphosphate, because only single samples were taken from each batch.

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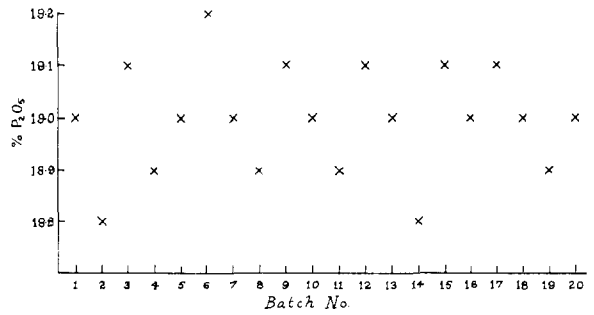

Figure 1. Variation in fertilizer quality

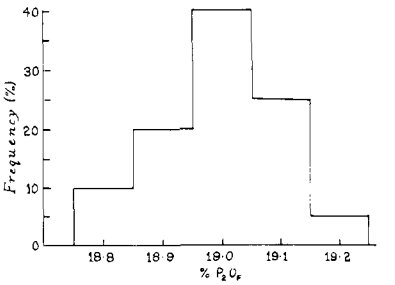

Figure 2. Histogram depicting variation in fertilizer quality

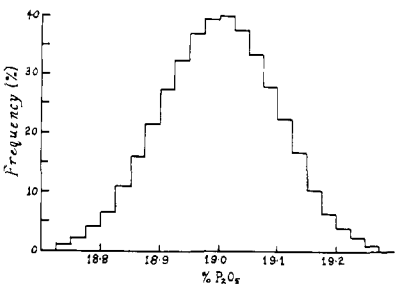

Figure 3. Histogram approximating theoretical distribution

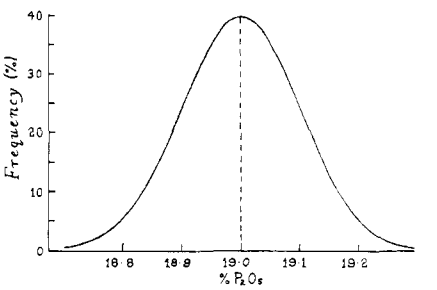

Figure 4. Normal distribution curve

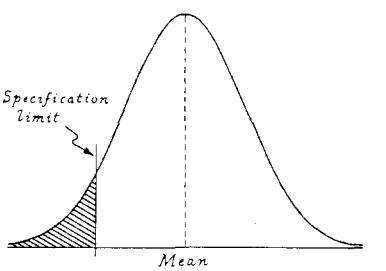

Figure 5. Shaded area gives risk of failing specification

If a number of samples had been taken from each batch and each sample analyzed, the larger number of analyses would allow a histogram as shown in Figure 3 to be constructed. This histogram more closely approximates the true variation in the superphosphate quality.

If the process of taking more and more samples for analysis is continued, the histograms ultimately approach a smooth theoretical distribution curve as shown in Figure 4.

It is convenient to use a theoretical distribution curve to describe the variation in quality of a fertilizer and to assume that the quality of the fertilizer is normally distributed. The normal distribution can be specified by its mean, or average, which is the position of the peak in the distribution curve (mean = 19.0% in Figure 4), and its standard deviation, which is a measure of the spread, or scatter, of the distribution about its mean. A wide flat distribution curve has a larger standard deviation than a narrow peaked curve.

Proportion Failing Specification. Suppose it is required to produce superphosphate that must not contain less than 19% P_2O_5. In good faith the manufacturer ensures that the mean content of his fertilizer is truly 19.0% P_2O_5. Then if Figure 4 represents the distribution of the phosphoric oxide content, it is seen from the symmetry of the curve about the mean of 19.0% P_2O_5 that the manufacturer will suffer a 50% chance of failing the specification on a snap sample.

To reduce the chances of failure, the manufacturer could produce material with a mean content greater than the specified value, as shown in Figure 5. Then chances of failing the specification would be given by the ratio of the shaded area to the total area under the curve.

Operating Characteristic Curves. The notion of defined risks of failure can be carried further to construct a graph from which can be read off the mean quality at which to aim for a defined risk of failing the specification.

To conform to the conventions adopted in acceptance sampling plans, the chances of passing the specification must be considered. This offers no difficulties, because if the chances of failure are p%, the chances of passing are (100 − p)%. In Figure 5 the chances of passing the specification are given by the ratio to the total area of the unshaded area on the right of the specification limit.

Consider a manufacturer to be producing superphosphate to the specification: not less than 19% P_2O_5 on a snap sample. If the mean phosphate content is 18.8% P_2O_5, as seen in *i* of Figure 6, *a*, his chance of passing the specification is small. This chance can be calculated and plotted as point *i* in Figure 6, *b*. If now the manufacturer increases the mean content to 18.9% P_2O_5, then, as shown in *ii* of Figure 6, *a*, the chance of passing on a snap sample is greater but still less than 50%. Again this chance can be calculated and plotted as point *ii* in Figure 6, *b*. When the manufacturer produces with a mean of 19.0% P_2O_5, he enjoys (or suffers) a 50% chance of the superphosphate passing on a snap sample. This gives point *iii* in Figure 6, *b*. The remaining points, *iv* and *v*, are now evident.

A smooth curve is drawn through the points in Figure 6, *b*, and found to be a cumulative normal distribution curve. This is called an operating characteristic curve. It is characteristic of the specification "not less than 19% P_2O_5" and the standard deviation of the distributions shown in Figure 6, *a*, all the distributions having the same standard deviation.

Using the operating characteristic curve, the manufacturer can read off at what average to aim in order to ensure a chosen large chance of the materials passing the specification (or small chance of its failing). Thus the manufacturer can produce with a defined risk.

The operating characteristic curve also gives the consumer's risk, because it shows the chances of his accepting material of a particular mean quality.

Acceptance Sampling Plans. The operating characteristic curve in Figure 6,*b*, enables a manufacturer to operate at a defined risk of failing on a snap sample. However, if he uses such a curve he will find that to reduce his risk of failure to a reasonably small level an uneconomically high grade material must be produced to pass the specification. The fault lies with taking a single snap sample.

To overcome this, a sampling inspection plan can be devised whereby a lot, or batch or consignment, of fertilizer is judged on the quality of several samples taken from the batch instead of a single snap sample.

There are two ways in which sampling inspection plans for fertilizers can be devised. The first kind of inspection plan requires a number of samples to be taken from a lot and each sample analyzed. From the analyses the mean and standard deviation of the sampled lot are calculated and from the mean and standard deviation the proportion of the lot which fails a specified limit is calculated. If this proportion of de-

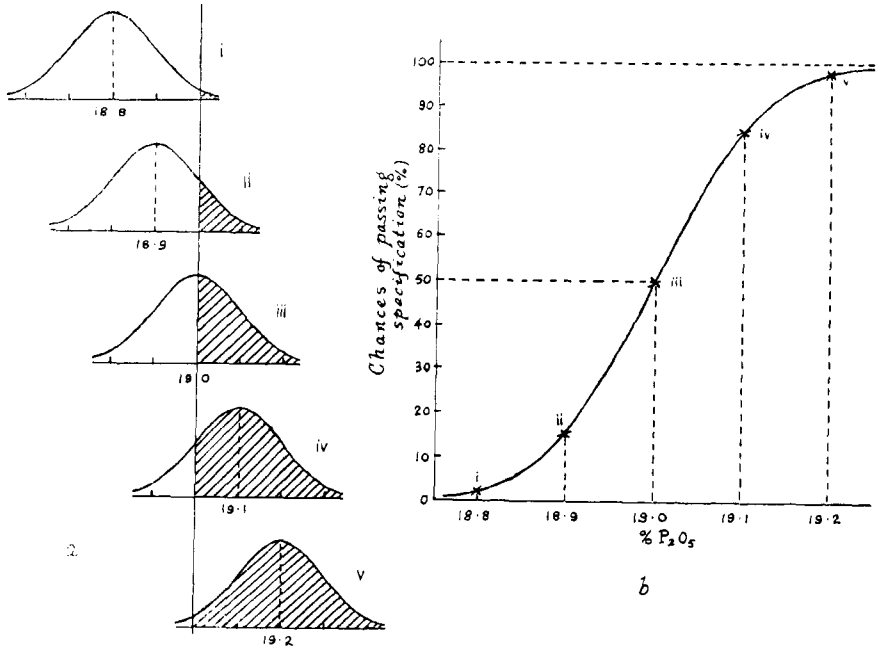

Figure 6. Construction of operating characteristic curve

fective material is too high, the lot is considered unacceptable and failed. This first kind of plan has many advantages, but it requires a larger number of samples than the second kind, which is outlined in the next paragraph. It also requires individual analyses of each sample. Because the quality of a fertilizer is judged on the results of chemical analyses, the plan is too costly to operate. If cheaper tests of fertilizer quality could be devised, the plan would be eminently suitable. This first type of plan is not discussed further in this paper.

In the second kind of sampling plan the number of samples to be taken is reduced by using a fixed standard deviation. A number of samples are taken and analyzed, either individually or composited, to find the mean content of the sampled lot. This mean is compared with a specified limit; if it is less than the limit; the sampled lot is failed. This second type of plan is not so powerful a tool as the first type, but it has the virtue of economy. If the samples can be well composited, it allows a large number of samples to be taken but requires only a few chemical analyses.

Only the second type of sampling plan is considered in this paper and it is used in two ways: to judge the quality of a lot and to judge the over-all performance of a manufacturer.

Although this paper discusses fertilizer sampling, the plans can just as usefully be applied to other chemicals which require costly analyses.

Use of Sampling Plans for Judging Lots

Throughout this second part of the discussion acceptance sampling plans will be devised to judge the quality of a lot, batch, or consignment of fertilizer. The results of sampling therefore apply only to the lot sampled.

Parameters of Sampling Plan. The principles developed above are applied to devise a sampling plan which will be based on the following parameters, or defined constants:

α = producer's risk of having a good quality fertilizer fail because bad samples are taken by chance

β = consumer's risk of accepting a poor quality fertilizer because good samples are taken by chance

σ = standard deviation of the lot sampled (assumed to be the same for all lots)

U_0 = measure of what is considered a good quality fertilizer

U_2 = measure of what is considered a poor quality fertilizer

From these parameters the following are calculated:

n = the number of samples which must be taken

C = the specification limit below which the mean of the n samples must not fall

Derivation of Mathematical Formulas. It is a consequence of the normal distribution that if n samples are taken from a batch of fertilizer having a standard deviation σ, then the mean, $\bar{x}$, of the n samples will have a standard deviation $\sigma_{\bar{x}}$, where

$$\sigma_{\bar{x}} = \sigma/n^{1/2} \qquad (1)$$

Suppose that a manufacturer has produced a batch of superphosphate which is normally distributed with a standard deviation, σ, about a true mean phosphate content equal to U_0% P_2O_5 as shown in Figure 7. If C is the specification limit below which the mean, $\bar{x}$, of n samples taken at random from the batch must not fall, then the probability, α, that the sampled batch will fail the specification because n bad samples are taken by chance is given by

$$\alpha = \{1/(2\pi)^{1/2}\sigma_{\bar{x}}\} \int_{-\infty}^{C} \exp\left[-\frac{1}{2}(\bar{x} - U_0)^2/\sigma_{\bar{x}}^2\right] d\bar{x}$$

Putting

$$t = (U_0 - \bar{x})/\sigma_{\bar{x}}$$

the integral transforms to

$$\alpha = -\{1/(2\pi)^{1/2}\} \int_{\infty}^{t\alpha} \exp\left[-\frac{1}{2}t^2\right] dt$$

where

$$t_\alpha = (U_0 - C)/\sigma_{\bar{x}} \qquad (2)$$

or, by virtue of the symmetry of the normal distribution,

$$\alpha = \{1/(2\pi)^{1/2}\} \int_{-\infty}^{t\alpha} \exp\left[-\frac{1}{2}t^2\right] dt \qquad (3)$$

As this integral has been tabulated as the cumulative normal distribution, α can be read off from those tables if t_α is known—that is, if U_0, C, and $\sigma_{\bar{x}}$ are known.

The probability, α, is referred to as the producer's risk because it gives the risk of failure through bad samples being taken by chance, even though the producer manufactured a batch of good fertilizer with a true mean U_0 which is above specification limit C.

Consumer's Risk of Accepting a Batch of Poor Quality Fertilizer. Suppose the batch of superphosphate is normally distributed with a standard deviation σ about a true mean phosphate content equal to U_2% P_2O_5, which is below the specification limit C% P_2O_5, as shown in Figure 8.

The probability, β, that the sampled batch will pass the specification because n good samples are taken by chance is given by

$$\beta = \{1/(2\pi)^{1/2}\sigma_{\bar{x}}\} \int_{c}^{\infty} \exp\left[-\frac{1}{2}(\bar{x} - U_2)/\sigma_{\bar{x}}^2\right] d\bar{x}$$

Putting

$$t = (\bar{x} - U_2)/\sigma_{\bar{x}}$$

the integral transforms to

$$\beta = \{1/(2\pi)^{1/2}\} \int_{t_\beta}^{\infty} \exp\left[-\frac{1}{2}t^2\right] dt \qquad (4)$$

where

$$t_\beta = (C - U_2)/\sigma_{\bar{x}} \qquad (5)$$

As the integral in Equation 4 has been tabulated as the cumulative normal distribution, β can be read off from those

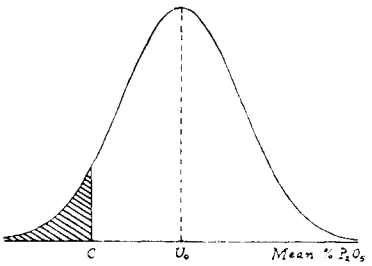

Figure 7. Shaded area gives producer's risk

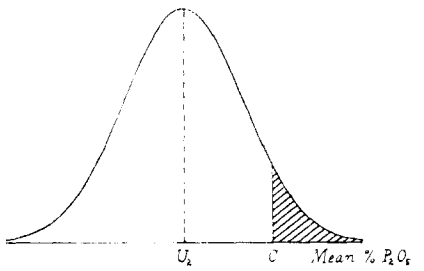

Figure 8. Shaded area gives consumer's risk

tables if t_β is known—that is, if C, U_2, and $\sigma_{\bar{x}}$ are known.

The probability, β, is referred to as the consumer's risk because it gives the risk that the consumer runs of accepting a batch of poor quality fertilizer, of which the true mean U_2 is below the specification limit, C, by the chance happening that n good samples may be selected at random.

Now the process must be reversed to determine the n and C which will give defined risks α and β. Equations 2 and 5 can be rewritten as

$$U_0 - C = t_\alpha \sigma_{\bar{x}} = t_\alpha \sigma/n^{1/2} \text{ by Equation 1}$$

$$C - U_2 = t_\beta \sigma_{\bar{x}} = t_\beta \sigma/n^{1/2} \text{ by Equation 1}$$

Adding these two equations,

$$U_0 - U_2 = (t_\alpha + t_\beta)\sigma/n^{1/2}$$

or

$$n = (t_\alpha + t_\beta)^2\sigma^2/(U_0 - U_2)^2 \qquad (6)$$

Thus if values are stipulated for α and β, values for t_α and t_β can be obtained from Equations 3 and 4, or from tables of the cumulative normal distribution, and then if σ, U_0, and U_2 are also stipulated, the number of samples, n, which must be taken can be calculated from Equation 6. The specification limit, C, can then be calculated from Equation 2, which can be rewritten as

$$C = U_0 - t_\alpha \sigma/n^{1/2} \qquad (7)$$

Specimen Sampling Plan for Straight Fertilizers. How many samples should be taken, and what should the specification limit be for a superphosphate sampling plan based on the following parameters?

α = 1%
β = 5%
U_0 = reputed phosphate content, or guaranteed analysis
U_2 = 92% of reputed phosphate content
σ = 4% of reputed phosphate content

Here $\alpha = 1\% = 0.01$, so that, from tables of the cumulative normal distribution, $t_\alpha = 2.326$. Similarly, $\beta = 0.05$, so that $t_\beta = 1.645$. Thus, from Equation 6,

$$n = (3.971)^2(0.04U_0)^2/(U_0 - 0.92U_0)^2$$
$$= (3.971)^2(0.04)^2/(1 - 0.92)^2$$
$$= 3.94$$

To the nearest whole number, $n = 4$. From Equation 7,

$$C = U_0 - 2.326(0.04U_0)/(3.94)^{1/2}$$
$$= U_0(1 - 0.04687)$$

i.e.,

$$C = 0.95313U_0$$

or

$$C \simeq 95.3\% \text{ of } U_0$$

The specimen sampling plan then is: "Take four samples at random from the batch and analyze these four samples; if the mean of the four analyses is less than 95.3% of the reputed phosphate content of the batch, then fail the batch."

Where a plan requires a large number of samples, these can be reduced to a smaller number for analysis by suitable methods of compositing.

Operating Characteristic of Specimen Sampling Plan. The specimen plan gives the risk, α, that the producer will run if the sampled lot has mean content U_0 and standard deviation σ. It also gives the risk, β, that the consumer runs if the true mean content is U_2. The producer's and consumer's risks for intermediate mean values are given by the operating characteristic curve, which can be calculated from the formula

$$t = (U - C)n^{1/2}\sigma \qquad (8)$$

which is obtained from Equation 2 by regarding U as a variable and expressing it as a percentage of the reputed content. Thus for the specimen plan

$$t = (U\% \text{ of } U_0 - 95.3\% \text{ of } U_0)(4)^{1/2}/(4\% \text{ of } U_0)$$
$$= (U - 95.3)2/4$$

i.e.,

$$t = (U - 95.3)/2$$

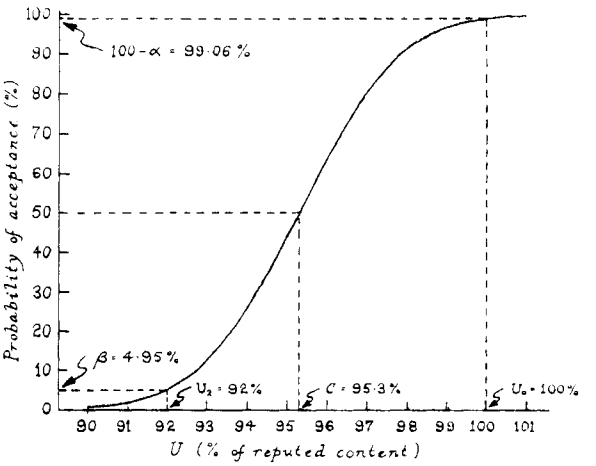

Figure 9. Operating characteristic curve of specimen sampling plan

From this last equation Table I is calculated. The operating characteristic curve is drawn in Figure 9. The ordinates of the curve give the probability of accepting a batch having a given real mean value U. The ordinate for $U = 92\%$ (when $U = U_2$) should be 5%, which is the value of β fixed in the specimen plan. However, it is 4.95% because n was rounded off from 3.94 to 4.

The ordinates subtracted from 100% give the producer's risk. Again, the risk for $U = 100\%$ (when $U = U_0$) should be 1%, which is the value of α fixed in the specimen plan. However, it is (100 − 99.06) = 0.94% because of the rounding off of n.

Risks for Mixed Fertilizers. So far only producer's and consumer's risks for a single-component, or straight, fertilizer have been considered. For mixed fertilizers a separate specification limit is applicable to each component of the mixture and there will be separate risks for each component. Because a producer will have the whole mixture failed if any one of its components fails, the over-all risk of failure is not the same as for any one component. Similarly, because the consumer will accept only a mixture which passes on all its components, the over-all consumer's risk is not the same as the risk of accepting any one component.

Consider a mixture made up of three components. Let α_1, α_2, and α_3 be the producer's risk that the first, second, and third components, respectively, fail, these three risks being expressed as fractions. Then, if α is the over-all producer's risk that the mixture fails on any one, two, or all three of its components, and it is expressed as a percentage,

α = 100{1 − (probability that mixture passes on all three components)} %
= 100{1 − (probability that it passes on first component) × (probability that it passes on second component) × (probability that it passes on third component)} %

Table I. Construction of Operating Characteristic Curve

U, %	U − 95.3, %	t	Probability of Acceptance, %
89	−6.3	−3.15	0.08
90	−5.3	−2.65	0.40
91	−4.3	−2.15	1.58
92	−3.3	−1.65	4.95
93	−2.3	−1.15	12.5
94	−1.3	−0.65	25.8
95	−0.3	−0.15	44.0
95.3	0	0	50.0
96	+0.7	+0.35	63.7
97	+1.7	+0.85	80.2
98	+2.7	+1.35	91.1
99	+3.7	+1.85	96.8
100	+4.7	+2.35	99.06
101	+5.7	+2.85	99.78
102	+6.7	+3.35	99.96

i.e.,

$$\alpha = 100\{1 - (1 - \alpha_1)(1 - \alpha_2)(1 - \alpha_3)\}\% \quad (9)$$

Similarly, for a two-component mixture,

$$\alpha = 100\{1 - (1 - \alpha_1)(1 - \alpha_2)\}\% \quad (10)$$

To find the over-all consumer's risk, let β_1, β_2, and β_3 be the consumer's risks of accepting the first, second, and third components, respectively, these risks being expressed as fractions. Then, if β is the over-all consumer's risk of accepting the whole mixture, and it is expressed as a percentage,

β = probability that the mixture passes on all three components
= (probability that it passes on the first component) × (probability that it passes on the second component) × (probability that it passes on the third component)

i.e.,

$$\beta = 100\beta_1\beta_2\beta_3\ \% \quad (11)$$

For a two-component mixture,

$$\beta = 100\beta_1\beta_2\ \% \quad (12)$$

Specimen Calculation of Component Risks. Consider a three-component mixture. What will the component producer's risks be for an over-all risk of 1%?

For simplicity, take $\alpha_1 = \alpha_2 = \alpha_3$. Then Equation 9 becomes

$$1\% = \alpha = 100\{1 - (1 - \alpha_1)^3\}\%$$

i.e.,

$$100(1 - \alpha_1)^3 = (100 - 1)\% = 99\%$$

or

$$(1 - \alpha_1)^3 = 0.99$$

so that

$$(1 - \alpha_1) = 0.99666$$

or

$$\alpha_1 = 0.00334\ (= 0.334\%)$$

What will the component consumer's risks be for an over-all risk of 5%? Taking $\beta_1 = \beta_2 = \beta_3$, Equation 11 becomes

$$5\% = \beta = 100\beta_1^3\ \%$$

therefore

$$\beta_1^3 = 0.05$$

or

$$\beta_1 = 0.3684\ (= 36.84\%)$$

This component risk of 36.84% is very high and appears to give little protection to the consumer. This is not true, because the consumer is protected by his over-all risk (5%) of accepting only a mixture which passes on all three components.

Using the component risks, the numbers of samples, the specification limits, and the operating characteristic curves for each component can be calculated by the procedure given in the calculation of the specimen plan above. By putting $\alpha_1 = \alpha_2 = \alpha_3$, $\beta_1 = \beta_2 = \beta_3$, and assigning the same parameters (σ, U_0, and U_2 as percentages) to each component, the numbers of samples and the specification limits (as percentages) become the same for each component. Then the sampling plan would be: "Take n samples at random from the batch of mixture and analyze these n samples (or a smaller number of good composites) for each component of the mixture; if the mean of the n analyses (or the mean of the composites) is less than C% of the reputed content for any one of the components, fail the whole batch."

Use of Sampling Plans for Judging Performance of Producer

Derivation of Mathematical Formulas. Consider a manufacturer to have produced over a certain period of time (say about 6 months) a large number of batches of fertilizer. There is no difficulty in talking of batches, because most manufacturers produce fertilizer to fill orders as they arise. Even if the manufacturer had been producing a particular fertilizer continuously during the 6 months, daily lots of production can be regarded as batches.

Suppose the fertilizer is superphosphate and it is required to judge the over-all quality of the superphosphate produced by a manufacturer during 6 months. To do this m batches can be selected at random from the whole production and n samples selected at random from each of these m batches. Of course, the batches will have to be selected and sampled as they are produced, judgment being reversed until after the mth batch is produced.

If σ = over-all standard deviation of the whole production
σ_1 = standard deviation between batches
σ_2 = average standard deviation within batches

so that

$$\sigma = (\sigma_1^2 + \sigma_2^2)^{1/2}$$

then the standard deviation of the mean, $\bar{x}$, of the mn samples from the m batches is

$$\sigma_{\bar{x}} = (\sigma_1^2/m + \sigma_2^2/mn)^{1/2} \quad (13)$$

Suppose that the whole 6 months' production is normally distributed about a true mean value equal to U_0, then as derived above,

$$\alpha = \{1/(2\pi)^{1/2}\} \int_{-\infty}^{t_\alpha} \exp\left[-\frac{1}{2}t^2\right]dt \quad (3)$$

where

$$t_\alpha = (U_0 - C)/\sigma_{\bar{x}} \quad (2)$$

or

$$t_\alpha = (U_0 - C)/(\sigma_1^2/m + \sigma_2^2/mn)^{1/2} \quad (14)$$

by virtue of Equation 13.

Similarly, if the whole 6 months' production is normally distributed about a true mean value equal to U_2, then

$$\beta = \{1/(2\pi)^{1/2}\} \int_{t_\beta}^{\infty} \exp\left[-\frac{1}{2}t^2\right]dt \quad (4)$$

where

$$t_\beta = (C - U_2)/\sigma_{\bar{x}} \quad (5)$$

or, by virtue of Equation 13,

$$t_\beta = (C - U_2)/(\sigma_1^2/m + \sigma_2^2/mn)^{1/2} \quad (15)$$

Equations 14 and 15 can be rewritten as

$$U_0 - C = t_\alpha\,(\sigma_1^2/m + \sigma_2^2/mn)^{1/2}$$

$$C - U_2 = t_\beta\,(\sigma_1^2/m + \sigma_2^2/mn)^{1/2}$$

Adding these two equations,

$$U_0 - U_2 = (t_\alpha + t_\beta)(\sigma_1^2/m + \sigma_2^2/mn)^{1/2}$$

or

$$n = \sigma_2^2(t_\alpha + t_\beta)^2/\{m(U_0 - U_2)^2 - \sigma_1^2(t_\alpha + t_\beta)^2\} \quad (16)$$

or

$$m = (n\sigma_1^2 + \sigma_2^2)(t_\alpha + t_\beta)^2/n(U_0 - U_2)^2 \quad (17)$$

From Equation 14,

$$C = U_0 - t_\alpha\,(\sigma_1^2/m + \sigma_2^2/mn)^{1/2} \quad (18)$$

Thus if values are fixed for α and β, values for t_α and t_β can be obtained from Equations 3 and 4, or from tables of the cumulative normal distribution. Then, if σ_1, σ_2, U_0, and U_2 are also fixed, the number of samples, n, which must be taken from a given number, m, of batches can be calculated from Equation 16, or the number of batches which should be sampled for a given n can be calculated from Equation 17. The specification limit, C, can then be calculated from Equation 18.

The above formulas apply to straight fertilizers. The same Equations 9, 10, 11, and 12 apply to the component risks for mixed fertilizers when the sampling plan is used to judge a manufacturer's performance.

Specimen Plan for Judging Performance of Manufacturer. How many samples should be taken from each of six batches (spread over 6 months, say) of a straight fertilizer and what should the specification limit be for a sampling plan based on the following parameters?

$$\alpha = 1\% \qquad \beta = 1\%$$

U_0 = long term reputed content, or guaranteed analysis
U_2 = 92% of reputed content
σ_1 = 3% of reputed content
σ_2 = 4% of reputed content

Because $\alpha = \beta = 1\% = 0.01$, from tables of the cumulative normal distribution, $t_\alpha = t_\beta = 2.326$. Thus from Equation 16, since $m = 6$,

$$n = (0.04U_0)^2(4.652)^2/\{6(U_0 - 0.92U_0)^2 - (0.03U_0)^2(4.652)^2\}$$
$$= (0.04)^2(4.652)^2/\{6(1 - 0.92)^2 - (0.03)^2(4.652)^2\}$$
$$= 1.83$$

Rounding off to the nearest whole number, $n = 2$. From Equation 18,

$$C = U_0 - (2.326)\{(0.03U_0)^2/6 + (0.04U_0)^2/6(1.83)\}^{1/2}$$
$$= U_0[1 - (2.326)\{(0.03)^2/6 + (0.04)^2/6(1.83)\}^{1/2}]$$
$$= U_0[1 - 0.0400]$$

i.e.,

$$C = 0.96U_0 \ (= 96\% \text{ of } U_0)$$

The specimen sampling plan would be: "Take two random samples from each of six batches, the selected batches being sampled, over a period of time, as they are produced, and analyze the 12 samples (or a smaller number of good composites); if the mean of the 12 analyses (or the mean of the composites) is less than 96% of the long-term reputed content, the manufacturer is producing material which is not acceptable."

The operating characteristic curve for the specimen sampling plan can be constructed from the formula

$$t = (U - C)/(\sigma_1^2/m + \sigma_2^2/mn)^{1/2}$$

which is obtained from Equation 14 by regarding U as a variable and expressing it as a percentage of the long-term reputed content.

Acknowledgment

The authors are indebted to F. L. Clark, research manager, African Explosives and Chemical Industries, Ltd., for the idea of using composited samples in sampling plans. This lends a great deal of power to plans for sampling chemicals with costly analyses because it removes the constraint on the number of samples which can be analyzed. D. J. Macleod of the same company suggested the idea of taking samples from a number of batches to judge a manufacturer's performance.

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ION ASSAYS

Simultaneous Determination of Phosphate and Sulfate Ions in the Presence of Metal Contaminations

LASZLO SZEKERES and ERZSÉBET BAKÁCS POLGÁR

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A method is presented for determining phosphate and sulfate ions simultaneously in the presence of calcium, magnesium, aluminum, iron, and fluoride ions as contaminants. The method is suitable for the rapid and simple determination of phosphate and sulfate ion content of phosphorus fertilizers.

A SIMPLE VOLUMETRIC METHOD (*5*), presented for the determination of phosphate and sulfate ions in the presence of each other, is suitable for the investigation of alkali salts. It is based on the following principle: The dissolved sample is adjusted with an ammonium hydroxide–ammonium chloride buffer to pH 10, ethyl alcohol is added, and the mixture is titrated with a standard solution of magnesium chloride and Eriochrome Black T as indicator. During the course of titration, crystalline magnesium ammonium phosphate is precipitated (*2*). At the equivalence point the excess magnesium ions react with the indicator to produce a color change from blue to purple-violet. Then the original blue color of the reaction mixture is recovered by adding a few drops of EDTA [(ethylenedinitrilo)tetraacetic acid, complexone III] and the sulfate ions are titrated with standard barium chloride solution (*4*). During this titration barium sulfate precipitates and, after the equivalence point is reached, magnesium ions are liberated from their complexonate bonds by the excess barium ions; this is indicated again by a color change from blue to purple-violet as in the first titration.

In a previous communication (*4*) an analogous measurement of arsenate ions with a standard solution of magnesium sulfate was suggested and was also applied to the determination of calcium arsenate (*6*). Essentially the same principle was utilized to mask certain ions which interfered in the investigation of phosphate fertilizers (*3*), as the earlier method suggested for the simultaneous determination of alkali sulfates and phosphates in the presence of Mg^{+2}, Ca^{+2}, Fe^{+3}, and Al^{+3} (*1*) was unsatisfactory for this purpose. Therefore, in the presence of these ions, as in the analysis of phosphorus fertilizers, the simultaneous determination of phosphates and sulfates was completed as follows:

Ferric and aluminum ions were masked with DCTA (1,2-diaminocyclohexanetetraacetic acid or complexone IV) in a solution whose pH was buffered between 2 and 3; then EDTA was added and the solution was adjusted to pH 10.0 to 10.5 with ammonium hydroxide, and with a buffer mixture of ammonium chloride and ammonium hydroxide solutions. EDTA formed stable complexes with Ca^{+2} and Mg^{+2} and the complexes of DTCA formed with iron and aluminum between pH 2 and 3 did not decompose either, if the pH of the medium was adjusted to 10 (*7, 8*). Although Ca^{+2} and Mg^{+2} may be masked with DCTA, this technique was abandoned because of the expense.

After the metal ions were masked, Eriochrome Black T was added as indicator and the solution was titrated

The above formulas apply to straight fertilizers. The same Equations 9, 10, 11, and 12 apply to the component risks for mixed fertilizers when the sampling plan is used to judge a manufacturer's performance.

Specimen Plan for Judging Performance of Manufacturer. How many samples should be taken from each of six batches (spread over 6 months, say) of a straight fertilizer and what should the specification limit be for a sampling plan based on the following parameters?

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A SIMPLE VOLUMETRIC METHOD (*5*), presented for the determination of phosphate and sulfate ions in the presence of each other, is suitable for the investigation of alkali salts. It is based on the following principle: The dissolved sample is adjusted with an ammonium hydroxide–ammonium chloride buffer to pH 10, ethyl alcohol is added, and the mixture is titrated with a standard solution of magnesium chloride and Eriochrome Black T as indicator. During the course of titration, crystalline magnesium ammonium phosphate is precipitated (*2*). At the equivalence point the excess magnesium ions react with the indicator to produce a color change from blue to purple-violet. Then the original blue color of the reaction mixture is recovered by adding a few drops of EDTA [(ethylenedinitrilo)tetraacetic acid, complexone III] and the sulfate ions are titrated with standard barium chloride solution (*4*). During this titration barium sulfate precipitates and, after the equivalence point is reached, magnesium ions are liberated from their complexonate bonds by the excess barium ions; this is indicated again by a color change from blue to purple-violet as in the first titration.

In a previous communication (*4*) an analogous measurement of arsenate ions with a standard solution of magnesium sulfate was suggested and was also applied to the determination of calcium arsenate (*6*). Essentially the same principle was utilized to mask certain ions which interfered in the investigation of phosphate fertilizers (*3*), as the earlier method suggested for the simultaneous determination of alkali sulfates and phosphates in the presence of Mg^{+2}, Ca^{+2}, Fe^{+3}, and Al^{+3} (*1*) was unsatisfactory for this purpose. Therefore, in the presence of these ions, as in the analysis of phosphorus fertilizers, the simultaneous determination of phosphates and sulfates was completed as follows:

Ferric and aluminum ions were masked with DCTA (1,2-diaminocyclohexanetetraacetic acid or complexone IV) in a solution whose pH was buffered between 2 and 3; then EDTA was added and the solution was adjusted to pH 10.0 to 10.5 with ammonium hydroxide, and with a buffer mixture of ammonium chloride and ammonium hydroxide solutions. EDTA formed stable complexes with Ca^{+2} and Mg^{+2} and the complexes of DTCA formed with iron and aluminum between pH 2 and 3 did not decompose either, if the pH of the medium was adjusted to 10 (*7, 8*). Although Ca^{+2} and Mg^{+2} may be masked with DCTA, this technique was abandoned because of the expense.

After the metal ions were masked, Eriochrome Black T was added as indicator and the solution was titrated

Table I. Simultaneous Titration of Phosphate and Sulfate Ions in Solutions of Known Concentration after Masking Metallic Ions with Complexing Agents

KH_2PO_4, Gram	K_2SO_4, Gram	Added to Sample, Mg. Ca^{+2}	Mg^{+2}	Al^{+3}	Fe^{+3}	F^-	DCTA,[a] Ml.	EDTA,[a] Ml.	Buffer, Ml.	EtOH in Titration of PO_4^{-3}, Ml.	$MgCl_2$[a] Consumed, Ml. Actual	Mean	KH_2PO_4 Found, Gram	Dev., %	EtOH in Titration of SO_4^{-2}, Ml.	$BaCl_2$[a] Consumed, Ml. Actual	Mean	K_2SO_4 Found, Gram	Dev., %
0.06685	0.08713	20	7.2	2.7	5.58	1.9	4	8	2	7	4.88 4.90	4.89	0.06655	−0.45	20	5.00 4.90	4.95	0.08626	−1.00
0.06685	0.08713	20	2.4	0.9	11.17	3.8	3	6	2	7	4.93 4.88	4.91	0.06682	0.00	20	5.00 4.95	4.98	0.08678	−0.40
0.06685	0.08713	20	2.4	0.9	11.17	1.9	3	6	2	7	4.88 4.90	4.89	0.06655	−0.45	20	5.00 5.00	5.00	0.08714	+0.12
0.06685	0.1730	20	7.2	1.8	11.17	1.9	5	6	3	10	4.85 4.90	4.88	0.06640	−0.68	25	10.00 9.90	9.95	0.1734	+0.23
0.1351	0.08713	20	7.2	1.8	11.17	1.9	5	6	3	10	9.90 9.85	9.88	0.1345	−0.44	25	5.00 5.00	5.00	0.08714	+0.12

[a] 0.1M solution.

with a standard solution of magnesium chloride until the transition color appeared, indicating the complete absence of free EDTA in the medium. After addition of ethyl alcohol, the phosphate ions were titrated with a standard solution of magnesium chloride, and the sulfate ions with a standard solution of barium chloride.

Calcium, magnesium, iron, and aluminum ions (as the chlorides) and sodium fluoride were added in amounts given in Table I to solutions of alkali phosphate and sulfate of known concentration. The results show that the accuracy of the measurements is satisfactory.

In the study of phosphorus fertilizers the sample was dissolved in hydrochloric acid, and phosphate and sulfate ions were determined in the solution by the suggested method. The values obtained were compared with gravimetric results (Table II).

Experimental

Reagents. A 0.1M standard solution of $MgCl_2$, prepared by dissolving magnesium oxide in hydrochloric acid. The solution may be standardized by complexometry.

0.1M standard solution of $BaCl_2$.

Buffer solution prepared by dissolving 50 grams of ammonium chloride and 400 ml. of concentrated ammonium hydroxide in 1000 ml. of water.

Indicator, Eriochrome Black T triturated with potassium chloride in a ratio of 1 to 100. Both substances should be finely powdered.

Procedure. About 10 grams of the carefully homogenized sample of superphosphate fertilizer were weighed and dissolved by boiling with about 250 to 300 ml. of 15% hydrochloric acid. After filtration, the solution was transferred to a 500-ml. measuring flask, made to volume, and 10-ml. aliquots of this stock solution were examined as follows. The solution was neutralized in the presence of methyl orange with concentrated ammonium hydroxide, then with 10% ammonium hydroxide until a slight turbidity appeared. Care must be taken that the solution remains definitely red. Then 3 to 5 ml. of 0.1M DCTA solution were added (according to the requirements), the mixture was boiled, about 9 to 10 ml. of 0.1M EDTA solution were added, and the solution was cooled. About 0.015 to 0.020 gram of Eriochrome Black T indicator was dissolved in the medium, then as much concentrated ammonium hydroxide was added dropwise (about 4 to 6 drops) as was needed to obtain a greenish blue tint. Next, 3 ml. of buffer solution were added, and the excess EDTA and DCTA were eliminated by adding 0.1M standard solution of magnesium chloride. The proper starting point for the subsequent titration was indicated by the appearance of the transition color. After this point was reached, about 5 ml. of ethyl alcohol were added and titration of phosphate ions with the standard 0.1M solution of magnesium chloride was started, using the same buret from which the magnesium chloride solution was added to attain the proper starting point. The first portion of the standard solution (50 to 75% of the expected total) was added at once, and then the medium was shaken until precipitation started (always within a minute) and the liquid turned greenish blue. Further precipitation of the magnesium ammonium phosphate during titration occurred immediately and titration was continued at the conventional rate until a stable purple-violet color appeared. The end point was easily observable by the color change.

Before titration of the sulfate ions, the original blue tint of the medium was recovered by adding a few drops of a 0.1M EDTA solution. In the presence of minute amounts of EDTA the color transition takes place before the equivalence point; therefore, small measured excesses (0.2 ml.) of EDTA solution were added and this volume was subtracted from the total amount of standard solution used. Then enough ethyl alcohol was added to obtain a solution of about 40% with respect to ethyl alcohol at the equivalence point. Sulfate ions were then titrated with 0.1M barium chloride solution under continuous shaking. The last drops of standard solution were added slowly until the purple-violet color of the indicator appeared.

Discussion

The presence of ammonium chloride proved to be favorable for the formation of the magnesium ammonium phosphate precipitate. Therefore, in all experiments summarized in Table I, 1 gram of ammonium chloride was added to the titrated solutions.

Addition of ammonium chloride is, however, not necessary if phosphorus fertilizers are analyzed, as they must be dissolved in hydrochloric acid and ammonium chloride is formed on neutralization with ammonium hydroxide.

Table II shows that, for determination of phosphate ion, the suggested method supplies equal results to those of gravimetric procedures, and the results attained for the sulfate ions exceed by 1% the gravimetrically determined value of the same.

Acknowledgment

The authors thank E. Körös and the Board of Directors of the Geigy firm for supplying samples of DCTA, and I. Csiky for valuable assistance in the experiments.

Table II. Phosphate and Sulfate Ions Found in Phosphorus Fertilizers by Suggested Method and by Gravimetry

Added to 10 Ml. of Superphosphate Stock Soln.			EtOH Added in Titration of		P_2O_5, %		EtOH Added in Titration		SO_4^{-2}, %		Dev. from Gravimetric Values, %	
DCTA,[a] ml.	EDTA,[a] ml.	Buffer, ml.	PO_4^{-3}, Ml.	MgC_2[a] Consumed Ml.	Authors' method	Gravimetry	of SO_4^{-2}, Ml.	$BaCl_2$[a] Consumed, Ml.	Authors, method	Gravimetry	PO_4^{-3}	SO_4^{-2}
4	9	3	5	6.45, 6.40, 6.43 } 6.43	22.96	22.96	20	7.20, 7.30, 7.40, 7.40, 7.45 } 7.33	35.43	34.98	0.00	1.29

[a] 0.1 *M* solution.

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PLANT TISSUE ANALYSIS

X-Ray Fluorescence Determination of Zinc in Plant Tissue

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X-ray emission spectroscopy has been applied to determination of zinc in a variety of plant species. Data are presented to show the lack of consistency in the intensity of the zinc emission maxima as a function of zinc concentration in plant tissue of complex and variable composition. Known amounts of zinc are added to samples and the original concentration is calculated on the basis of the response to the known added zinc.

INTERPRETATION of factors affecting intensity of emission maxima and compensation for them are of primary concern in applying x-ray emission spectroscopy to the quantitative analysis of chemical elements. Compensation for interferences can often be calculated or otherwise easily determined in relatively simple systems of limited elemental components. Evaluation and compensation for interferences in complex systems such as soils and plant tissue present a much more difficult problem.

As reviewed by Liebhafsky (*9*), variations in the intensity of fluorescent x-ray maxima of elements result from absorption of excitation energy and of secondary emission energy by the sample and enhancement of emission maxima of an element as a result of excitation by higher energy secondary radiation emanating from other elements in the sample. Besides these factors which affect intensity of emission maxima directly, variable matrix compositions contribute to variations in background radiation levels. Variable absorption and enhancement together with variable background levels in samples of different composition require most careful evaluation and compensation in quantitative fluorescence analysis.

Compensation for Interferences. The magnitude of absorption interference in relatively simple chemical systems can often be calculated from known absorption coefficients, so that suitable corrections can be made (*4*). Alternatively, in simple systems, standard curves may be prepared in which elemental emission intensity is plotted against concentration of interfering elements (*5*). A commonly used technique is the addition of internal standards which are affected in the same manner as the elements being analyzed (*3, 5*). Suitable correction can be applied to the emission intensity of the unknown on the basis of variation in intensity of known concentration of internal standard.

An interference compensating technique employed by Kemp and Andermann (*7*), Andermann and Kemp (*1*), and Brandt and Lazar (*2*) utilizes scattered radiation as an internal standard in emission intensity measurements. In theory, scattered radiation of specific wave length can be selected which is affected by absorption in nearly the same way as the emitted radiation of an element being analyzed. A ratio of an element's emission intensity to intensity of the scattered radiation at the selected wave length will be constant so long as the interferences result only from instrument variability and absorption of secondary emission energy.

The ratio technique can be valid only if interferences affect emission and scatter intensity proportionately—not in situations where scatter intensity varies independently of elemental emission intensity, or where there is variable absorption of primary excitation energy.

It would appear that any variable affecting the intensity of an element's emission maxima in a sample could conveniently be measured and compensated for by adding a known quantity of the element concerned and relating the increase in intensity obtained by the addition to that originally present. Such a technique has been applied in analysis of inorganic mineral samples (*3, 10*). The present investigation is concerned with instrumentation and a comparison of methods of evaluating concentration levels of zinc in tissue of various plant species. The method of analysis described combines precision and accuracy with speed suitable for routine analyses on a large scale.

Experimental

Instrumentation. A spectrograph (General Electric XRD-5) equipped with a tungsten target x-ray tube (Machlett AEG-50), lithium fluoride analyzing crystal, 0.005-inch Solar slit, and krypton-filled proportional counter detector was used. The optimum voltage

Table II. Phosphate and Sulfate Ions Found in Phosphorus Fertilizers by Suggested Method and by Gravimetry

Added to 10 Ml. of Superphosphate Stock Soln.			EtOH Added in Titration of		P_2O_5, %		EtOH Added in Titration		SO_4^{-2}, %		Dev. from Gravimetric Values, %	
DCTA,[a] ml.	EDTA,[a] ml.	Buffer, ml.	PO_4^{-3}, Ml.	MgC_2[a] Consumed Ml.	Authors' method	Gravimetry	of SO_4^{-2}, Ml.	$BaCl_2$[a] Consumed, Ml.	Authors, method	Gravimetry	PO_4^{-3}	SO_4^{-2}
4	9	3	5	6.45, 6.40, 6.43 } 6.43	22.96	22.96	20	7.20, 7.30, 7.40, 7.40, 7.45 } 7.33	35.43	34.98	0.00	1.29

[a] 0.1 *M* solution.

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PLANT TISSUE ANALYSIS

X-Ray Fluorescence Determination of Zinc in Plant Tissue

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X-ray emission spectroscopy has been applied to determination of zinc in a variety of plant species. Data are presented to show the lack of consistency in the intensity of the zinc emission maxima as a function of zinc concentration in plant tissue of complex and variable composition. Known amounts of zinc are added to samples and the original concentration is calculated on the basis of the response to the known added zinc.

INTERPRETATION of factors affecting intensity of emission maxima and compensation for them are of primary concern in applying x-ray emission spectroscopy to the quantitative analysis of chemical elements. Compensation for interferences can often be calculated or otherwise easily determined in relatively simple systems of limited elemental components. Evaluation and compensation for interferences in complex systems such as soils and plant tissue present a much more difficult problem.

As reviewed by Liebhafsky (*9*), variations in the intensity of fluorescent x-ray maxima of elements result from absorption of excitation energy and of secondary emission energy by the sample and enhancement of emission maxima of an element as a result of excitation by higher energy secondary radiation emanating from other elements in the sample. Besides these factors which affect intensity of emission maxima directly, variable matrix compositions contribute to variations in background radiation levels. Variable absorption and enhancement together with variable background levels in samples of different composition require most careful evaluation and compensation in quantitative fluorescence analysis.

Compensation for Interferences. The magnitude of absorption interference in relatively simple chemical systems can often be calculated from known absorption coefficients, so that suitable corrections can be made (*4*). Alternatively, in simple systems, standard curves may be prepared in which elemental emission intensity is plotted against concentration of interfering elements (*5*). A commonly used technique is the addition of internal standards which are affected in the same manner as the elements being analyzed (*3, 5*). Suitable correction can be applied to the emission intensity of the unknown on the basis of variation in intensity of known concentration of internal standard.

An interference compensating technique employed by Kemp and Andermann (*7*), Andermann and Kemp (*1*), and Brandt and Lazar (*2*) utilizes scattered radiation as an internal standard in emission intensity measurements. In theory, scattered radiation of specific wave length can be selected which is affected by absorption in nearly the same way as the emitted radiation of an element being analyzed. A ratio of an element's emission intensity to intensity of the scattered radiation at the selected wave length will be constant so long as the interferences result only from instrument variability and absorption of secondary emission energy.

The ratio technique can be valid only if interferences affect emission and scatter intensity proportionately—not in situations where scatter intensity varies independently of elemental emission intensity, or where there is variable absorption of primary excitation energy.

It would appear that any variable affecting the intensity of an element's emission maxima in a sample could conveniently be measured and compensated for by adding a known quantity of the element concerned and relating the increase in intensity obtained by the addition to that originally present. Such a technique has been applied in analysis of inorganic mineral samples (*3, 10*). The present investigation is concerned with instrumentation and a comparison of methods of evaluating concentration levels of zinc in tissue of various plant species. The method of analysis described combines precision and accuracy with speed suitable for routine analyses on a large scale.

Experimental

Instrumentation. A spectrograph (General Electric XRD-5) equipped with a tungsten target x-ray tube (Machlett AEG-50), lithium fluoride analyzing crystal, 0.005-inch Solar slit, and krypton-filled proportional counter detector was used. The optimum voltage

Table I. Effect of Sample Packing on Zinc Emission Intensity and Emission to Scatter Ratio

F−S		F/S	
Loose-pack, c.p.s.	Briquet, c.p.s.	Loose-pack, c.p.s.	Briquet, c.p.s.
14.5	15.0	1.29[a]	1.29[b]
3.3	2.5	1.06	1.05
8.1	9.0	1.16	1.17
10.8	10.8	1.22	1.21
18.5	19.5	1.38	1.36
3.5	2.7	1.07	1.05
4.7	3.9	1.09	1.07
33.5	35.3	1.62	1.62
3.6	3.0	1.07	1.06
12.6	12.8	1.25	1.24
66.5	73.1	2.29	2.31
9.5	10.2	1.18	1.18
68.0	73.0	2.30	2.30

[a] 1.286 ± 0.023. [b] 1.289 ± 0.014.

Standard deviations as shown were obtained by analysis of 20 subsamples.

Table II. Effect of Addition of Zinc to Samples of Unknown Zinc Content

	Unknown		Unknown + 40γ Zn/G.			
Tissue	F − S, c.p.s.	F/S	F − S, c.p.s.	F/S	ΔF − S, c.p.s.	ΔF/S
Apricot	3.9	1.09	32.0	1.73	28.1	0.64
Prune	8.2	1.17	40.8	1.82	32.6	0.65
Almond	16.7	1.36	45.9	2.00	29.2	0.64
Apple	89.9	2.51	128.8	3.17	39.9	0.66
Apple	59.5	2.01	97.4	2.66	37.9	0.65
Apple	18.2	1.33	55.1	1.99	36.9	0.66
Quince	38.7	1.60	78.0	2.25	39.3	0.65
					Mean	0.65 ± <0.01[a]
Corn	9.9	1.17	41.8	1.72	31.9	0.55
	21.3	1.37	46.1	1.90	24.8	0.53
	23.2	1.47	48.2	1.98	25.0	0.51
	12.4	1.26	41.0	1.82	28.6	0.56
	1.0	1.02	33.9	1.57	32.9	0.55
					Mean	0.54 ± 0.02[a]
Milo	11.7	1.25	36.9	1.79	25.2	0.54
	8.2	1.18	37.2	1.72	29.0	0.54
	6.8	1.14	32.5	1.66	25.7	0.52
	15.3	1.32	36.9	1.82	21.6	0.50
	17.6	1.36	41.0	1.85	23.4	0.49
					Mean	0.52 ± 0.02[a]
Tomato	5.7	1.11	31.6	1.69	25.9	0.58
	9.4	1.20	34.7	1.75	25.3	0.55
	9.9	1.22	32.6	1.74	22.7	0.52
	9.4	1.18	37.5	1.78	28.1	0.60
	8.7	1.19	33.7	1.75	25.0	0.56
					Mean	0.56 ± 0.03[a]

[a] Standard deviation of $\Delta F/S$ values.

and current for generation of exciting x-rays were found to be essentially the highest attainable with the instrument. Somewhat lower than maximum voltage and current yield higher emission to scatter ratios, but lower the actual intensity for the zinc emission maxima. This latter factor becomes important when analysis of small quantities of the element is involved. Throughout this investigation the instrument was operated at 50 kv. and 48 ma. A wave length discriminating reverter set to yield approximately 70% of maximum intensity at the zinc wave length was used in measurement of both emission and scatter as a means of discriminating against scatter. The wave length at which scatter radiation was measured was chosen because it is close to the wave length of zinc emission and it occurs at a point free of any possible interfering elemental emission maximum. The angle at which the scatter radiation is measured is empirical, but in this type of analysis it must be as close as possible to the angle where the zinc wave length is measured and yet far enough removed so as not to be affected by the zinc radiation. The first order zinc K_α maximum was measured at 41.80° 2 theta and the scatter radiation at 41.00° 2 theta.

Preparation of Samples and Counting Technique. Samples of dried plant material were ground to pass a 20-mesh stainless steel screen in a Wiley mill. Standards for reference were obtained by chemical analysis of reference samples using the Zincon method (*11*) and by addition of known amounts of zinc to reference samples. When zinc was added, the following procedure was used.

Sufficient acetone was added to 10 grams of dried, ground plant tissue (containing an unknown quantity of zinc) in a Mullite mortar just to wet the entire mass without excess. Aliquots of zinc sulfate solution were added to give the desired, added concentrations of zinc. After thorough mixing, the samples were placed in a 60° C. oven, until excess acetone and moisture were removed, and then remixed thoroughly in the dry state.

During analysis of samples, radiation intensity was measured in terms of time required for registration of 10,000 counts in order to stay within a standard deviation of 1% for a given intensity measurement (*8*). These values were converted to counts per second. Emission intensity values, F, as reported, represent the intensity of zinc emission plus scatter at 41.80° 2 theta.

A preliminary investigation of the effect of briquetting samples of fruit tree foliage under a pressure of 14,000 p.s.i. as contrasted to packing the samples by hand in a Bakelite sample holder of 3-mm. depth with a stainless steel spatula is summarized in Table I. Briquetting increased the precision of analyses, but because the precision obtained with loose-packed samples was sufficient for present purposes, subsequent data were obtained on loose-packed samples. The thickness of the samples was sufficient to mask any scatter from the holder.

Expression of Results and Calculations. For the purpose of calculation, the intensity of the scatter at the 2 theta angle where the zinc maximum is detected must be known. This was accomplished by measuring the scatter intensity at 41.80° 2 theta and at 41.00° 2 theta from materials known to be free of zinc. Their ratio established the intercept for zero zinc concentration and also provided a constant multiplier for the calculation of the actual emission above scatter intensity. The ratio for pure calcium carbonate, silicic acid, starch, and filter paper was found to be a consistent value of 0.91. This value was used in the subsequent calculations.

In analyses involving the method of addition of known quantities of zinc, the initial concentration of zinc was calculated in one of two ways:

$$X = \frac{Y[(F/S)\mu - 0.91]}{(F/S)y - (F/S)\mu} \quad (1)$$

or

$$X = \frac{Y(F - 0.91S)\mu}{(F - 0.91S)y - (F - 0.91)\mu} \quad (2)$$

where X = unknown zinc concentration in the plant sample
Y = zinc added, γ/gram
F = radiation intensity, c.p.s. at 41.80° 2 theta
S = radiation intensity, c.p.s. at 41.00° 2 theta
μ = unknown sample
y = unknown sample plus Y
0.91 = F/S at zero zinc concentration

Equation 1 utilizes the emission to scatter ratio, whereas Equation 2 utilizes the increase in emission intensity effected by the known quantity of zinc added.

Results and Discussion

Consistent with the observation of Brandt and Lazar (*2*), a plot of emission intensity ($F-S$) as a function of concentration of an element in different samples gives a linear relationship (with a correlation coefficient, r, of

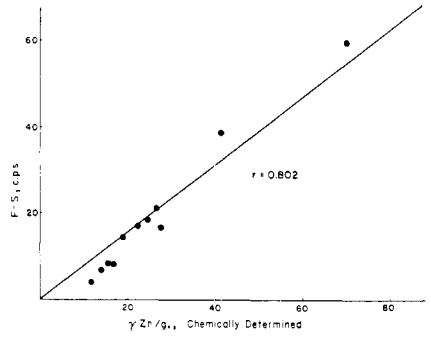

Figure 1. Emission intensity as a function of zinc concentration in deciduous tree foliage

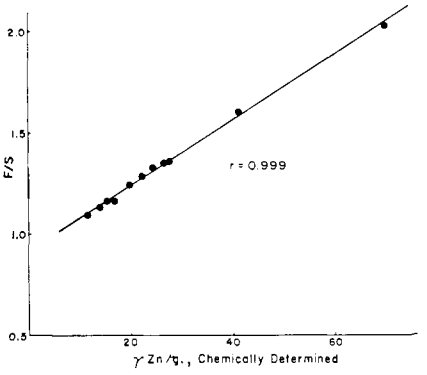

Figure 2. Emission to scatter ratio as a function of zinc concentration in deciduous tree foliage

0.802) as exemplified by the analysis of deciduous fruit tree foliage (Figure 1). When these same data are plotted using the ratio of intensity of zinc emission to scatter (F/S) as a function of concentration, a linear relationship with much better precision (r = 0.999) is obtained (Figure 2). This supports the conclusion that interferences within different samples are variable and that the interferences affect both the zinc radiation and the scatter radiation in similar manner within the range of concentrations encountered in this study. Similar plots of data in which increments of zinc were added to tissue samples established the validity of plotting emission to scatter ratio as a function of concentration within a particular sample.

A linear relationship between ratio and zinc concentration is not necessarily maintained between tissue of different species of plant. Table II shows the effects of addition of 40 γ of zinc per gram of tissue to a number of plant species. Some very significant relationships are noted in these data. Addition of the zinc to the deciduous fruit tree foliage resulted in an increase in the intensity of zinc emission above scatter of from 28.1 to 39.9 c.p.s. In addition, scatter intensity varied considerably from sample to sample. In spite of variations in emission and scatter intensities, however, the increase in emission to scatter ratios ($\Delta F/S$) remained nearly constant (between 0.64 and 0.66). This represents a variability of only ± 0.6 γ of zinc per gram of tissue at this level. The constancy of ratio increase with added zinc indicates that zinc emission is affected in the same way as the scatter within this group of similar species. The data also indicate that interferences are extremely variable within this class of materials. Figure 2, which shows the relationship between F/S and zinc concentration in various deciduous fruit tree species, as determined chemically, shows also a $\Delta F/S$ of 0.64 per 40 γ of zinc per gram of tissue.

Variability in emission to scatter ratio as a function of zinc concentration between classes of plant materials is illustrated by results obtained with cereal, vegetable, and deciduous fruit tree species. Milo, corn, and tomato foliage responded to the addition of zinc in similar order of magnitude, but the response was different than that exhibited by deciduous fruit tree species.

Despite relatively large differences in intensity of zinc emission due to the addition of 40 γ of zinc per gram of tissue, actual intensity above scatter ($F-S$) can be used to calculate the zinc content of the original material using Equation 2. This technique would yield precise results in that interferences would be minimized, because the unknown samples and unknown plus zinc differ in composition only in the zinc content and the small amount of sulfur and oxygen added as sulfate. However, this does require the addition of zinc to every sample, a procedure which, as shown above, may not be necessary when dealing with similar types of plant material, especially where routine assay of a particular plant species is involved.

The results obtained with tissues of different species support the contention that factors other than absorption are operative in analysis of plant materials. Lower emission to scatter ratios obtained with corn samples as compared to fruit tree foliage may be due to variations in scatter level between the two classes of material. The data obtained do not define the reason for the difference noted, however.

Application of the x-ray emission method of analysis of zinc in a number of plant tissue samples is illustrated in Table III. Results obtained by both procedures outlined above agree well with results by chemical analysis.

Table III. Comparison of Zinc Concentration in Deciduous Tree Foliage as Determined by Chemical Analysis and by X-Ray Emission Methods

Chemical Method, γ/G.	X-Ray Emission by Addition	
	F/S^a, γ/g.	$F-S^b$, γ/g.
11.6	11.9	11.1
98.4	100	94.4
69.8	68.3	68.6
18.9	19.4	19.1
24.4	25.5	25.1
22.2	22.8	22.1
26.5	26.3	27.2
41.2	41.8	45.1
13.9	13.1	13.9
16.8	14.9	15.1
15.5	15.2	15.7
27.5	27.3	28.6

[a] Calculated by Equation 1.
[b] Calculated by Equation 2.

Conclusions

Scatter radiation can be used as an internal standard in zinc analyses of plant materials. Reasonable accuracy in determination of zinc in plant samples can be attained from a standard curve, or by calculation, from data obtained by correlating ratio of zinc emission to scatter with zinc concentration in a particular kind of plant material. Such a relationship obtained with one kind of material cannot necessarily be applied to another. For high accuracy, a known quantity of zinc should be added in any case and the amount of zinc in a sample calculated either on the basis of emission to scatter ratio increase or actual emission intensity increase per unit of zinc added.

Results obtained in zinc determination may readily apply to determination of other elemental constituents of plants.

Acknowledgment

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Chemical and Biochemical Analyses on Brain Tissue Preparations during the Epileptiform-Like Activity of Dieldrin and Other Cerebral Convulsants

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This investigation was undertaken to determine the etiological factors associated with cerebral convulsions. Because many conditions are capable of producing the same end result in animals—cerebral convulsions—it was believed that these could have some common feature. Convulsing animals had a mixture of betaine CoA esters released in their brains through damage of brain mitochondria. Similar results could not be obtained with normals. It was concluded that because these betaine CoA esters possess acetylcholine-like properties, their release from brain mitochondria may be an important step in elucidating the etiology of epileptiform-like convulsions.

CHLORINATED HYDROCARBONS as a group can produce in mammals, as a consequence of administration or ingestion, most or all of the following symptoms: nervousness and excitability, excessive blinking, cold skin (or fur), loss of appetite, muscular weakness, fine tremors due to muscular fibrillation, paralysis, and finally convulsions which could terminate in death (*26, 27*).

The most commonly used chlorinated hydrocarbons for insecticidal purposes are: dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin, chlordan, benzene hexachloride, lindane, and methoxychlor. All these substances are extremely insoluble in water but soluble in fat and organic solvents, and on incorporation into the animal body, they are usually deposited and stored in the fat (*10, 17*). Consequently, their metabolism and excretion could be either a slow or a rapid process, dependent in some manner on the rate of metabolism of the body fat stores (*17*). It is perhaps because of these properties that, depending on the amount of the agent incorporated within the fat stores of the body, toxic symptoms in mammals may immediately be apparent or be delayed for several days or months.

In this paper, chemical and biochemical studies on the epileptiform-like activity of dieldrin and other chemically unrelated cerebral convulsants are discussed. The LD_{50} of dieldrin in rats is 50 to 75 mg. per kg. (*18*), which probably makes it less toxic than aldrin, which has an LD_{50} of 40 to 50 mg. per kg. (*6*). Depending on the dosage administered, the symptoms of dieldrin poisoning may be delayed for several days. In vitro studies by McArdle, Hosein, and Denstedt (*21*) showed that the addition of 25 mg. of dieldrin (as a solution in olive oil) to rat brain cortex slices did not inhibit oxygen consumption by this tissue in the presence of various substrates. If, however, the brain slices were preincubated with the dieldrin for 30 minutes at 37.5° C., subsequent measurement indicated inhibition of the oxygen consumed by the tissue when it was incubated in the presence of various substrates. This inhibition was found to be due to the action of dieldrin on various dehydrogenases and on cytochrome b. Other studies by these authors showed that dieldrin could inhibit anaerobic glycolysis and the hydrolysis of acetylcholine (ACh) by brain acetylcholinesterase. Dieldrin also was found to increase the activity of the nonspecific serum esterases, confirming the earlier findings of Crevier, Ball, and Kay (*9*).

Effects of Dieldrin on the Rat

Other studies in our laboratory (*13*) have shown that the intraperitoneal injection of 200 mg. of dieldrin to normal adult rats produced violent convulsions which were fatal. These large quantities of dieldrin were necessary in order to produce convulsions within a reasonably short time.

About 25 minutes after intraperitoneal injection of the drug, the animal goes into convulsions which may persist from about 30 seconds to 1 minute. About 20 minutes after this convulsion, a second one of similar duration usually takes place. The third convulsion may appear about 15 minutes after the second, the duration being slightly more than 1 minute. The fourth convulsion occurs about 10 minutes after the third, and invariably the fifth is fatal. This latter convulsion occurs about 5 minutes before death and persists throughout this time interval. At the time of each convulsion, the animal salivates profusely. Gowdey *et al.* (*11*) were able to produce similar effects in cats (convulsions and salivation) and reported that application of dieldrin to the chorda tympani did not elicit salivation. From these results it appeared to us that the profuse salivation produced on dieldrin administration was due not to dieldrin itself, but probably to some substance released in the brain of the animal as a result of treatment with this agent.

Precipitation of Reineckate

One of the main purposes of our investigation was to determine whether some substance was released in brain as a consequence of the dieldrin administration which could be responsible for the salivation and the convulsions.

In experiments performed in our laboratory (*13, 15*), 10 groups of 20 hooded rats of either sex and weighing about 200 grams were injected intraperitoneally with 1 gram of dieldrin per kg., in order to produce convulsions in a relatively short time. During the last 5 minutes of life when the convulsions were continuous, the animals were decapitated and the excised brains were immediately homogenized in ice-cold water containing $10^{-5}M$ eserine. After centrifugation at 2000 r.p.m. (1500 g) for 15 minutes, the supernatant was removed and recentrifuged at 18,000 g for 20 minutes. The volume of this latter supernatant was then reduced to 5 ml. by vacuum evaporation at 40° C. The concentrated extract was

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This is the fifth paper in a Symposium on the Mechanism of Action of Pesticide Chemicals, presented at the 137th Meeting of the American Chemical Society. The other four papers were published in the July–August issue of the JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY.

deproteinized with an equal volume of cold methanol. Dilute ammonium hydroxide was then added to the protein-free methanol-water extract so as to bring the pH of the solution to 8.5 to 9.0, as suggested by Bregoff, Roberts, and Delwiche (*5*). Any precipitate formed at this time was removed by centrifugation. Then 3 ml. of 5% reineckate solution in methanol were added to the clear supernatant and the solution was left in the refrigerator for 3 hours. The precipitate formed was collected by centrifugation, washed with 1-propanol, and finally dissolved in a minimum quantity of an acetone-water (50–50) mixture.

In certain experiments, the supernatant from the alkaline reineckate precipitation was adjusted to pH 1.2 by the addition of 6*N* hydrochloric acid and the precipitate formed on leaving this acid solution in the cold was treated like the precipitate obtained in alkaline medium. In either case, the individual acid or alkaline reineckate precipitate dissolved in the acetone-water mixture was treated as described by Banister, Whittaker, and Wijesundera (*3*) with just sufficient saturated silver sulfate solution to remove the soluble reineckate. Any excess of silver was removed by careful addition of just sufficient 10% barium chloride and any excess barium was removed by addition of a saturated solution of sodium sulfate. The clear supernatant was then reduced to dryness by vacuum evaporation and the solid residue was extracted twice with absolute methanol. The volume of the pooled methanol extracts was reduced to 0.1 ml. under nitrogen gas at 40° C., this was applied as a band on a strip of Whatman No. 1 filter paper $1^1/_2$ inches wide and 20 inches long, and the chromatogram was developed in a butanol-water system (*3*). The developed chromatogram was air-dried and sprayed with a 1% solution of iodine in ethanol (*4*).

The reineckate precipitate formed in alkaline solution contained material which had R_f values of 0.10 to 0.14, 0.21, 0.30, and 0.50 to 0.70. Material contained in the acid-precipitated reineckate fraction had an R_f of 0.1. Aqueous brain extracts made from 20 normal animals in a similar manner contained no acid-reineckate precipitable material, while the alkaline reineckate precipitate contained only material which had R_f values of 0.10 to 0.14, 0.21, and 0.30.

From these results it appeared that as a consequence of the administration of dieldrin, certain substances could be separated from the brain extract of the convulsing animals which could not be found in normal brain extract. Further experiments confirmed the earlier findings of Banister *et al.* (*3*), who used ox spleen in their experiments, that the substances on our chromatograms with values of 0.10 to 0.14 and 0.21 were choline, ACh, and propionyl choline, respectively. We observed, as did these workers, that the band with R_f 0.50 to 0.70 contained substances which were physiologically active as tested on the frog rectus preparation. The material from the acid reineckate precipitation which had an R_f of 0.1 was devoid of physiological activity.

Bregoff *et al.* (*5*) have shown that this method of reineckate precipitation enables the separation of conjugated betaines from free betaines. Reineckate precipitation in alkaline medium enables the removal of the conjugated betaines from solution, while the free betaines are precipitated in acid media. The results from our experiments with extracts made from convulsing animals indicated that both free and conjugated betaines might have been liberated in the brain of these animals. Again, as the conjugated betaine fraction contained substances which were similar in physiologic action to ACh, it was believed that these substances could well be associated with the initiation and propagation of the convulsions, since topical application of ACh on the eserinized cat brain cortex elicits epileptiform activity (*22*).

Because the intraperitoneal injection of dieldrin caused the release of certain substances in brain which could not be found in aqueous extracts of normal animals prepared in a similar manner, it was of interest to investigate whether this effect was limited to dieldrin or whether other agents and conditions which are known to cause cerebral convulsions would produce similar results. Metrazol (50 mg. per kg.), camphor (1 gram per kg.), and ammonium chloride (250 mg. per kg.) were each administered intraperitoneally to groups of 20 rats at a time. A similar number of rats were made to convulse with electroshock, by the application of the necessary threshold voltage delivered through the leads of a physiological stimulator. During the convulsions, the animals were decapitated and aqueous brain extracts were made and analyzed by paper chromatography as described above. In every instance, results similar to those obtained with dieldrin were obtained. It appeared, therefore, that the release of these substances in brain may well be associated with convulsions of the epileptiform type.

Identification of Substances from Brain Extracts

The next phase of the investigation was to identify these substances which were found in the brain extracts derived from convulsing animals and shown to be present in both the alkaline- and acid-precipitable reineckate fractions. The material in the acid-precipitable reineckate fraction, possibly free betaines with an R_f of 0.1, was eluted from the pooled paper strips with a methanol-water (90–10) mixture and the eluate was analyzed using the procedure described by Linneweh (*19*) for the separation and identification of betaines by forming their gold salts. The gold derivatives were prepared by boiling the eluate with 100 mg. of gold chloride in 1% HCl. The salts formed on cooling were then separated on the basis of their solubility in 1% HCl, as described by Carter and others (*8*). The separated crystals were dried and were found to melt at about 152°, 182°, and 211° C., all with decomposition. On determining the melting points of the gold derivatives of authentic crystalline carnitine, γ-butyrobetaine, and crotonbetaine, similar values were obtained. When the crystals formed from the eluate of the chromatograms were mixed with those of authentic crystalline material, there was no depression of the melting points. From these results it was concluded that the material contained in the acid-precipitable fraction of the aqueous brain extract originally made from convulsing animals was comprised of a mixture of free betaines—namely, γ-butyrobetaine (gBB), crotonbetaine, and carnitine. This was further confirmed by determining the R_f values of authentic samples of these substances in the butanol-water system, which was found to be 0.1.

The eluate of the band with an R_f of 0.1 was found to be physiologically inactive. As we had identified the substances on this chromatogram, it was of interest to investigate the influence of these crystalline betaines on the frog rectus preparation and on the intact animal. These substances were all without effect on the frog rectus preparation, and intracranial injections of up to 1 mg. into unanesthetized rats produced no observable response. On the other hand, the intracranial injection of less than 100 μg. of the methyl, ethyl, or choline esters of these betaines caused violent and fatal convulsions in the unanesthetized rat. In addition, these betaine esters caused immediate contraction of the frog rectus preparation and this effect was abolished by atropine. From these results it appeared that betaine esters were capable of producing convulsions in animals and that their pharmacologic effects were similar to those of ACh (*13*). The similarity of the actions of betaine esters and those of ACh has been described by several authors (*7, 25*).

The presence of these betaines has not previously been reported in brain tissue, although their presence in muscle extracts has been known for a long time (*12*). Because the free betaines

have been found to be without physiologic activity but their esters were active (*7, 13, 25*), it seemed reasonable to surmise that these substances might be normally present in brain as esters, especially in view of the fact that we were unable to detect any free betaines in aqueous extracts made from normal animals. In such a case, it was also possible that the free betaines could have been liberated from their naturally occurring esters during the preparation of the extract and the subsequent manipulation required by the reineckate fractionation procedure. Accordingly, an aqueous extract was made from the brains of normal animals and divided into two parts; 1 mg. of the ethyl ester of gBB (gBBE) was added in a saline solution to one part of the extract. The entire extraction procedure for the isolation of free betaines was repeated with both samples. Acid reineckate–precipitable material (free gBB) was found in the fraction to which the gBBE was added but none in the companion control sample. These results indicated that the free betaines previously isolated from the aqueous brain extract made from convulsing animals could have been liberated from some easily hydrolyzable betaine ester precursor during the extraction process and accordingly, their presence in the extract could have been an artifact, as a consequence of hydrolysis.

As it has been reported that alkaline reineckate precipitation selectively removes conjugated betaines from solution (*5*), the next investigation was to determine the identity of the material found in this fraction with R_f 0.50 to 0.70 derived from the aqueous extract made from the brains of convulsing animals, since none of this material could be found in similar extracts made from normal animals.

The material in this band obtained from several chromatograms was eluted with methanol-water and tests were performed on the pooled eluate. A positive hydroxamic reaction was obtained when the eluate was treated with hydroxylamine and ferric chloride (*14*), indicating that some carboxylic acid derivative was present (*20*). Accordingly, a portion of the eluate was hydrolyzed by boiling with 0.1*N* HCl for 10 minutes. The solution was cooled and neutralized and its volume reduced to 0.5 ml. by vacuum evaporation. A portion of the concentrated hydrolyzate was applied as a spot on paper and developed in the butanol-water system as described above. Spraying the developed chromatogram with 1% iodine in ethanol revealed a spot with an R_f of 0.1. The material in this spot was eluted and gold derivatives were prepared as described above. The melting points of the crystals formed were determined and compared with those previously shown in this paper. Similar values were obtained. On the basis of results obtained in these and previous experiments, it was concluded that the same three betaines were also present in the alkaline reineckate fraction of the extract made from the brains of convulsing animals, and that these betaines were conjugated in some manner, probably through the carboxylic acid group, since a positive hydroxamic test was obtained and alkaline reineckate precipitation selectively removed conjugated betaines.

Since the eluate of the band derived from alkaline reineckate precipitation had an R_f of 0.50 to 0.70 and its acid-hydrolyzed product had an R_f of 0.1 in the same system, it appeared that the chemical group which was released by hydrolysis significantly altered the mobility of the betaines in this system. The R_f value of the methyl and ethyl derivatives of gBB was determined and found to be 0.21 in the butanol-water system (*13*). It, therefore, appeared that the group released by hydrolysis of the naturally occurring conjugated betaines may not have been of the hydrocarbon type. Aliquots of the acid hydrolyzate of the eluate were then applied as a spot on paper for two-dimensional chromatography using propanol-water (70–30) and phenol-water (3–1) systems, respectively. When the developed chromatogram was sprayed with 1% ninhydrin, a number of spots were observed, one of which was identified as that of β-alanine. Since this amino acid has an R_f of less than 0.1 in butanol-water, it was assumed that it could have been part of the structure of the conjugated betaine. The amino acid does form part of the structure of coenzyme A, a substance which forms thiol esters with carboxylic acids (*24*). Coenzyme A contains several substances, two of which are ribose and phosphoric acid as phosphate (*1*). Chemical analyses on the acid hydrolyzate of the concentrated eluate revealed the presence of both of these substances. Further identification of the other substances normally present in the coenzyme A structure was vitiated by chromogenic material. The CoA derivative of gBB has been synthesized enzymatically and found to have an ACh-like activity as tested on the frog rectus preparation (*16*). This material is precipitable by alkaline reineckate and is easily hydrolyzed in acid or alkaline media when heated. It has an R_f of 0.55 in the butanol-water system.

It was concluded that the aqueous extracts made from brains of animals which had received either convulsant drugs or electroshock contained a mixture of minute quantities of the CoA derivatives of gBB, crotonbetaine, and carnitine. Because of the severe losses involved during the entire manipulation, it is very difficult to say precisely the relative proportion of these esters in the extracts.

Origin of CoA Betaine Derivatives

Because CoA derivatives of betaines have been shown to be present in aqueous extracts made from the brains of convulsing animals and they could not be found in extracts prepared similarly from normal animals, the next phase of the investigation was to determine their origin in this tissue preparation. Further investigation (*15*) revealed that these substances are normally present in brain, but are released only when the tissue homogenate is treated with agents such as chloroform, ether, and ethanol. These substances were found to be present within the mitochondria. Similar results have been obtained with normal muscle mitochondria (*16*).

It appeared, therefore, that in our aqueous extracts made from the brains of convulsing animals, these betaine esters could have been released from the mitochondria, presumably through the action of the agents and conditions used to initiate convulsions in our experimental animals. If this were the case, we should expect to find more damaged mitochondria in brain preparations made from convulsing animals than in those made from normals. A number of enzyme systems are located exclusively within the mitochondria, and rupture of these bodies results in increased activity of these enzyme systems (*23*). This method of enzyme assay has been to determine the extent of mitochondrial damage under our experimental conditions, by measuring the succinoxidase activity of the isolated mitochondria. Brain mitochondria were isolated following the procedure described by Balazs and Lagnado (*2*). As there are many environmental factors which rapidly cause mitochondria to swell and eventually burst (*23*), we have measured the succinoxidase activity of mitochondria isolated from animals treated with convulsant agents and other conditions previously described, and from normal controls, for only 12 minutes in the presence of the substrate. In this way we were able to determine the succinoxidase activity of the isolated mitochondria before there was further rupture of the intact structures. The results shown in Table I are derived from experiments in which animals treated with cerebral convulsants and electroshock served as experimentals, and normal untreated animals as controls. The enzyme activity is reported in terms of microliters of oxygen per minute consumed by mitochondria contained in 1 gram of fresh brain. Table I shows that in all the mitochondrial preparations derived from the experimental animals, there was increased

succinoxidase activity as compared with the controls, especially within the first minutes of the incubation, which we assume is indicative of greater mitochondrial damage.

Conclusions

The results of these experiments seem to indicate that as a consequence of the administration of the cerebral convulsants used in this work or electroshock, to animals, the mitochondria in the brain is damaged. This, in turn, could lead to the detachment and release of the CoA derivatives of the betaines into a new environment. These betaine esters are structurally similar to ACh, having the same quaternary ammonium nitrogen and carbonyl groups. In addition, the bond distance between these two functional groups in the two molecules is not very different. Hence, it is not surprising to find that the betaine esters are pharmacologically active substances. It is therefore possible that these betaine esters could attach themselves to the same receptors in brain as does ACh itself. Miller *et al.* (*22*) have observed that the topical application of ACh to the eserinized cerebral cortex can elicit the electrical activity normally associated with convulsions of the epileptiform type. It would appear therefore that betaine esters released in brain as a consequence of the administration of cerebral convulsants could act in a manner similar to that observed on the topical application of ACh to the cerebral cortex.

Table I. Damage of Brain Mitochondria in Vivo by Cerebral Convulsants as Assayed by Succinoxidase Activity

(μl. of oxygen consumed by mitochondria in 1 gram of fresh brain per minute)

Time, Min.	Agent	Control	Expr.	Diff.	Agent	Control	Expr.	Diff.
0–3	NH_4Cl	5.6	9.6	4.0	Camphor	7.1	11.6	4.5
3–6	(4.5 g.)[a]	6.2	9.0	2.8	(4.0 g.)[a]	6.0	7.1	1.1
6–9		7.8	8.6	2.4		5.3	6.4	1.1
9–12		5.8	7.3	1.5		4.9	4.9	0
0–3	Dieldrin	7.5	14.3	6.8	Electroshock	13.2	20.0	6.8
3–6	(7.0 g.)[a]	7.8	9.2	1.4	(5.0 g.)[a]	15.0	16.9	1.9
6–9		7.9	9.3	1.4		11.2	11.6	0.4
9–12		7.9	10.2	2.3		13.0	13.0	0

[a] Weight of fresh brain from which mitochondria were isolated.

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INSECT RESISTANCE TO INSECTICIDES

Biochemical Factors in the Acquired Resistance of Houseflies to Organophosphate Insecticides

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THE RAPID DEVELOPMENT of resistance to certain organophosphates is proving to be a limiting factor in their use and a deterrent to the search for new insecticides of this type (*9, 10*). Houseflies have been most frequently used for investigations on the mechanism of this resistance. Although strains of houseflies have developed resistance to almost all the organophosphates, the resistance pattern varies with the strain (*11, 20, 22, 28, 31, 36, 48*, and others). The varying resistance pattern in different strains, along with genetical studies (*35, 37*), indicates that houseflies may have more than one mechanism of resistance to organophosphates. The resistance mechanism is even more complex, since selection with organophosphate insecticides can result in resistance to carbamates (*17, 18, 20, 29*) and *vice versa* (*32*). In addition, exposure to either phosphates or carbamates may result in high side resistance to chlorinated hydrocarbon insecticides (*27, 31, 32*), whereas the reverse situation does not hold (*27, 31*), although DDT-resistant flies developed organophosphate resistance faster than normal flies (*26*).

Many experimental approaches have been used in studying the mechanism of organophosphate resistance in houseflies. Attempts have been made to

succinoxidase activity as compared with the controls, especially within the first minutes of the incubation, which we assume is indicative of greater mitochondrial damage.

Conclusions

The results of these experiments seem to indicate that as a consequence of the administration of the cerebral convulsants used in this work or electroshock, to animals, the mitochondria in the brain is damaged. This, in turn, could lead to the detachment and release of the CoA derivatives of the betaines into a new environment. These betaine esters are structurally similar to ACh, having the same quaternary ammonium nitrogen and carbonyl groups. In addition, the bond distance between these two functional groups in the two molecules is not very different. Hence, it is not surprising to find that the betaine esters are pharmacologically active substances. It is therefore possible that these betaine esters could attach themselves to the same receptors in brain as does ACh itself. Miller *et al.* (*22*) have observed that the topical application of ACh to the eserinized cerebral cortex can elicit the electrical activity normally associated with convulsions of the epileptiform type. It would appear therefore that betaine esters released in brain as a consequence of the administration of cerebral convulsants could act in a manner similar to that observed on the topical application of ACh to the cerebral cortex.

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Many experimental approaches have been used in studying the mechanism of organophosphate resistance in houseflies. Attempts have been made to

The metabolic fate of radiolabeled Diazinon, malathion, and methyl parathion was studied in three housefly strains, two of which were resistant to organophosphates. Rate differences between the strains in cuticle penetration, phosphorothionate oxidation, or phosphate hydrolysis did not appear to explain the resistance. The lesser in vivo cholinesterase inhibition in resistant than susceptible flies treated with the same dose of the organophosphate probably results from some other mechanism than detoxification. Flies treated with the organophosphate immediately after decapitation were similar to whole flies in the symptomology of poisoning and degree of resistance. Evidence is presented for a "factor" in the thorax and/or abdomen which contributes to resistance by reducing rate of cholinesterase inhibition without destroying active antiesterase organophosphate.

correlate resistance with behavioristic avoidance (*19, 23, 44, 45*), lipoid content of the tarsi and thoracic ganglia (*41*), rate of insecticide penetration through the cuticle (*12, 25*), rate differences in oxidation of the phosphorothionates or persistence differences for the antiesterase phosphates formed (*25, 31, 36, 38*), rate differences in the hydrolysis of the phosphates (*6, 30, 31, 38*), the in vivo inhibition of cholinesterase (*38, 47*), and accumulation of acetyl choline (*39*), the activity (*5, 16, 31, 38*) or sensitivity (*6, 31, 38*) of the cholinesterase to organophosphates as assayed in vitro, and the level of aliesterase activity (*5, 6, 37*).

Most of the resistance mechanisms proposed depend on rate differences in the penetration or hydrolysis of the organophosphate. A critical test of these hypotheses was made with radiolabeled insecticides of high specific activity. Another hypothesis was also considered whereby the resistance might be due to a factor protecting the cholinesterase of resistant flies from inhibition by the organophosphate.

Methods and Materials

Housefly strains. Three strains of organophosphate-resistant houseflies (*Musca domestica* L.) were investigated: R-A, the Rutgers A strain which developed resistance under Diazinon pressure (*20*); R-I, the Italian strain which developed resistance under Diazinon pressure (*11, 43*); and R-P, the Orlando Tropical P strain which developed resistance in the field under Diazinon, Dipterex, malathion, and parathion pressure and was further selected in the laboratory under parathion pressure (*28, 48*). The resistant flies were reared through the fourth generation in our laboratory without insecticide pressure. Susceptible (S) flies were the C.S.M.A. (1948) strain. The larval medium and rearing technique have been described (*46*). Adult female flies were used the first day after emergence. Prior to testing, these day-old flies were fed skim milk diluted with an equal volume of water.

Insecticides. Isotopic exchange (*13*) was used to obtain the labeled organophosphorothionates of high specific activity. Krueger and O'Brien's procedure (*24*) was used for preparing labeled malathion in 58% yield with a specific activity of 6000 c.p.m. per μg. Diazinon (*25, 40*) was obtained in 48% yield with a specific activity of 4900 c.p.m. per μg. The synthesis of methyl parathion-P^{32} (*40*) gave about 40% yield and a specific activity of 4000 c.p.m. per μg. Solvent extraction and partition chromatography were used for purifying the radiolabeled compounds (*40*) until they were identical in infrared spectra (10% solutions in carbon tetrachloride or carbon bisulfide, Baird infrared spectrophotometer, sodium chloride optics) with the authentic compounds. The LD_{50} values for flies 24 hours after injection were identical for the radiolabeled and the authentic nonlabeled compounds.

The nonlabeled insecticides used were of the highest purity obtainable from the manufacturers.

Administration of Insecticides and Toxicity Studies. Houseflies were injected through the mesoscutum with a glass needle drawn to an 0.3-mm. tip fitted on an 0.05-ml. microsyringe driven by a micrometer. Propylene glycol was used as the insecticide solvent, each fly receiving 0.08 μl. Topical applications on the ventral tip of the abdomen were made with acetone solutions of the toxicants and 1.3 μl. of acetone per fly (*33*). The mortalities occurring in controls treated with solvent alone were 3.6% at 4 hours and 5.8% at 24 hours with the topical application and 0.8% at 24 hours with injection. In a separate study the compounds were applied topically to whole flies or flies which had just been decapitated. Results from the decapitation studies are reported only at 4 hours after treatment, because at this time the control mortality was 9.7% and by 24 hours complete mortality had occurred. Following injection or topical application, flies were held in gauze-covered 1 × 3 inch shell vials with 10 flies per vial. A small amount of sucrose was added to the vials to prevent starvation. LD_{50} values as micrograms of toxicant per gram of fly were estimated from logarithm-probit mortality plots.

Cuticle Penetration. The rate of cuticle penetration was determined following topical application of acetone solutions of the labeled insecticides to the ventral tip of the abdomen. Methyl parathion was applied at 0.5 and 5.0 μg. per gram, malathion at 10 μg. per gram, and Diazinon at 4.0 μg. per gram. With the 4-hour penetration experiments, the mortality in the S strain was no more than 50%, except with the high methyl parathion level, which yielded complete mortality. The resistant strains (R-A and R-P) showed no symptoms of poisoning except with the high methyl parathion level, where hyperactivity and partial paralysis of the flies occurred within 4 hours.

The amount of cuticle penetration was determined by washing the surface of 1 gram of treated flies with highly refined kerosine (Deobase) at various intervals after topical application. The inside of the vial used to hold the flies was also washed, the washes were combined, and the total radioactivity was determined. The difference between the amount of insecticide recovered and the amount applied was assumed to have penetrated into or through the cuticle. All experiments were duplicated.

In vivo Detoxication Studies. The in vivo fate of the radiolabeled insecticides was determined following injection at the same insecticide levels used for the penetration studies. These dosages yielded no more than 50% mortality among susceptible flies and no poisoning symptoms with the resistant flies, with the exception of the 5.0 μg. per gram of methyl parathion which gave complete mortality of the S flies within 4 hours but only symptoms with the R-A and R-P strains.

For determining the nature of the metabolites at various time intervals after injection of the P^{32} insecticides, a procedure was used which allowed almost quantitative separation of the original phosphorothionate, its oxygen analog, and the hydrolysis products of either of these.

One gram of treated flies and approxi-

mately 7 grams of Celite were homogenized in 50 ml. of acetone in a cooled Lourdes blender. The homogenate was filtered and the residue with the Celite rehomogenized with 50 ml. of benzene. This second homogenate was filtered, and the filtrates were combined and extracted with 50 ml. of water. The layers were separated and aliquots were removed, evaporated, and counted for P^{32} content. The solvent was evaporated from the remaining organic solubles, and the residue was dissolved in hexane and chromatographed on an alumina column (*25*) with 150 ml. of benzene to elute the original phosphorothionate, 150 ml. of chloroform to elute the phosphate oxidation products, and finally with 100 ml. of methanol to ascertain that all the radioactivity had been eluted from the column.

When this procedure was used, more than 90% of the radioactivity was extracted from flies immediately after injection of the labeled compound, and subsequent recovery from the alumina column was also greater than 90%. The losses in these two steps probably contributed to the lack of complete recovery in determining the separate amounts of the various metabolites. All experiments were made in duplicate. Radioactive measurements were made on dried samples in an automatic gas flow counter with suitable corrections for self-absorption and isotopic decay.

The levels of oxygen analog derived from injected nonlabeled parathion, methyl parathion, and malathion (0.5, 0.5, and 10 μg. per gram, respectively) were also determined in separate experiments by extraction of the inhibitor and assay of the recovered anticholinesterase agents against housefly head cholinesterase. At 0, 1, and 4 hours after injection, the flies were immediately frozen in an acetone–dry ice mixture and subsequently homogenized in this same acetone. The acetone was then evaporated, equal volumes of water and chloroform were added, and the neutral phosphoric esters were extracted into chloroform. The anticholinesterase agents in aliquots of this chloroform were then assayed against fly head homogenates. The aliquot used was one predetermined to yield a maximum of 60 to 80% cholinesterase inhibition.

Rate Studies on Fly Cholinesterase Inhibition. The activity of fly head cholinesterase was determined at various times after topical application of the three organophosphates (*33*) with the S, R–A, and R–P strains. With this technique the interference from excess inhibitor (*3*) was minimal (*34*).

In vitro studies on the rate of cholinesterase inhibition followed the procedure of Aldridge and Davison (*1*).

The enzyme preparation in 1.60 ml. of bicarbonate buffer was placed in a 22-ml. double-side-arm Warburg flask. The organophosphate in 0.20 ml. of buffer was placed in one side arm and the substrate in 0.20 ml. in the second side arm. When the side arms were tipped, the final concentrations were $0.01M$ for acetyl choline, $1 \times 10^{-6}M$ for malaoxon, and $1 \times 10^{-7}M$ for paraoxon. After gassing with a 95% carbon dioxide–5% nitrogen mixture and equilibration at 25° C. in the Warburg bath for a total of 10 minutes, the first side arm was tipped to initiate the reaction of the inhibitor with the cholinesterase. When the desired time for inhibition had elapsed, the substrate was tipped in to terminate the inhibition reaction and initiate the assay of the residual cholinesterase activity.

All in vitro rate studies were made at 25° C. and results were calculated as b_{30} values (microliters of carbon dioxide evolved in 30 minutes). Enzyme sources were prepared from both susceptible (S) and resistant (R–A and R–P) flies and included homogenates of whole flies, heads from flies, and thoraces plus abdomens from flies. The enzyme concentration was standardized at 1.5 fly equivalents (or part thereof such as head, or thorax plus abdomen) per ml. The in vitro rate studies were varied by using the heads from one strain of flies as the cholinesterase source and the thorax plus abdomen from another strain as modifiers of the inhibition rate. In certain cases the homogenized thoraces plus abdomens were heated at 100° C. for 5 minutes and then cooled before use.

The stability of the organophosphate inhibitor on incubation with fly homogenates was also studied under the concentration, buffer, pH, and temperature conditions involved in the normal Warburg assay. After various incubation times of the fly homogenates with $1 \times 10^{-6}M$ malaoxon or $1 \times 10^{-7}M$ paraoxon at 25° C., 2.0-ml. aliquots were withdrawn and extracted with an equal volume of chloroform and the anticholinesterase agents recovered in the chloroform were assayed against a fresh fly head homogenate at 37° C. The aliquot size was standardized to give 50 to 60% cholinesterase inhibition. The difference between inhibitor recovered at zero time and that recovered with varying incubation times was assumed to represent the loss of the inhibitor under the conditions utilized in studying the in vitro rate of cholinesterase inhibition.

Results

Degree of Resistance. The degree of resistance—i.e., the 24-hour LD_{50} of the resistant strain divided by the LD_{50} of the susceptible strain—of the R–A, R–P, and R–I strains did not vary greatly when determined by topical application or injection of the insecticides. With

Table I. Characteristics of Housefly Strains Studied

Characteristic	Housefly Strain S	R–A	R–P	R–I
Av. wt. adult ♀ mg.	22	22	22	20
ChE activity of heads $-b_{30}$/head at 37° C.	41	42	39	35
Sensitivity to Compds.[a]	LD_{50} μg./g.	Degree of Resistance[b]		
Topical, 24 hr.				
Diazinon	3.6	37	15	17
Malaoxon	1.8	8	10	4
Malathion	15	9	7	7
Methyl parathion	0.9	6	7	4
Paraoxon	0.5	3	6	44
Parathion	0.7	9	16	44
Isolan	15	13	10	..
Sevin	45	25	10	..
Injected, 24 hr.				
Diazinon	6	30	6	21
Malathion	12	6	11	3
Methyl parathion	0.5	11	13	16

[a] Replications. After approximate LD_{50} values were found in survey runs, final values were determined with 1.2 to 1.4 factor dosage increments and 20 flies at each dose. Final values for topical LD_{50} were based on 2 to 4 separate dosage series about the LD_{50} for the S and 1 to 3 series for the R strains. A single series was used in arriving at final injected LD_{50} values.

[b] $\frac{LD_{50} \text{ resistant strain}}{LD_{50} \text{ susceptible strain}}$.

Table II. LD_{50} Values for Strains 4 Hours after Topical Treatment of Houseflies with and without Decapitation[a,b]

Insecticide	Whole Flies S	R–A	R–P	Decapitated Flies S	R–A	R–P
Diazinon	4	175	385	4	150	360
Malaoxon	1.9	18	20	1.8	17	20
Malathion	14	180	120	12	180	100
Methyl parathion	0.9	6	7	0.9	6	7
Paraoxon	0.6	2	4	0.6	2	4
Parathion	0.9	8	12	0.8	7	11
Isolan	45	210	175	45	210	140
Sevin	65	1400	670	65	1400	670

[a] LD_{50} as μg./g. disregarding weight loss on decapitation.

[b] After approximate LD_{50} values were found in survey runs, final values were determined with 1.2 to 1.4 times dosage increments and 20 flies at each dosage. Reported values are based on 1 to 3 dosage series.

topical application the resistance was almost as great with the oxygen analogs (malaoxon and paraoxon) as with their corresponding phosphorothionates (malathion and parathion). The resistance pattern in each of the strains (Table I) was similar to that recorded in the literature (*11*, *20*, *28*, *43*, *48*).

Flies decapitated just prior to treatment were similar to intact flies in their susceptibility to the toxicants based on LD_{50} values 4 hours after topical application (Table II). The symptoms of poisoning at 4 hours after treatment were the same for whole or decapitated flies on the basis of the proportion of normal, hyperactive, paralyzed, and dead individuals. The site of action involved in initiating the poisoning symptoms and that governing the degrce of resistance to these insecticides appears to be primarily localized in the thorax and/or abdomen.

These studies were made on flies from the second through the fourth generations without insecticide pressure. Little loss in the degree of resistance occurred by the fourth generation with the R–A, R–P, and R–I strains, whereas March (*31*) has noted that in other strains the phosphate resistance drops off rapidly once removed from insecticide pressure.

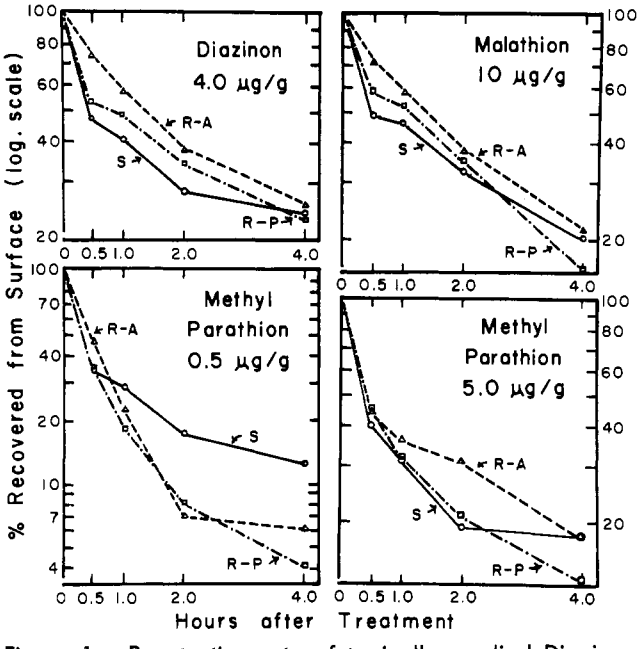

Figure 1. Penetration rate of topically applied Diazinon, malathion, and methyl parathion into three housefly strains

Penetration as a Factor in Resistance. The penetration rate was not a major factor contributing to the resistance based on the tolerance of the resistant flies studied to the toxicants following either topical application or injection (Table I). Penetration following topical application in this study may have been greatly aided by the solvent or disruptive action of the acetone on the cuticular constituents.

Results from penetration rate studies with radiolabeled Diazinon, malathion, and methyl parathion following topical application to the ventral tip of the abdomen are given in Figure 1. The data are plotted as a logarithmetic function of the percentage of applied insecticide recovered against hours, on the assumption that a certain constant fraction of the insecticide penetrated during each equal time interval. By averaging the data for the three fly strains and considering the slope of the line from 0.5 to 4 hours, it was found that the time for half penetration with methyl parathion was 45 minutes at 0.5 and 100 minutes at 5.0 µg. per gram, with Diazinon it was 110 minutes at 4.0 µg. per gram, and with malathion it was 130 minutes at 10 µg. per gram. The results with methyl parathion at 5.0 µg. per gram cannot be directly compared with the other compounds and dosages, since the susceptible flies were paralyzed and died during the course of the experiment. Extrapolation of these curves to zero time indicated that 40 to 48% of the methyl parathion and 30 to 32% of the Diazinon and malathion had penetrated at a faster rate, possibly through the action of the solvent.

Diazinon and malathion penetrated more rapidly into the susceptible than into the R–A and R–P strains, the major difference occurring within the first 30 minutes. This is in agreement with a published report comparing the S and R–A strains with Diazinon at 30 µg. per gram (*25*). The strain differences with methyl parathion were less clear-cut. Insufficient data are available to make precise correlations of degree of resistance (Table I) and differences in penetration rate (Figure 1), but radiotracer penetration studies do not implicate penetration as the major factor contributing to the resistance.

Detoxication of Organophosphate as a Factor in Resistance. Studies on the rate of degradation and excretion for the radiolabeled insecticides following injection into the different fly strains are presented in Table III. Recovery of the applied radioactivity was good, considering that unextractable radioactivity remaining in the fly residue and that not eluting from the alumina column in the positions for the unhydrolyzed phosphorothionate or phosphate were not included in the percentage recovery figures. Averaging all data gave recoveries of

Table III. Degradation and Excretion of Radiolabeled Phosphorothionates after Injection into Three Housefly Strains

(Results as % of administered dose recovered in fraction indicated)

Insecticide, Dose and Fraction	Hours after Injection of Phosphorothionate											
	Susceptible				Resistant A				Resistant P			
	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
Diazinon, 4 µg./g.												
Org. sol., P = S	72	57	40	23	72	50	35	18	77	40	30	18
Org. sol., P = O	3.7	6.2	3.7	3.0	2.5	5.0	3.7	3.2	5.0	6.2	3.7	2.5
Water sol.	25	48	38	35	28	48	50	45	23	48	48	43
Excreted	0.1	0.2	5	20	0.1	1	5	23	0.1	1	8	20
Malathion, 10 µg./g.												
Org. sol., P = S	63	45	23	17	61	53	23	8	65	50	25	9
Org. sol., P = O	0.6	0.6	0.6	1.1	0.2	0.3	0.7	0.7	0.3	0.9	0.5	0.8
Water sol.	22	33	51	35	29	43	65	21	22	33	47	20
Excreted	1	2	3	30	3	5	10	51	2	3	5	36
Methyl parathion, 0.5 µg./g.												
Org. sol., P = S	36	24	22	18	34	30	24	24	46	36	30	28
Org. sol., P = O	6	2	2	2	4	2	4	4	6	4	2	2
Water sol.	10	62	44	18	40	58	50	26	16	80	52	24
Methyl parathion, 5.0 µg./g.												
Org. sol., P = S	36	26	22	16	30	24	18	16	32	26	22	18
Org. sol., P = O	6	5	2	2	6	5	4	2	6	3	2	2
Water sol.	26	60	56	52	28	58	42	42	24	62	52	52
Excreted	2	16	14	24	2	28	38	40	4	24	36	42

95% for Diazinon, 85% for malathion, 96% for the high level of methyl parathion, and 73% for the 0.5 μg. per gram of methyl parathion dosage where excretion data were not available for consideration. The percentage recovery, particularly with the low level of methyl parathion, varied considerably within this experiment, as indicated by the following ranges calculated from Table III: Diazinon 81 to 111%, malathion 66 to 101%, and methyl parathion (0.5 μg. per gram) 38 to 120% and (5.0 μg. per gram) 66 to 115%.

The rates of destruction of the injected phosphorothionate and formation of water-soluble metabolites (hydrolysis products) were not greatly different between the susceptible and resistant strains. Only with the high dose of methyl parathion were large excretion differences evident and at this dose the paralysis of the susceptible flies probably accounted for their low excretion compared with the active resistant flies.

No great difference occurred between the strains in the percentage of the injected dose that existed as radiolabeled oxygen analog at any time after treatment (Table III). Possible differences in in vivo levels of oxygen analogs were also examined by injecting the S, R–A, and R–P flies with 0.5 μg. per gram of parathion or methyl parathion and 10 μg. per gram of malathion and recovering the unhydrolyzed materials by extraction with acetone and partitioning into chloroform from water for subsequent assay of extracted cholinesterase inhibitors against fresh fly head homogenates. Little or no strain difference was found in the amount of inhibitor recovered from the S, R–A, and R–P flies treated with parathion or methyl parathion and the S and R–P flies treated with malathion. The extracts from flies homogenized immediately after treatment yielded no cholinesterase inhibition with the aliquot assayed. Parathion at 1 hour yielded 80% inhibition and at 4 hours yielded 30% inhibition, and corresponding values with methyl parathion were 80% at 1 hour and 30% at 4 hours, and with malathion were 65% at 1 hour and 50% inhibition at 4 hours with each of the S and R strains studied. A greater instability for the oxygen analogs was indicated by these studies on recovery of the anticholinesterase agents than was found with the radiotracer experiments (Table III).

Cholinesterase Inhibition as a Factor in Resistance. This study confirmed a previous report (*17*) that the cholinesterase inhibition in the heads of organophosphate-poisoned flies correlates with resistance. The data from methyl parathion applied topically at 5.0 μg. per gram (Figure 2) illustrate the lesser inhibition and milder symptoms occurring in the resistant strains. Studies with methyl parathion at 0.5 μg. per gram, malathion at 10 μg. per gram, and Diazinon at 4.0 μg. per gram showed less than 5% inhibition at any time after treatment with the R–A and R–P strains, while the greatest degree of inhibition occurred at 0.5 hour with the S strain, when values of 9, 24, and 16% inhibition, respectively, for the three organophosphates were observed.

The initial rate of in vitro inhibition by malaoxon and paraoxon was greater for the cholinesterase in homogenates of whole susceptible flies than in homogenates of whole resistant flies (Figure 3), even though the initial cholinesterase activity was nearly the same in both cases. This rate difference with strain was not evident when only the heads were studied, confirming previous reports (*31*, *38*). When the experiments illustrated in Figure 3 were repeated using the heads of S flies in both cases combined with either the S or R thoraces plus abdomens, similar rate differences were noted. In such studies about 75% of the cholinesterase activity was contributed by the heads, and the remainder by the combined thoraces plus abdomens. The cholinesterase in homogenates of heads was inhibited more rapidly than that in homogenates of whole flies, a difference which was greatly reduced or lost by boiling the thoraces plus abdomens before adding to the fresh head homogenate. Comparisons were also made between boiled and unboiled thoraces plus abdomens from S or R flies mixed with fresh heads from S flies. In both cases (S and R flies) the esterase was more rapidly inhibited by the organophosphate in the presence of the boiled thoraces plus abdomens, and the difference in rate resulting from boiling was greater with the R than with the S flies.

The difference in inhibition rate of the cholinesterase in homogenates of S and R flies was not due to more rapid hydrolysis of the inhibitor by the R-fly homogenates, since comparable amounts of the inhibitor could be recovered by chloroform extraction of the S or R fly homogenate–organophosphate incubation mixtures (Table IV). It appears probable that in resistant flies there is a higher concentration of some "factor" in the thorax and/or abdomen capable of reducing the rate of cholinesterase inhibition by organophosphates without hydrolyzing the inhibitor.

Discussion

Several differences were found between the resistant and susceptible houseflies which might contribute to the survival of the resistant forms when exposed to certain organophosphate insecticides.

Rate differences in cuticle penetration may be a contributing factor (*12*, *25*, *31*), since a somewhat slower penetration of Diazinon and malathion occurred with the resistant than with the susceptible flies (Figure 1). Penetration did not appear to be the most critical factor, since similar degrees of resistance and resistance patterns resulted with topical

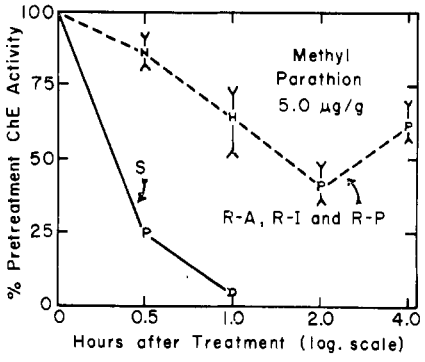

Figure 2. Brain cholinesterase activity and symptomology of surviving houseflies after topical treatment with methyl parathion

Symptoms for over 50% of the flies at the selected posttreatment times. N normal, H hyperactive, P paralyzed, D dead. Results on cholinesterase inhibition with resistant flies indicated as the range for the three strains. Each point with each strain is based on 2 to 4 replicates.

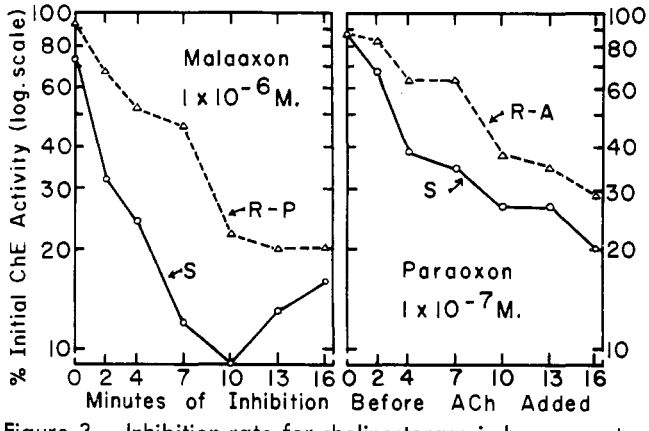

Figure 3. Inhibition rate for cholinesterase in homogenates of whole S and R flies by malaoxon and paraoxon at 25° C.

Each point based on 4 determinations

Table IV. Extent of Malaoxon and Paraoxon Breakdown on Incubation in pH 7.45 Bicarbonate Buffer with Homogenates of Resistant and Susceptible Houseflies

Substrate and Fly Strain	% ChE Inhibition from $CHCl_3$ Extract Made after Indicated Minutes of Incubation			
	0	5	20	80
Malaoxon, $1 \times 10^{-6} M$				
S	63	61	63	57
R-P	61	60	63	59
Paraoxon, $1 \times 10^{-7} M$				
S	54	48	49	51
R-A	51	48	49	50

application and injection of the organophosphate (Table I), although the resistance to injected toxicants found in the present study was greater than that reported in the literature (*11*, *31*, *38*).

Possible differences in efficiency of phosphorothionate oxidation to form the antiesterase phosphate analogs cannot account for the resistance, because the tolerance to the oxygen analogs is of the same order of magnitude as that to their phosphorothionates (Table I; *31*, *36*, *38*). Radiotracer studies on phosphorothionate oxidation failed to show strain differences in rate of loss of the phosphorothionate or formation of the phosphate analog (Table III), confirming the bioassay results.

The rate of in vivo hydrolysis following administration of the radiolabeled phosphorothionate was not greatly different between the resistant and susceptible flies (Table III). As comparable levels of the oxygen analogs were recovered with all the strains (Table III), it appears likely that the oxygen analogs were degraded no more rapidly by the resistant than the susceptible flies. No strain difference was noted in the total anticholinesterase activity recovered by chloroform extraction from flies treated with three phosphorothionates including parathion (see results), confirming the lack of difference noted in rate of paraoxon destruction by a susceptible and resistant strain by a slightly different analytical method (*30*).

The brain cholinesterase from resistant and susceptible flies is similar in in vitro susceptibility to inhibition by organophosphates (*31*, *38*; see Results), but this same cholinesterase is much more greatly inhibited in vivo by the same organophosphate dose in susceptible than in resistant strains (Figure 2; *10*, *47*) to yield a greater acetylcholine accumulation in the susceptible than in the resistant flies (*39*). The head can be removed from the fly without altering the symptoms of organophosphate poisoning or the degree of resistance (Table II), indicating that the site of action involved in initiating the poisoning symptoms and that governing the degree of resistance to these insecticides are primarily localized in the thorax and/or abdomen.

With whole fly homogenates, a slightly larger amount of certain organophosphates is required to inhibit the cholinesterase from susceptible than from resistant flies, possibly because of the aliesterase which is present in higher concentration in the susceptible forms preferentially reacting with these organophosphates (*6*). Treatment of head or whole fly homogenates by a method which partially inactivates the aliesterase (pH 8.3, 37° C., 1 hour) increases the susceptibility of the cholinesterase in these homogenates to organophosphates, again presumably by removing the aliesterase which preferentially reacts with the organophosphate. The aliesterase activity and susceptibility relationships tend to favor the survival of the susceptible rather than the resistant strains (*6*). This discrepancy has been explained by the assumption, with no more than meager correlative evidence, that mutation of a single gene in the resistant forms has resulted in the modification of the aliesterase to a phosphatase capable of destroying the organophosphate (*6*).

Despite much speculation regarding possible detoxifying enzymes involved in resistance, only three reports to date have presented data favoring such a hypothesis. In one of these studies (*38*), the degree of in vivo cholinesterase inhibition following injection of parathion or paraoxon was interpolated from a standard curve prepared with fly homogenates and paraoxon in vitro to derive the degree of detoxification. A large difference in detoxification rate was found between the R and S strains. This indirect analytical procedure yielded a far greater apparent efficiency in oxidation of the phosphorothionate (85%) than radiotracer studies (Table III; *24*, *25*). Further, when R or S flies were injected with parathion, no difference was noted in the amount of paraoxon that could be recovered by chloroform extraction of the flies at various times after treatment (see results). When whole fly homogenates (*6*) or sections of decapitated flies (*31*) are incubated with such antiesterases as malaoxon, paraoxon, Diazoxon [*0,0*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphate], or DDVP (*0,0*-dimethyl 2,2-dichlorovinyl phosphate) and the persisting antiesterases after 1 to 4 hours determined by their inhibitory activity on an added fly head cholinesterase source, lesser amounts of anticholinesterase agents are recovered from the resistant strains. The time and substrate concentration relationships (*6*, *31*) indicate, but do not necessarily establish, the involvement of an enzymatic detoxification reaction. In any case, the detoxifying system in resistant flies is probably unable to reduce the total amount of organophosphate rapidly enough to a harmless level (*6*).

An alternative mechanism to detoxification might be a "factor" present in higher concentration in the resistant forms which protects the cholinesterase from inhibition by the organophosphate. Such a factor might act by reducing the rate of the inhibition process or increasing the rate of reactivation of the inhibited cholinesterase. Evidence for the existence of such a factor is presented in the results section and Figure 3. The nature of this factor in the thorax and/or abdomen of resistant flies is not known.

Several mechanisms are evident by which a factor might protect cholinesterase both in vivo and in vitro from inhibition by organophosphates without destroying the organophosphate. The first mechanism would be one of localization where the organophosphate was unavailable for reaction with the cholinesterase because of differences in lipide concentration or characteristics, extent of protein binding, or loss of the organophosphate by reaction with proteins other than cholinesterase. Another mechanism would be a direct protection of the cholinesterase as by substrates or competitive inhibitors, so that the active sites could not be approached by the organophosphate. A third mechanism would involve an enzymatic (*8*) or nonenzymatic factor serving to dissociate or destroy the inhibitor bound to cholinesterase before the more difficultly reversible stage of phosphorylation occurred. Finally, the factor might act as a reactivator for the phosphorylated cholinesterase. The potentiality of such mechanisms has been shown directly or indirectly in studies involving fly cholinesterase (*2–4*, *33*, *34*, *42*) and many other cholinesterase sources, but they have usually not been applied specifically to the problem of insect resistance to organophosphate insecticides.

The side resistance of organophosphate-resistant houseflies to carbamate insecticides indicates a possible involvement of a similar resistance mechanism. On the basis of studies concerning carbamate insecticide degradation by mammalian enzymes (*7*, *14*, *15*, *21*), it appears unlikely that similar detoxification mechanisms are involved for the phosphate and carbamate antiesterase agents. However, some factor altering the mechanism of cholinesterase inhibition might be effective against both types of inhibitors in contributing to resistance.

Further work is in progress on the nature of the factor in resistant flies which reduces the rate of cholinesterase inhibition by certain organophosphates, and on the correlation of this factor with resistance.

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INSECTICIDE RESIDUES IN MILK

Excretion of Co-Ral in the Milk of Dairy Cattle

R. D. RADELEFF, Animal Disease and Parasite Research Division

H. V. CLABORN, Entomology Research Division, U. S. Department of Agriculture, Kerrville, Tex.

Co-Ral, *O* - (3 - chloro - 4 - methylumbelliferone) *O,O* - diethyl phosphorothioate, known also as Bayer 21/199, has shown considerable promise as a systemically active insecticide against the cattle grubs *Hypoderma lineatum* (DeVill.) and *H. bovis* (Deg.) (*1, 2*) and as a contact insecticide against a number of other external parasites (*5*). Recent reports indicate that it may have activity against certain helminths (*3*).

The use of Co-Ral on lactating dairy cattle could be recommended only if Co-Ral were absent from their milk. The present series of studies was initiated to determine the amount of Co-Ral which might appear in milk of cattle sprayed with the material.

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Through the courtesy of the Bayer Chemical Co., Leverkusen, Germany, and the Chemagro Corp., Kansas City, Mo., Co-Ral labeled with phosphorus-32 was made available to the Animal Disease and Parasite Research Division. This material was chromatographically demonstrated to be of at least 99.5% purity and at the time of shipment demonstrated a specific activity of 4.6 mc. per gram.

The radioactive material was diluted with ordinary Co-Ral and made up into a 20% emulsifiable concentrate by dissolving the chemical in a mixture of 65 parts of xylene, and 10 parts of Triton X-100.

This emulsifiable concentrate was then diluted to 0.5 and 0.75% concentrations with tap water immediately before application to the cows.

Acknowledgment

The authors are indebted to J. R. Busvine, A. J. Forgash, G. C. LaBrecque, and C. N. Smith for providing cultures of resistant houseflies on repeated occasions, and to P. V. Stone of the Wisconsin Alumni Research Foundation for maintaining a constant available supply of susceptible houseflies. Malaoxon [*O,O*-dimethyl *S*-(1,2-biscarboethoxy)ethyl phosphorothiolate] was kindly provided by R. W. Young, American Cyanamid Co., Stamford, Conn.

The authors further acknowledge the assistance of W. C. Dauterman in preparing the radiolabeled malathion and methyl parathion and of Janet L. Mengle in performing the bioassays. A discussion with K. van Asperen and F. J. Oppenoorth led to the experiments on the decapitation of houseflies. The initial stages of this study were made with the counsel and assistance of R. D. O'Brien.

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Through the courtesy of the Bayer Chemical Co., Leverkusen, Germany, and the Chemagro Corp., Kansas City, Mo., Co-Ral labeled with phosphorus-32 was made available to the Animal Disease and Parasite Research Division. This material was chromatographically demonstrated to be of at least 99.5% purity and at the time of shipment demonstrated a specific activity of 4.6 mc. per gram.

The radioactive material was diluted with ordinary Co-Ral and made up into a 20% emulsifiable concentrate by dissolving the chemical in a mixture of 65 parts of xylene, and 10 parts of Triton X-100.

This emulsifiable concentrate was then diluted to 0.5 and 0.75% concentrations with tap water immediately before application to the cows.

Co-Ral, O-(3-chloro-4-methylumbelliferone)O,O-diethyl phosphorothioate, also known as Bayer 21/199, is an effective systemic and contact insecticide for livestock use. To determine whether it would be excreted in milk of sprayed cattle, dairy cows were sprayed with 0.5 and 0.75% concentrations. Maximum organo-soluble extractive (Co-Ral plus other organo-soluble compounds) was approximately 0.2 and 0.25 p.p.m., respectively, for the 0.5 and 0.75% concentrations, reached 5 hours after treatment. These levels declined gradually over 7 days, being only a trace at 10 days.

Table I. Excretion of Phosphorus-32 and Organo-soluble Phosphorus-32 Compounds in Milk Following Spray Treatment of Cows with Phosphorus-32–Labeled Co-Ral

		Total P^{32} in Whole Milk, P.P.M.[a]		Organo-soluble P^{32} Labeled Compounds, P.P.M.[a] (4% Butterfat Basis)	
Date	Time	0.5% spray	0.75% spray	0.5% spray	0.75% spray
8/30 (5 hr.)	P.M.	0.544	0.522	0.196	0.245
8/31	A.M.	0.783	0.833	0.126	0.158
	P.M.	1.219	1.427	0.121	0.154
9/1	A.M.	1.177	1.666	0.064	0.082
	P.M.	1.311	2.024	0.069	0.082
9/2	A.M.	1.039	1.858	0.037	0.063
	P.M.	1.086	1.963	0.034	0.064
9/3	A.M.	1.061	1.769	0.034	0.036
	P.M.	0.975	1.819	0.033	0.040
9/4	A.M.	0.855	1.555	0.019	0.024
	P.M.	0.778	1.453	...	...
9/5	A.M.	0.719	1.303	...	...
	P.M.	0.678	1.372	...	...
9/6	A.M.	0.664	1.294	0.012	0.014
	P.M.	0.625	1.470	...	...
9/9	A.M.	0.375	0.661	...	...
9/10	A.M.	0.336	0.504	0.001	0.003
9/13	A.M.	0.244	0.444	...	...
9/16	A.M.	0.244	0.299	...	...
9/20	A.M.	0.114	0.230	...	...
9/23	A.M.	0.069	0.119	...	...

[a] Co-Ral equivalents.

Experimental Animals

Two Jersey cows, approximately 6 years of age, were selected, as near alike in weight, size, hair coat, and milk production as possible. Both cows were in excellent health and were maintained on good quality feeds and average dairy conditions.

Methods

Each cow was sprayed with 2 liters of emulsion, using suitable precautions for protection of personnel. Material in excess of that held in the hair was collected and its quantity determined radiometrically.

The cow receiving the 0.5% spray retained 16.03 mg. per kg., while the one receiving 0.75% retained 22.9 mg. per kg.

Each cow was milked by machine behind a protective shield of plastic sheeting. The operator was specially trained to handle the necessary procedures in a minimum of time.

Milk samples were collected at each milking, beginning 5 hours after spraying through the first week, then from the morning milk only at intervals through the 25th day. Weight of the milk produced was recorded and the percentage of butterfat was determined at each sampling time.

Total phosphorus-32 was determined on the whole milk samples. Organo-soluble compounds containing phosphorus-32 were determined following extraction.

Extraction. Two hundred milliliters of the milk sample was diluted with an equal volume of ethyl alcohol and extracted three times with a 75 to 25 *n*-hexane–ethyl ether mixture, using 200 ml. of the ether mixture for the first extraction and 100 ml. each for the other two extractions. The ether extracts were combined and washed with 125 ml. of water. The extracts were dried over anhydrous sodium sulfate and filtered. The ether was removed as completely as possible and the fatty residue was dissolved in 200 ml. of *n*-hexane and extracted in a separatory funnel with four 50-ml. portions of acetonitrile. Each portion of acetonitrile was drained through another separatory funnel containing 50 ml. of *n*-hexane. The acetonitrile was distilled off to a volume of 10 ml. The residue was transferred to a 125-ml. glass-stoppered Erlenmeyer flask with a side stem calibrated at 5 ml. using 50 ml. of *n*-hexane to make the transfer. The solvent was evaporated on a steam bath, or hot plate at low heat, to a volume of less than 5 ml. The volume was then made up to exactly 5 ml. with benzene and the flask stoppered tightly to prevent evaporation of the solvent. Aliquots were taken from this 5-ml. volume for radiometric assay. Recoveries of radioactive Co-Ral were essentially 100% by this method.

Results

Milk yield, butterfat percentage, total phosphorus-32, extractable labeled material, and percentage extracted are given in Table I. The greatest excretion of organo-soluble material was observed at the 5-hour sampling, being approximately 0.2 p.p.m. for the 0.5% spray and 0.25 p.p.m. for the 0.75% spray. The quantity declined steadily throughout the next 14 days. A greater percentage of the total radioactivity was extracted at the 5-hour period than at any later time. The extractable portion declined rapidly in the first 48 hours, then more slowly during the remaining time.

The total organo-soluble material was not sufficient for our paper chromatographic identification; therefore, it can only be presumed that a portion of the extractive was Co-Ral.

An experiment has been described (*4*) in which a Holstein cow was sprayed with a 0.75% concentration of Co-Ral and the total radioactivity in milk was similar to that obtained in our study. The authors report a very small fraction of this total as Co-Ral, the highest value being equivalent to that obtained by us 48 hours after treatment. Those results are not necessarily contradictory of our findings, since the formulation, spraying, and animals differed considerably between the two tests.

Total phosphorus-32 in the milk increased rapidly during the first 48 hours, then declined gradually during the following 3 weeks.

Conclusions

Co-Ral, labeled with phosphorus-32, applied dermally to lactating dairy cows, results in excretion of phosphorus-32 in the milk in both organo-soluble and organo-insoluble forms. Significant quantities of both forms appear in milk within 5 hours after the treatment. The maximum quantity of organo-soluble material was found 5 hours post-treatment, the amount declining rapidly. Organo-insoluble phosphorus-32 reached maximum levels 48 hours post-treatment, then declined slowly over a 3-week period.

Total organo-soluble substances, calculated but not identified as Co-Ral, did not exceed 0.2 p.p.m. for a 0.5% spray nor 0.25 p.p.m. for a 0.75% spray.

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INSECTICIDE RESIDUES

Meat and Milk Residues from Livestock Sprays

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and

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Before an insecticide can be recommended for use on livestock, studies must be made to determine whether it will contaminate meat and dairy products. Results of residue studies show that all the chlorinated hydrocarbon insecticides, Co-Ral, and malathion were excreted in the milk after spray treatments. Studies were also made on DDT, TDE, methoxychlor, chlordan, gamma chlordan, heptachlor, dieldrin, lindane, Strobane, toxaphene, malathion, and Co-Ral in the fat of beef cattle following spray treatments.

CHLORINATED hydrocarbons and some of the phosphorus insecticides are fat-soluble and when sprayed on animals may be absorbed through the skin and stored in fatty tissues or they may also be excreted in milk. Therefore, before an insecticide can be recommended for use on livestock, it must be determined whether the dosages used will contaminate meat and milk. The U. S. Department of Agriculture has supported studies on residues in meat and milk at Kerrville, Tex., for the last 12 years. These studies have shown that a number of effective insecticides cannot be recommended for use on livestock because of their residues.

Several of these studies have already been reported in the literature (*4, 6, 7, 17*). The purpose of this paper is to summarize them and, where possible, compare the residues in both meat and milk resulting from the use of various insecticides under analogous conditions.

If the insecticides are stored in the animal's body, they will be found in fatty tissues and excreted in the butterfat of milk because of their solubility in fats and insolubility in water. The small concentrations found in other tissues can usually be attributed to the fat content of the tissue (*17*). In a sample of milk the amount of insecticide is proportional to the amount of butterfat.

In some instances, it is not enough to analyze for the insecticide used, because contamination may be caused by the storage of toxic metabolites in tissues. Davidow and Radomski (*9*) have shown that heptachlor (*3a*,4,5,6,7,8,8 - heptachloro - 3*a*,4,7,7*a* - tetrahydro - 4,7-methanoindene) is converted within the animal's body to heptachlor epoxide, which is stored in fatty tissues. Bann *et al.* (*2*) presented evidence showing that aldrin (1,2,3,4,10,10 - hexachloro - 1,4,4*a*, 5,8,8*a* - hexahydro - 1,4 - *endo* - *exo*-5,8 - dimethanonaphthalene) is converted by metabolic processes to dieldrin (1,2,3,4,10,10 - hexachloro - 6,7 - epoxy-1,4,4*a*,5,6,7,8,8*a* - octahydro - 1,4-*endo* - *exo* - 5,8 - dimethanonaphthalene), which is stored in fat.

Although it has complicated chemical analysis, the storage of insecticides in the fat has simplified residue studies. With the biopsy technique developed by Radeleff (*15*), it has been possible to take samples of abdominal fat from the same animals at various intervals after spray treatments. This has reduced the number of experimental animals needed and has permitted following the level of insecticides in the fat of individual animals.

The analytical methods used were found to give satisfactory recoveries before any test samples were analyzed.

Various solvents were used for extractions, but no extraction procedure was considered satisfactory that did not extract fat from the tissues or butterfat from milk.

The spray concentrations used were those determined to be the most practical for insect control. Both single and multiple treatments representing frequent and prolonged or seasonal applications were included.

Experimental

Meat Contamination. Hereford calves were used for most of the meat contamination studies. Except where otherwise specified, they were in good condition and fed on a fattening diet during the experiments. Fat samples were taken from all animals before

Conclusions

Co-Ral, labeled with phosphorus-32, applied dermally to lactating dairy cows, results in excretion of phosphorus-32 in the milk in both organo-soluble and organo-insoluble forms. Significant quantities of both forms appear in milk within 5 hours after the treatment. The maximum quantity of organo-soluble material was found 5 hours post-treatment, the amount declining rapidly. Organo-insoluble phosphorus-32 reached maximum levels 48 hours post-treatment, then declined slowly over a 3-week period.

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Table I. Residues in Fat of Cattle Following a Single Spray Treatment with Various Insecticides

Insecticide	Dosage, %	Time Interval,[a] Weeks	Maximum Residue, P.P.M.	Duration of Detectable Residues, Weeks	Reference
DDT	0.5	2	11.2	>27	(*4*)
TDE	0.5	2	11.0	>27	(*4*)
Methoxychlor	0.5	2	2.8	<10	(*4*)
Lindane	0.03	2	<2.5	...	(*4*)
	0.075 dip	1	21.8	8	
	0.075	1	17.3	8	
Co-Ral	0.5	1	0.40[b]	2	

[a] Time after spraying.
[b] These results were corroborated by Chemagro Corp. using method described by Anderson (*1*).

Table II. Residues in Fat of Cattle Following Multiple Spray Treatments with Various Insecticides

Insecticide	Dosage, %	Number of Sprays	Interval, Weeks	Time of Maximum Residue,[a] Weeks	Maximum Residue, P.P.M.	Duration of Detectable Residues, Weeks	Reference
Gamma chlordan	0.5	6	2	2	24.0	<16	(*4*)
Chlordan	0.5	6	2	2	20.7	>16	(*4*)
DDT	0.5	6	3	3	35.2	>36	(*4*)
Dieldrin	0.05	4	3	3	24	>28	(*4*)
Heptachlor	0.5	6	2	2	19.3	>16	(*4*)
Lindane	0.03	6	3	3	<2.5	...	(*4*)
Methoxychlor	0.5	6	3	3	2.4	<12	(*4*)
TDE	0.5	6	3	3	28.4	>36	(*4*)
Strobane	2.0	6	2	2	28.5	>14	(*4*)
	0.5	12	2	2	6.6	>6	(*4*)
Toxaphene	0.5	12	2	2	16.0	6	(*4*)
Malathion	0.5	16	1	1	<0.5	...	(*5*)

[a] Time after last spray treatment.

treatment. Also, a group of control animals were used in each experiment.

The spraying and dipping operations were carried out with emulsions or suspensions of an appropriate chemical. Suspensions were prepared from wettable powders supplied by the manufacturers, and emulsions from both laboratory and commercially supplied concentrates. In spraying, each animal was wet as thoroughly as possible. In dipping, the entire animal was submerged. Residues were determined from single spray treatments and from multiple treatments that simulated the continuous use of the insecticides during a season of insect control.

Single Treatments. Residue studies from single spray applications were made on DDT, TDE, methoxychlor, lindane, Co-Ral [*O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate], and Delnav [2,3-*p*-dioxanedithiol *S,S*-bis (*O,O*-diethyl phosphorodithioate)].

DDT AND TDE. They were determined by the method described by Schechter, Pogorelskin, and Haller (*19*), methoxychlor by the method described by Claborn and Beckman (*5*), lindane by the method of Davidow and Woodard (*10*) or by the method of Schechter and Hornstein (*18*). Co-Ral was determined by the method of Claborn *et al.* (*6*) and by radiochemical analysis using the phosphorus-32–labeled insecticide. Delnav was determined by radiochemical analysis using phosphorus-32–labeled Delnav.

A summary of these results is shown in Table I. References to previously published material are given in the tables. Other experiments which could not be tabulated are briefly described below.

LINDANE. Twelve hogs were sprayed with 0.06% lindane, six with an emulsion, and six with a suspension. Two untreated animals were kept for controls. One hog from each group was slaughtered at 1, 2, 4, and 6 weeks after spraying, and fat samples were taken for analysis. In the group sprayed with the suspension, the lindane residue in the fat was 0.47 p.p.m. after 1 week, 0.14 p.p.m. after 4 weeks, and none could be detected after 6 weeks. In the emulsion-sprayed group, 0.66 p.p.m. was found after 1 week, but none after 4 weeks.

Four sheep and four goats were dipped in a 0.025% suspension of lindane. Fat samples were taken from all animals before dipping, and from alternate pairs at 2-week intervals after treatment. The lindane residue averaged 4.22 p.p.m. after 2 weeks, decreased to 0.26 p.p.m. at 8 weeks, and could not be detected at 12 weeks.

CO-RAL. Two calves were sprayed with phosphorus-32–labeled Co-Ral. One animal, sprayed with 25 mg. per kg. and slaughtered after 1 week, had an apparent Co-Ral residue of 0.12 p.p.m. in the fat. The other animal, sprayed with 58 mg. per kg. and slaughtered 2 weeks later, had only 0.04 p.p.m. in the fat. Higher residues were indicated in the kidney and liver, but apparently these residues were not Co-Ral, but some phosphorus-containing metabolites.

DELNAV. A calf was sprayed with 16.2 mg. per kg. of phosphorus-32–labeled technical Delnav. The animal was slaughtered 7 days after treatment and tissues were taken for analysis. Radioactivity counts indicated the following residues in the tissues: omental fat, 1.51 p.p.m.; renal fat, 1.07 p.p.m.; muscle, 0 p.p.m.; liver, 0.20 p.p.m.; and kidney, 0.07 p.p.m.

Multiple Treatments. Studies of residues resulting from multiple spray treatments were made on DDT, TDE, methoxychlor, lindane, chlordan (1,2,4,-5,6,7,8,8 - octachloro - 2,3,3*a*,4,7,7*a*-hexahydro-4,7-methanoindene), gamma chlordan, heptachlor, dieldrin, Strobane (a mixture of chlorinated terpenes with about 66% chlorine), toxaphene, and malathion {*S* - [1,2, - bis(ethoxycarbonyl)ethyl] *O,O*-dimethyl phosphorodithioate}.

The analytical methods for the first four insecticides were the same as indicated above, and malathion by the method described by Norris *et al.* (*13*), but the residues from all the other compounds were estimated from total chlorine analysis. The insecticides were extracted from the fat with hot nitromethane, the organically bound chlorine was reduced with sodium, and the chlorides were titrated amperometrically with silver nitrate.

The results from these studies are summarized in Table II. Again, other experiments which could not be tabulated are briefly described below.

EXCESSIVE TREATMENTS WITH DDT. Earlier experiments had shown that the storage of DDT in the fat reached a maximum after six applications. An experiment was then run to determine the residues that would result from spray treatments over an extended period of time (*17*). Fat samples were taken from six 6-month-old calves with unknown history and analyzed for DDT. Before treatment, the DDT content ranged from 5 to 75 p.p.m. and averaged 45 p.p.m. The calves were sprayed five times with 0.5% DDT emulsion at 3-week intervals and then, after 5 days, were given 28 treatments at 2-week intervals. The DDT content of the fat samples taken 2 weeks after the last treatment ranged from 76 to 89 p.p.m. and averaged 84 p.p.m. The one animal that had 75 p.p.m. at the start had only 81 p.p.m. after 33 additional sprayings.

Another group of six calves, fat samples from which were negative for DDT

before treatment, were sprayed 28 times at 2-week intervals. Fat samples taken from five of the animals 2 weeks after the last treatment contained from 80 to 103 p.p.m. of DDT, and averaged 88 p.p.m., 4 p.p.m. higher than for the first group.

COWS AND CALVES SPRAYED WITH DDT. Eight Hereford cows with young calves were used for this experiment. All the cows and four of the calves were sprayed five times at 28-day intervals with 0.5% DDT, half of these animals with an emulsion and the other half with a suspension. The other four calves were not sprayed. Each calf lived upon the milk supplied by its mother. All the animals were slaughtered 28 days after the last spraying. An average of 52 p.p.m. of DDT was found in the fat from the calves that were getting DDT by both absorption and ingestion, and 25 p.p.m. from those that were getting it only from the milk. More DDT was stored in the fat of the calves that were not sprayed than in the fat of their mothers, which averaged only 15 p.p.m.

Four calves in good condition were sprayed with 0.5% emulsion of chlordan 12 times at 2-week intervals. Four other calves in very poor condition were given the same treatment. Fat samples were taken 2, 8, and 16 weeks after the last spraying. Two weeks after the last spraying the poor cattle had 28 p.p.m. of chlordan in the fat, and the fat cattle had 21 p.p.m. Fourteen weeks later the residues were 3.9 and 4.5 p.p.m., respectively.

Milk Contamination

The dairy cows used were Jerseys in full lactation. They were sprayed all over, 2 quarts for each cow. They were milked by machine and the samples taken from the full yield. Care was taken to avoid mechanical contamination of the milk. The butterfat of each sample was determined and the results were calculated and adjusted to a uniform butterfat content of 4%. Milk samples were taken from each cow before spraying and at intervals after treatment, and untreated animals were included in each experiment.

Residue studies were made on milk from cows sprayed with DDT, dieldrin, methoxychlor, Dilan—1 part of 1,1-bis(*p*-chlorophenyl) - 2 - nitropropane plus 2 parts of 1,1 - bis(*p* - chlorophenyl)-2 - nitrobutane — malathion, Strobane, toxaphene, Co-Ral- and Perthane [1,1-dichloro - 2,2 - bis(*p* - ethylphenyl)-ethane].

The method used for the determination of DDT is described by Schechter *et al.* (*19*); the total chlorine method for dieldrin, by Carter (*3*); the method for methoxychlor, by Prickett *et al.* (*14*); for Dilan, by Jones and Riddick (*11*, *12*), with modification by Cundiff (*8*); for malathion, by Norris *et al.* (*13*); and for Perthane, by Weiss (*20*). Co-Ral was determined by a radiochemical method using phosphorus-32–labeled Co-Ral, and toxaphene and Strobane by the total chlorine method described under Meat Contamination.

Table III. Residues in Milk from Cows Sprayed with Various Insecticides

Insecticide	Concentration of Spray, %	Time of Maximum Residue,[c] Days	Maximum Residue, P.P.M.	Duration of Detectable Residue, Days	Reference
DDT	0.5[a]	2	2.8	>21	(*6*)
Dieldrin	0.5[a]	3	7.0	>21	(*6*)
Methoxychlor	0.5[a]	1	0.70	<21	(*6*)
	0.5[b]	2	0.44	<21	(*6*)
Dilan	0.5[b]	2	0.75	>14	(*6*)
Malathion	0.5[a]	5[d]	0.08	3	(*5*)
	1.0[a]	5[d]	0.20	3	(*5*)
	0.5[b]	5[d]	0.27	3	(*5*)
	1.0[b]	5[d]	0.36	3	(*5*)
Strobane	0.5[a]	1	0.74	14	(*4*)
	0.5[b]	2	0.61	14	(*4*)
Toxaphene	0.5[a]	2	0.61	14	(*4*)
	0.5[b]	2	0.74	14	(*4*)
Co-Ral	0.5[a]	5[d]	0.20	14	(*16*)
	0.75[a]	5[d]	0.25	14	(*16*)
Perthane	0.5[a]	3	0.17	<21	(*4*)

[a] Emulsion.
[b] Suspension.
[c] Time after spraying.
[d] Hours.

The results of these studies are summarized in Table III. Other experiments, which for various reasons could not be tabulated, are described separately.

An experiment with methoxychlor was conducted on a herd basis, since there had been some evidence with other insecticides that lower residues result when larger groups of animals are sprayed with commercial equipment. Two herds were sprayed with a 0.5% suspension of methoxychlor. The samples were analyzed by the method described by Claborn and Beckman (*5*). Pooled milk samples from one herd contained 0.18, 0.13, 0.11, and 0.05 p.p.m. of methoxychlor after 1, 2, 3, and 5 days, and less than 0.05 p.p.m, after 7, 14, and 21 days. Similar data were obtained on another herd.

In other experiments, two dairy cows were sprayed twice daily with 1 ounce of a 2% oil solution of toxaphene and two cows were sprayed in the same manner with Strobane. Samples of milk were taken at frequent intervals during the 21-day spray period. The two insecticides caused about the same contamination. On the third day after the first spraying, the contamination reached 0.3 p.p.m. and, with few exceptions, remained at this level for the remainder of the 21 days.

Acknowledgment

The authors express their appreciation to the Hercules Powder Co. and to the Humble Oil and Refining Co. for their cooperation in the toxaphene studies; to the B. F. Goodrich Chemical Co. for its cooperation in the Strobane studies; to the Velsicol Chemical Corp. for its cooperation in the chlordan, gamma chlordan, and heptachlor studies; to the American Cyanamid Co. for its cooperation in the malathion studies; and to the Chemagro Corp. for its cooperation in the studies on Co-Ral.

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INSECTICIDE RESIDUES

Determination of Heptachlor and Heptachlor Epoxide in Soil

R. T. MURPHY and W. F. BARTHEL

Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture, Gulfport, Miss.

An improved method is described which makes possible the determination of 0.01 p.p.m. of heptachlor and heptachlor epoxide in soil with satisfactory accuracy. Soil from plots treated with 0.25, 0.50, 1, and 2 pounds per acre of heptachlor was analyzed 3 and $5^1/_2$ months after treatment. A second group of samples was taken after 8 months from an area which was treated with two different granular formulations. A third group of samples was taken in a treated area where biological control was incomplete, in an attempt to correlate chemical and biological data. The initial loss of insecticide from the soil appears to be rapid, and is followed by a much slower loss and conversion of a portion of heptachlor to heptachlor epoxide.

RECENT WORK in this laboratory demonstrated a need for a method to determine quantitatively heptachlor and heptachlor epoxide residues as low as 0.01 p.p.m. in soil. Since this is below the limits of present published methods (*2*, *4*, *10*), a study was undertaken to find a more sensitive procedure.

There were two possible approaches to the problem. The first would involve finding a reagent capable of yielding a more intense color than does the Polen-Silverman reagent (*7*) for both heptachlor and its epoxide. Davidow's (*1*) and Radomski's (*8*) work was in this direction, but did not attain the necessary sensitivity. The second approach would make use of a much larger soil sample with suitable elimination of the interfering substances. The latter method was selected for this study.

Table I. Insecticide Residues[a] of Four Different Dosages of Heptachlor after 3 and $5^1/_2$ Months; Series I

Lb./	3 Months[b]			3 Months[c]			$5^1/_2$ Months[c]		
Acre	Hept.	Epox.	Total	Hept.	Epox.	Total	Hept.	Epox.	Total
$^1/_4$	<0.1	0.2	0.2	<0.01	0.16	0.16	<0.01	0.17	0.17
	<0.1	0.2	0.2	<0.01	0.12	0.12	0.01	0.17	0.17
Av.	<0.1	0.2	0.2	<0.01	0.14	0.14	0.01	0.17	0.17
$^1/_2$	<0.1	0.2	0.2	<0.01	0.24	0.24	0.01	0.26	0.26
	0.1	0.2	0.2	<0.01	0.12	0.12	0.01	0.27	0.28
Av.	0.1	0.2	0.2	<0.01	0.18	0.18	0.01	0.26	0.27
1	0.1	0.6	0.7	0.04	0.62	0.66	0.04	0.67	0.71
	0.1	0.4	0.5	0.05	0.33	0.38	0.03	0.33	0.37
Av.	0.1	0.5	0.6	0.05	0.48	0.52	0.03	0.50	0.54
2	0.2	0.8	1.0	0.20	0.78	0.98	0.07	0.86	0.75
	0.3	0.9	1.2	0.28	0.86	1.14	0.08	1.06	1.14
Av.	0.3	0.8	1.1	0.24	0.82	1.06	0.08	0.96	1.04

[a] All values represent p.p.m. in the top inch of soil.
[b] Data from use of 50-gram soil samples without acid wash.
[c] Data from use of 250-gram soil samples with acid wash.

Experimental

Series I. Ten plots measuring 200 × 400 feet were set up in a large pecan grove. Two of these were not treated and were used as check plots throughout the experiment. The others were treated in duplicate with 0.25, 0.50, and 1 pound of heptachlor per acre, using 10 pounds of 2.5, 5, and 10% heptachlor granules, respectively, and with 2 pounds of actual heptachlor per acre, using 20 pounds of granules which contained 10% of heptachlor. The granules were applied by a Buffalo turbine mounted on the back of a jeep. The plots were treated on May 25, 1959, and sampled on August 29 and on November 2, 1959, 3 and $5^1/_2$ months, respectively, after treatment. Results are given in Table I.

Series II. An airfield of approximately 500 acres was divided into two treatment areas of similar size. Five 1-acre plots were selected throughout each treatment area, from which the soil samples were taken. Before treatment, blank samples were taken from these plots for use on the recovery curve. On one treatment area, a granular formulation containing 10% oil and 10% heptachlor was applied at 10 pounds per acre. On the other treatment area, granules which were formulated with no oil were applied at the same dosage. After 8 months, the five plots of each area were sampled and the residues were determined.

Series III. Samples were also taken from plots of another location which was treated with 10% oil-containing granules at the same time and dosage as in Series II. At the time of sampling, active ant mounds were found in the treated area. These mounds appeared to be in rows parallel to the line of flight taken by the aircraft while applying the insecticide. Samples were taken along

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Av.	<0.1	0.2	0.2	<0.01	0.14	0.14	0.01	0.17	0.17
$^1/_2$	<0.1	0.2	0.2	<0.01	0.24	0.24	0.01	0.26	0.26
	0.1	0.2	0.2	<0.01	0.12	0.12	0.01	0.27	0.28
Av.	0.1	0.2	0.2	<0.01	0.18	0.18	0.01	0.26	0.27
1	0.1	0.6	0.7	0.04	0.62	0.66	0.04	0.67	0.71
	0.1	0.4	0.5	0.05	0.33	0.38	0.03	0.33	0.37
Av.	0.1	0.5	0.6	0.05	0.48	0.52	0.03	0.50	0.54
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Av.	0.3	0.8	1.1	0.24	0.82	1.06	0.08	0.96	1.04

[a] All values represent p.p.m. in the top inch of soil.
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Series III. Samples were also taken from plots of another location which was treated with 10% oil-containing granules at the same time and dosage as in Series II. At the time of sampling, active ant mounds were found in the treated area. These mounds appeared to be in rows parallel to the line of flight taken by the aircraft while applying the insecticide. Samples were taken along

these rows of ant mounds to see if it could be demonstrated that skips in application could be chemically detected. Results are shown in Table II.

Reagents

Heptachlor standard solution containing 10 γ per ml. of pentane made from analytical grade heptachlor (Velsicol Chemical Corp., 300 East Grand Ave., Chicago 11, Ill.).

Heptachlor epoxide standard solution containing 10 γ per ml. of pentane made from analytical reference grade heptachlor epoxide (Velsicol Chemical Corp.).

Polen-Silverman reagent (*7*), prepared at least one month in advance, using the method suggested by Ordas *et al.* (*5*).

Benzene–isopropyl alcohol mixture, containing 4 parts of benzene to 1 part of isopropyl alcohol.

Pentane, pure grade (Phillips Petroleum Co.), further purified by the method suggested by Polen (*6*). This method involves passing 10 liters of pentane through a 5-liter percolator containing 500 grams of 60/100-mesh activated Florex (Floridin Co.) between two plugs of glass wool. The first and last 500 ml. are discarded and the middle fraction is collected and stored in glass containers. The Florex was previously activated for 48 hours at 135° C.

Carbon black, technical lamp black, Monsanto.

Fuming sulfuric acid, 7%, made by adding 1 part of concentrated sulfuric acid to 1 part of 15% fuming sulfuric acid. Caution: exothermic reaction.

Special Equipment

Extraction tumbler suitable to rotate concentrically eight 1-gallon paint cans held in a horizontal position.

Snyder distilling column, three-bulb, borosilicate glass, with 24/40 ground-glass joints.

Chromatographic columns of the type described previously (*9*), with the exception that a coarse-porosity sintered-glass disk was necessary.

Constant temperature oil bath (100° C.) equipped with rack to hold at least eight test tubes.

Centrifuge tubes, borosilicate glass, 15-ml., graduated in 0.1 ml., which were used as reaction tubes.

Bausch & Lomb Spectronic 20 or an equivalent spectrophotometer which is suitable for reading wave lengths of 567 and 410 mμ.

One-half-gallon fruit jars, equipped with standard snap lids and rings.

Sampling Procedure

Fifty cores were taken diagonally across each plot, using a 2-inch diameter auger set for a 2-inch depth. This method was substituted for random sampling to obtain a more representative sample. Since the plots were treated in strips, small overlaps and skips that could occur would be included in the sample. Random sampling would not give consistently representative samples with this type of treatment without resorting to a much greater number of cores with resultant handling problems.

As only a 1-inch core was needed, every core was measured and cut with a knife exactly 1 inch below the ground level. The lower part of the core was discarded, except random samples which were saved in separate containers. Two sources of error made it necessary to take deeper cores than needed. First, the grass formed a pad of inconsistent thickness, and second, the grass roots made it impossible to break the core at the required depth. These two errors combined could result in as much as a 50% error, using an auger set for a 1-inch depth. Taking cores of greater length and cutting them overcame these difficulties. The cores were then placed in 25-pound lard cans for storage until extraction.

The mixing procedure was accomplished by manually grating the moist cores through $^1/_4$-inch wire mesh, making sure to strip the soil from the grass and roots as completely as possible. The grass and roots were discarded and the soil was collected in 25-pound lard cans. It was mixed further by hand-stirring and by sieving through wire mesh a second time.

Analytical Procedure

The moisture content was determined by drying a weighed amount of soil on the steam bath and reweighing. For analysis, an unheated sample of 500 grams (based on the dry weight of the soil) was placed in a 0.5-gallon jar and 1000 ml. of a 3 to 1 mixture of pentane and isopropyl alcohol was added. The jars were tightly sealed and placed on the extraction tumbler for 4 hours. After removal from the tumbler, they were kept sealed until analysis was begun.

Five hundred milliliters of the extract was filtered into a 1000-ml. Erlenmeyer flask, which was then fitted with a Snyder column and the extract evaporated on the steam bath to about 250 ml. It was then transferred quantitatively to a 500-ml. separatory funnel, where it was washed twice with 200-ml. portions of distilled water to remove the isopropyl alcohol. Any emulsions which formed were broken by the addition of small amounts of a saturated sodium bicarbonate solution. The pentane layer was then dried, in a 500-ml. Erlenmeyer flask, over anhydrous sodium sulfate.

It was then poured into a clean, dry 500-ml. separatory funnel, and the volume increased to 300 ml. with pentane. Thirty-five milliliters of 7% fuming sulfuric acid was added slowly and the separatory funnel was shaken moderately for 1 minute. After separation, the sulfuric acid layer was drawn off. Another 35-ml. portion of fuming sulfuric acid was added, and the mixing process was repeated. Vigorous shaking causes emulsions which are difficult to break. After the second sulfuric acid wash there may be a small amount of emulsion left in the separatory funnel. This may be broken with the careful addition of water, one drop at a time, making sure to allow for the dissipation of heat between drops. Caution: Never attempt this addition of water if more than 1 inch of emulsion is present in the bottom of the separatory funnel.

When the emulsion was completely broken and the sulfuric acid layer was discarded, the pentane layer was washed with two separate 150-ml. portions of distilled water. After discarding the water, the pentane layer was dried over anhydrous sodium sulfate in a 500-ml. Erlenmeyer flask. The dried sample was then decanted into another 500-ml. Erlenmeyer flask, and the sodium sulfate was washed with pentane several times.

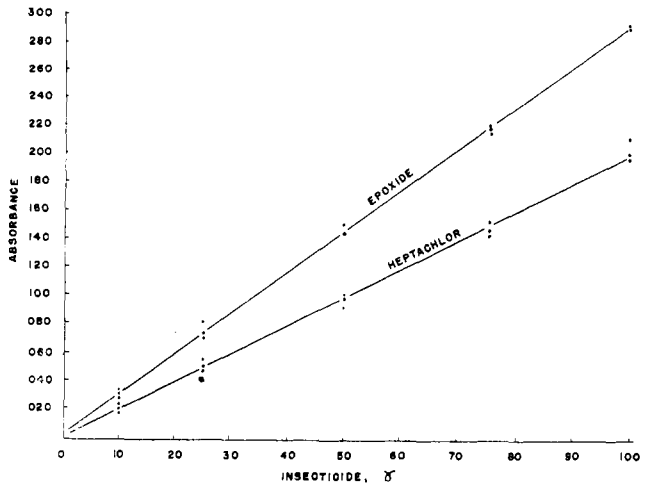
Table II. Determination of Heptachlor and Heptachlor Epoxide in Soil from Series of Field Plots[a]

Plot No.	Heptachlor, P.P.M.	Epoxide, P.P.M.	Total,[b] P.P.M.	% of Original
	Series II (without oil)			
1	0.25	0.15	0.40	13
2	0.13	0.14	0.27	9
3	0.11	<0.1	0.16	4
4	0.18	0.15	0.33	11
5	0.54	0.23	0.77	26
			Av.	12
	Series II (with oil)			
1	0.32	0.20	0.52	17
2	0.18	<0.1	0.23	8
3	0.17	<0.1	0.22	8
4	0.13	0.18	0.31	10
5	0.13	0.13	0.26	9
			Av.	10
	Series III (with oil)			
1	<0.1	0.40	0.45	15
2	0.10	0.36	0.46	15
3	0.10	0.16	0.26	9
4	0.22	0.28	0.50	17
			Av.	14
	Series III (skips in application)			
1	0.10	0.18	0.28	9
2	<0.1	<0.1	0.10	2
3	0.11	<0.1	0.16	4
			Av.	5
	Checks[c]			
1	0.12	0.92	1.04	95
2	0.14	1.00	1.14	104
3	0.50	0.47	0.97	97
4	0.48	0.53	1.01	101
5	0.17	0.25	0.42	105
6	0.22	0.23	0.45	112
			Av.	102

[a] 50-gram soil samples were used for analysis with the sulfuric acid wash eliminated, giving a lower limit of 0.1 p.p.m.

[b] In calculation of total p.p.m., the figure <0.1 was given an arbitrary value of 0.05 p.p.m.

[c] Following amounts of insecticide added to blank soil for checks: checks 1 and 2, 0.10 p.p.m., heptachlor and 1.00 p.p.m. heptachlor epoxide; 3 and 4, 0.50 p.p.m. heptachlor and 0.50 p.p.m. heptachlor epoxide; 5 and 6, 0.20 p.p.m. heptachlor and 0.20 p.p.m. heptachlor epoxide.



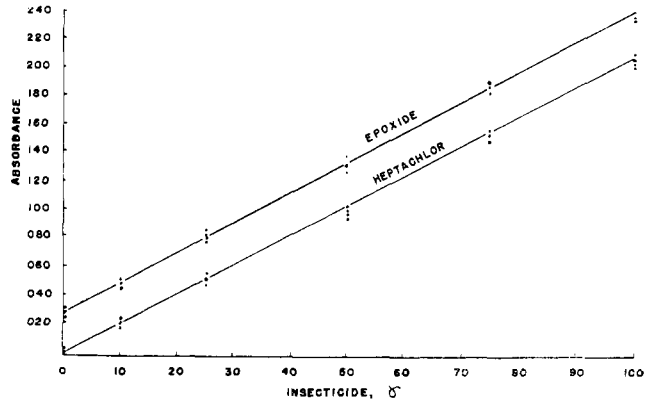


Figure 1. Standard calibration curve for heptachlor and heptachlor epoxide

The sample was then evaporated to approximately 20 ml. on the steam bath by using a Snyder column.

When the residues of both heptachlor and its epoxide are above 0.2 p.p.m., the fuming sulfuric acid wash may be eliminated from the procedure by using a 50-gram soil sample weight (100 ml.). If the acid wash is eliminated, the sample should be washed with water three times to remove all alcohol prior to introduction into the chromatographic column. Table I shows a comparison between samples run with and without the acid wash. The results in Table II were obtained without the acid wash.

The chromatographic columns were filled, using dry materials. A 1/4-inch layer of sodium sulfate next to the sintered-glass disk stopped the disk from becoming clogged with Florisil. Ten grams of Florisil (60/100-mesh, Floridin Co.) was poured into the column and gently tapped to smooth the top of the powder. Ten grams of 15 to 2 Florisil-carbon mixture was added next, with the same tapping procedure. On top of this was added another 5 grams of Florisil. A 1/2-inch layer of sodium sulfate was added to protect the column from any moisture or mixing action of the added solvents.

The column was washed with 100 ml. of 5% ether in pentane, followed by 100 ml. of pentane. The sample was then added and washed into the column with two 20-ml. portions of 2% benzene in pentane. As soon as the sample was added, the collection of the heptachlor fraction in a 500-ml. Erlenmeyer flask was begun. When the liquid level approached the top of the sodium sulfate layer, 100 ml. more of 2% benzene in pentane was added to the column and collected as part of the heptachlor fraction.

When the column again approached dryness, 225 ml. of 5% ether in pentane was added to elute the heptachlor epoxide. The flasks were changed immediately after this addition, and the heptachlor epoxide fraction was collected.

Both the heptachlor and heptachlor epoxide fractions were evaporated, using Snyder columns, to approximately 5 ml. on the steam bath. It is extremely important that the samples do not go to dryness on the steam bath, as the insecticide is lost quickly at this temperature. The samples were quantitatively transferred to graduated 15-ml. borosilicate glass centrifuge tubes, using pentane as the washing solvent. The volumes were then reduced to 0.5 ml. by evaporation in a 40° C. water bath using a gentle flow of dry air. One milliliter of Polen-Silverman reagent was added and the tubes were placed in a 100° C. constant-temperature bath for exactly 15 minutes. Then the tubes were removed and cooled under the tap to room temperature, with care not to allow water inside the tubes.

The volume was increased to 6 ml. with the benzene–isopropyl alcohol mixture. The heptachlor was read at the wave length of 567 mμ while the epoxide was read at 410 mμ. The readings were then compared with the recovery curves which were previously prepared by using blank soil samples containing known amounts of insecticide.

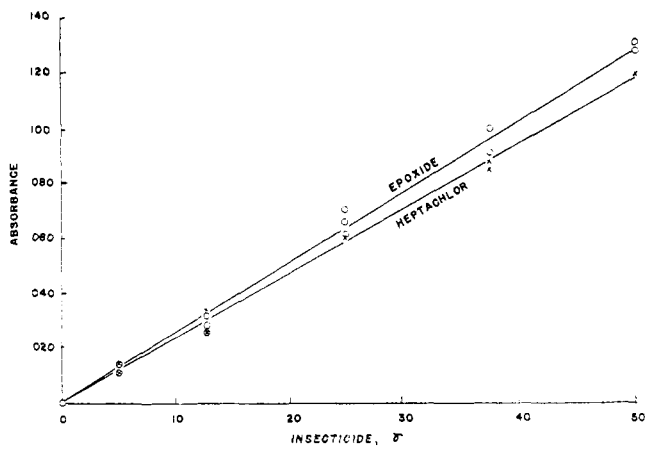


Figure 2. Recovery of heptachlor and heptachlor epoxide in 50 grams of soil

Figure 3. (Left). Recovery curves of heptachlor and heptachlor epoxide in 250 grams of soil

Calibration and Recovery Curves

A calibration curve was prepared by placing 1.0, 2.5, 5.0, 7.5, and 10.0 ml. of standard solutions of heptachlor and heptachlor epoxide in the graduated centrifuge tubes. They were evaporated and the color was developed exactly as the procedure described. The absorbance was then plotted against concentration (Figure 1).

A recovery curve was prepared by adding known amounts of the insecticides to the soil blanks. The samples were carried through the entire procedure described above. The amount of insecticide added, in micrograms, was plotted against the absorbance (Figures 2 and 3). Addition of known amounts of the insecticides to soil extract gave results identical with those obtained when the insecticides were added prior to extraction. Several types of soil were analyzed and all gave consistent values on blank determinations.

Unless a method of analysis can be provided which will completely eliminate color due to the reagents and the untreated soil, the use of a standard with accompanying standard curves as presented in this paper becomes of major importance. The purpose in preparing

a standard curve, using soil extract from untreated soil to which are added known amounts of heptachlor and epoxide, is to provide a reliable standard to which unknown readings may be compared. Consequently, in Figure 2, if the blanks from the soil were subtracted from the epoxide curve, the curve would go through the origin.

With the use of this technique an epoxide reading (Figure 2) must be greater than 0.030, with the soil used in this study, to be indicative of the presence of any epoxide at all. Values above 0.030 can be read directly as epoxide. The precision of this procedure is greater at higher epoxide levels, but this does not detract from its utility, as this limitation is characteristic of many methods. In Figure 3 the soil blank has been subtracted from the epoxide curve.

Discussion

The sampling procedure employed in these tests represents the best of several methods which were tried. At first, 3-inch-long cores were taken for analysis (*3*), but as the insecticide was found only in the top inch, it was diluted by a factor of 3, making the lower limit of the test too high to be of maximum value. Analysis of the second inch showed no trace of heptachlor or epoxide on any of the plots tested.

The mixing procedure was checked by extracting three samples from the same group of cores. The results compared within 5% of each other. Subsequent work on other soil experiments has verified the ability to obtain a uniform sample by this method. It is important that the soil be extracted while still moist. An experiment on loss of insecticide from stored soil samples showed as high as 25% loss in 10 days when the soil was allowed to dry. There is a negligible loss when the soil is stored in tightly sealed cans.

Certain factors in the operation of the columns were found critical. If the column is not packed too tightly, it will flow approximately 5 ml. per minute. This rate gives a clean separation in a relatively short time. Not more than four columns should be attempted at one time, as each column must be watched constantly to keep it from going dry.

The adsorbance of the Florisil does not appear to be uniform from one batch to another. For this reason, it is necessary to run preliminary tests to determine the exact polarity of solutions needed to elute the insecticide from the column for every new batch of Florisil.

An expedient in the development of color is the use of graduated centrifuge tubes as reaction tubes. These tubes allow for the adjustment of the final volume, so that the color concentration is within the limits of the curve. If it is necessary to use other than a 6-ml. total volume, the micrograms found should be multiplied by the fraction $X/6$, where X = milliliters of final volume.

Conclusions

The lower limit of the method is approximately 2.5 γ; the largest amount of soil which could be analyzed was 250 grams. The lowest concentration of insecticide in soil which could be accurately detected was 0.01 p.p.m.

The over-all results of the analyses shown in Table I are in agreement with the theory that a rapid loss of insecticide occurs immediately following application. They also show that once the insecticide becomes "fixed" in the soil there is very little loss, even in warm weather.

Table II shows no significant difference between residues left by two formulations. The check plots of series III show some disagreement with those in series II, perhaps caused by a more dense grass cover on series III plots.

In series III there was 64% less insecticide on the suspected skips than on the average of the treated plots. This shows that the described method is sufficiently sensitive to give correlation with biological data.

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INSECTICIDE RESIDUES

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W. F. BARTHEL, R. T. MURPHY, W. G. MITCHELL, and CALVIN CORLEY

Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture, Gulfport, Miss.

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In the current program for eradication of the imported fire ant, *Solenopsis saevissima richteri*, from 21,000,000 acres in nine southern states, the problem of the residual effectiveness of heptachlor when applied to soil is of considerable importance. Heptachlor is the most effective insecticide tested so far against the imported fire ant. Lofgren and Stringer (*4*) reported that heptachlor, on 24-hour exposure, has an LD_{50} for the imported fire ant of 0.04 p.p.m. in the soil, heptachlor epoxide, a metabolic product, has an LD_{50} of 0.015 p.p.m., while that for an alternate insecticide, dieldrin, was 0.045 p.p.m. Since these laboratory studies are so much at variance with recommended application rates (originally 2 pounds of heptachlor per acre, giving 6 p.p.m. in the top inch of soil, were recommended), a study was undertaken to determine what happened to heptachlor after application to the soil.

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The all-inclusive term "breakdown" has been used to account for loss of effectiveness of insecticide residues following soil treatment. This study was set up to determine the factors involved in loss of heptachlor residue and possible means of controlling this loss. Much of the loss can be accounted for by vaporization of the insecticide. A number of additives may be used to lower loss by vaporization. An increase in soil residues results from the use of some of the additives used.

Table I. Analysis of Soil for Heptachlor and Heptachlor Epoxide at Various Intervals, Following Granular Application to the Surface[a,b]

When Sampled	Insecticide Found, P.P.M. Heptachlor	Heptachlor epoxide	Total	Insecticide Remaining, %
1 week, 5-24-59	1.4	<0.1	1.4	24
1 month, 7-18-59	0.6	0.2	0.8	14
3 months, 9-15-59	0.2	0.4	0.6	10
6 months, 1-14-60	0.2	0.5	0.7	12

[a] 5.76 p.p.m. of heptachlor actually applied to top inch of soil. All concentrations are based on parts per million in top inch of soil.
[b] Date of application June 17 to 18, 1959.

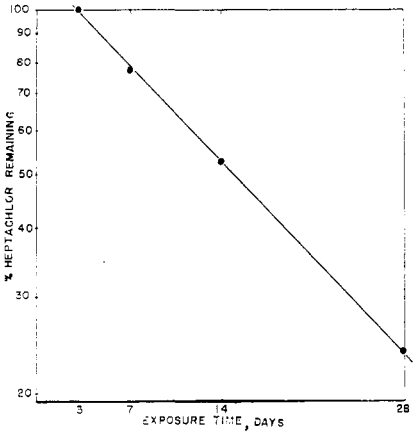

Figure 1. Volatilization of heptachlor from 10% heptachlor granules
Exposed at 70° to 100° F. on August 25, 1959

Table II. Insecticide[a] Remaining Following Application[b] of Heptachlor Granules

Solvent, %	Insecticide Remaining, % 1 week	1 month	3 months	6 months
5	24	12	15	14
10	24	14	10	12
20	24	16	11	12

[a] Heptachlor plus heptachlor epoxide.
[b] Applied June 17 and 18, 1959.

Table III. Percentage of Insecticide[a] Remaining in the Soil Following Granular Application[b] of Vegetable and Animal Fat Formulations with Heptachlor

Additive	Insecticide Remaining, % 1 week	1 month	3 months	6 months
Corn oil	32	12	12	15
Peanut oil	38	21	14	12
Neat's foot oil	32	16	18	18
Tallow	29	12	13	16

[a] Heptachlor plus heptachlor epoxide.
[b] Applied June 17 and 18, 1959, at the nominal rate of 1.25 pounds per acre.

Table IV. Effect of Additives on Insecticide[a] Remaining in Soil Following Granular Application of Heptachlor

Additive	Insecticide Remaining, % 1 week	1 month	3 months	6 months
Aroclor	44	32	29	34
Rosin	28	30	22	21
Heavy aromatic naphtha	24	14	10	12

[a] Heptachlor plus heptachlor epoxide.

it is not possible to determine their total recovery of insecticide, but it appears to be less than 10% of that applied, if they sampled the top 3 inches of the treated soil.

Young and Rawlins (*7*) made a study of persistence of heptachlor in the soil, when the insecticide was incorporated in the soil, and reported a loss of 74% from field test plots in 21 months. On surface application of heptachlor emulsion, they reported losses as high as 92% in the first week. They checked this work with bioassay and obtained good agreement between specific chemical methods and bioassay, although they did not determine the heptachlor epoxide.

In the author's first experiment, heptachlor was applied at a nominal rate of 2 pounds per acre [20 pounds of 10% of government specification (*1*) granules] to test plots. At intervals of 1 week and 1, 3, and 6 months, soil samples were taken from the treated plots and analyzed for heptachlor and heptachlor epoxide by the method of Murphy and Barthel (*5*). Results of this test are given in Table I.

The difference between the 3- and 6-month samples reflects the usual analytical variation. The data show that there is an initial rapid loss of heptachlor, followed by a long period in which there is little loss of insecticide and conversion to heptachlor epoxide of a portion of that remaining. The concentration of epoxide was almost constant after 3 months in this test.

When such a rapid loss of insecticide following granular application was noted, a study was made to determine the rate of loss of heptachlor from intact granules. For this study, 10-gram samples of 10% heptachlor granules were spread out over shallow pans and exposed to air under a metal roof, which protected them from rain, yet permitted free circulation of air. A recording thermometer indicated a variation in temperature between 70° and 100° F. The duration of the test was 28 days with two pans being taken for duplicate analyses at 3, 7, 14, and 28 days. The granules were weighed immediately before analysis, and the loss in weight corresponded to the loss of oil solvent plus insecticide. The data are plotted in Figure 1. The loss was apparently by volatilization which commenced as soon as the oil solvent used in the formulation had evaporated.

The loss from intact granules can largely be attributed to volatilization, and since it seemed to follow the loss of the oil solvent, an experiment was set up to determine the effect of various percentages of this oil on the loss of heptachlor, when the granules were applied to the soil. For this study, granules were formulated containing 5, 10, and 20% heavy aromatic solvent (alkylated naphthalenes) and 10% heptachlor on the usual Attapulgus clay granules. The nominal application rate was 12.5 pounds of these 10% granules per acre. The concentration in the top inch of soil in parts per million of heptachlor was calculated from the weight actually applied per square foot. After analysis, the percentage loss of insecticide could be determined.

In Table II, data are presented showing percentage of heptachlor plus heptachlor epoxide remaining in the soil following application of granules formulated with various amounts of oil on Attapulgus clay. The percentage of oil did not affect the loss of insecticide. This may mean that the oil evaporates

so rapidly that it has little effect on the long-term residue. In this study, and others, no heptachlor or heptachlor epoxide has been detected in the second inch of soil and leaching has been ruled out as a factor in the loss of heptachlor.

A second experiment was set up to determine the effect of some nonvolatile vegetable and animal oils on the residual life of heptachlor in the soil. It was thought that the presence of oils with low vapor pressures would tend to suppress the vapor pressure of the heptachlor. The materials used and results obtained are listed in Table III. In this work four vegetable and animal oils were substituted for mineral oil and formulated with heptachlor on Attapulgus clay at the rate of 10% heptachlor, 10% oil, and the balance clay and deactivator. Application and calculations were carried out as previously described.

The use of vegetable and animal oils gave a slightly greater residue of insecticide in the soil than that of mineral oils, but not as great as might be anticipated from the difference in vapor pressures. The apparent rapid loss of insecticide from the nonvolatile oil might be explained by considering that there is a partitional separation of the heptachlor from the oil on the soil surface followed by normal loss of insecticide by volatilization.

It was of interest to compare some nonvolatile solids as additives for the heptachlor formulation since these would keep partitional separation at a minimum. For this preliminary study, rosin and Aroclor 5460, a chlorinated biphenyl were used. Hornstein and Sullivan (*3*) had previously reported prolonged residual effects with lindane from the use of Aroclor 5460. Formulation of the additives and heptachlor with clay was carried out in the usual manner, except that a cosolvent, methylene chloride, was used. After formulation, the methylene chloride was allowed to evaporate. The heptachlor and solid additives each amounted to 10% of the finished formulation.

The retention percentages of insecticide, using these solid additives as compared with normal heavy aromatic naphtha solvent, are given in Table IV. The solid additives retained about twice the amount of insecticide on the soil as did the normal heavy aromatic naphtha.

Throughout the analytical studies one consistent and striking behavior of the residues was noted. The heptachlor epoxide content seldom exceeded 10% of the amount of heptachlor applied. This was also observed (*6*) in an earlier study involving the dosage rates from 0.125 to 2 pounds per acre.

In Table V are listed the heptachlor, the heptachlor epoxide, and the total concentration in parts per million remaining at the end of 6 months, as well as the parts per million of heptachlor applied to test plots using formulations with various additives. The original application was made on June 18 to 19, 1959. Samples were taken for analyses on January 14, 1960.

Table V. Heptachlor and Heptachlor Epoxide Content of Plots Treated with Granular Heptachlor after 6 Months

Additive	Heptachlor Applied in Top Inch, P.P.M.	Insecticide Remaining, P.P.M. Hept.	Hept. epox.	Total
Aroclor	4.05	1.03	0.36	1.39
Rosin	4.35	0.53	0.40	0.93
Neat's foot oil	4.98	0.49	0.39	0.88
Corn oil	4.05	0.22	0.39	0.61
Tallow	3.48	0.18	0.37	0.55
Peant oil	4.26	0.11	0.42	0.53
Heavy aromatic naphtha	5.76	0.18	0.50	0.68

The data in Table V show a 10-fold variation in heptachlor content without significant difference in heptachlor epoxide in the treated soil. This may indicate that the epoxide formed is proportional to the amount of heptachlor applied, while the amount of heptachlor remaining is dependent on many factors that may influence loss by volatilization.

One important factor affecting loss by volatilization is ground cover. To check this, two series of plots were examined. Both series were in Bahia pasture treated with 0.25, 0.50, 1, and 2 pounds of heptachlor per acre in duplicate plots of 1 acre each. Both series were sampled and analyzed after $4^1/_2$ months. The first series, located on the Moran pasture, was covered with short grass at the time of application. The other series on the Ritchie pasture was long grass in a pecan grove and provided good ground cover. Another difference in these plots, which was thought inconsequential at the time, was that the Moran plots were treated with 20- to 60-mesh clay granules, while the Ritchie plots were treated with 16- to 30-mesh clay granules. The finer mesh on the Moran plots may have favored more rapid loss because of the greater surface area of the granules. Rainfall and weather factors were similar.

In Table VI are data showing the heptachlor and heptachlor epoxide content of soil from these two series of plots. These data were further evidence that factors favoring volatilization of the heptachlor (poor ground cover, fine mesh granules) coincide with rapid disappearance of the insecticide from the soil. In the Ritchie series, the highest conversion to heptachlor epoxide was observed of any field test conducted so far in this work. The maximum conversion was from 15 to 20% of the amount of heptachlor applied.

Table VI. Analysis[a] of Soil from the Moran-Ritchie[b] Tests Following Granular Application of Heptachlor

Application, Lb./Acre	Residue After $4^1/_2$ Months, P.P.M. Hept.	Hept. epox.	Hept.	Hept. epox.
	Moran		Ritchie	
0.25	<0.01	0.03	<0.01	0.17
0.50	<0.01	0.02	<0.01	0.26
1	<0.01	0.04	0.03	0.50
2	0.01	0.08	0.08	0.96

[a] For comparison purposes 0.25 lb./acre of heptachlor application is 0.75 p.p.m. of heptachlor in top inch; 0.50 lb. is 1.5 p.p.m.; 1 lb. is 3 p.p.m.; and 2 lb. are 6 p.p.m. All values are based on samples from top inch.

[b] Moran plots treated on April 15, 1959; sampled on August 28, 1959. Ritchie plots treated on May 25, 1959; sampled on November 2, 1959.

An attempt was made to study the possibility of decomposition of heptachlor as a factor in its loss from soil. At the low concentrations studied, no soils could be found with a sufficiently low blank to permit the use of the total chloride method. The many total chloride analyses run in conjunction with the specific method indicated no apparent accumulation of decomposition products of heptachlor in the soil.

In this study, the major loss of heptachlor was concluded to be by volatilization. The other significant loss was by conversion of heptachlor to heptachlor epoxide. This was found to reach a maximum of 20% of the amount applied. Losses due to decomposition and leaching were too small to be detected by the methods of analysis used.

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Insecticidal Residues in Various Crops Grown in Soils Treated with Abnormal Rates of Aldrin and Heptachlor

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A Carrington silt loam soil was treated with aldrin and heptachlor at rates of 5 and 25 pounds per 5-inch acre. The soil and crops grown on it were analyzed by colorimetric analyses, paper chromatography, and a bioassay procedure for residues of the applied aldrin and heptachlor, or their oxidation products: dieldrin and heptachlor epoxide. Data are presented on radishes, beets, potatoes, onions, carrots, cucumbers, lettuce, Lima beans, peas, and alfalfa. The potentiality of translocation from soils into crop tissues is demonstrated under these abnormal conditions of high insecticide dosages. However, it appears likely that treatment of soil with insecticide levels normally used for insect control would yield none or only trace amounts of insecticidal residues within the crop tissues investigated.

During recent years various workers have considered insecticidal residues within crops which were grown in insecticide-contaminated soils (*1, 3, 5, 15, 16*). Recently (*6*) it was shown that under extreme experimental conditions several chlorinated hydrocarbon insecticides were translocated into pea vines from a quartz sand treated at 30 p.p.m.

To obtain more data under actual field conditions, loam plots were treated in 1958 and 1959 with aldrin and heptachlor. Various crops grown on these soils were analyzed for insecticidal residues.

Procedure

Soil Treatment and Sampling. In May 1958, twelve 30 × 40 foot plots (Carrington silt loam) were staked out, leaving 20-foot buffer strips in between. Two insecticides, two application rates, and two replicates each were used for a total of eight experimental plots. Aldrin (A) or heptachlor (H) was applied at rates of 5 and 25 pounds per 5-inch acre. The four remaining ones were used as controls, although they were treated with solvent-emulsifier solutions which differed from the aldrin or heptachlor formulations only in that the insecticides were absent. Application rates of 5 and 25 pounds per 5-inch acre were chosen in order to have residue levels within the range of the sensitivity of the analytical methods. It was felt that in such a way more accurate data might be obtained. In addition, it was intended to find out if higher soil residue levels (treatment with 25 pounds per acre) would result in relatively higher residues within the crops.

The insecticidal application involved a thorough mixing of a measured amount of an emulsifiable concentrate with 20 gallons of water. Each experimental plot was subdivided into five 6 × 40 foot strips in the south-north direction and five 8 × 30 foot strips in the east-west direction. Two-gallon quantities of the diluted emulsion were then spread as uniformly as possible with a sprinkling can over each of the 10 strips. This crosswise treatment was used to attain a more uniform application. Immediately after treatment all plots were rototilled to a depth of 4 to 5 inches.

Soils to which insecticides had been applied at 5 pounds per 5-inch acre in 1958 were retreated at the same rate in 1959. However, the treated areas in the second year of the experiment were reduced to 30 × 24 feet, because the remainder (30 × 16 feet) of each plot was in alfalfa which had been seeded one year previously. After retreatment of the four plots to which 5 pounds per 5-inch acre of aldrin or heptachlor had been applied in 1958, all 12 plots were rototilled to a depth of 4 to 5 inches.

Soil samples were collected in 1958 immediately after the insecticide application, as well as 2 and 4 months later. In 1959, however, samples were collected in the spring (after treatment) and fall only. The sampling area had a size of 30 × 24 feet which was not covered with alfalfa. Only the initial soil sample in 1958 was collected from the total area treated (30 × 40 feet).

Sampling was done with a soil auger, and 30 cores (3/4 inch in diameter, 6 inches long) were collected from each 30 × 24 foot area. Only the initial soil sample in 1958 consisted of 40 cores.

Crop Growth and Crop Sampling. In May 1958 and in May 1959 various crops were seeded in 30-foot rows of all treated and untreated plots. The crops grown during 1958 were radishes (Early Scarlet Globe), beets (Detroit Dark Red), potatoes (Russet Sebago), onions (Yellow Globe Danvers), carrots (Red Cored Chantenay), cucumbers (Straight Eight), lettuce (Great Lakes), Lima beans (Fordhook 242), and alfalfa (Vernal) (Figure 1). In 1959 the same crops were grown, except that peas (Wilt Resistant Alaska) were grown instead of Lima beans.

To eliminate the sampling problem, all the edible parts of a particular crop grown in one 30-foot row were collected at harvest time and brought into the laboratory for processing. Each edible plant part was then brushed in warm water, to remove adhering soil particles. After that, the crop was rinsed with acetone by means of a wash bottle and immediately thereafter rinsed with warm water. The clean crop parts were macerated with a food grinder, the ground material was mixed, and aliquots were placed in plastic bags within 1-quart ice cream cartons, frozen, and held until extraction. Seeds of beans and peas were not washed, but ground, and then processed as described. Alfalfa grown on plots

Figure 1. View of experimental plots in 1958

treated in May 1958 was sampled on June 9, 1959. The plants were 10 to 12 inches high and the harvest included all the plant parts more than 1 to 2 inches above the soil. From each plot between 2500 and 3500 grams of plant material was obtained by random sampling and harvesting by a procedure which ensured no surface contamination from the soil. After that the plants were passed through a food grinder and handled as described above.

Each potato tuber, after having been cleaned, was cut in two equal parts. One half was then peeled, resulting in peels and pulp. All the unpeeled portions, representing "whole potatoes," were ground, mixed, and subsampled for freezing and future analyses. The same was done with the peels as well as with the pulp, in order to find out what percentage of the total amount of residues being found within whole potatoes might be located in either the potato peels or the potato pulp.

Analytical Methods

Colorimetric Analyses. The handling of the soil samples, including extraction, clean-up, and analysis, was done as previously described (*7–9*).

When crops were analyzed, the frozen plant material was thawed and 300-gram aliquots were mixed with 600 to 700 grams of anhydrous sodium sulfate, and kept overnigh in a refrigerator. Then the extraction of the crop material as well as the purification of the extracts was performed by a described procedure (*6*).

The extracts of the 1959 crops, grown in aldrin-treated soils, were separated by column chromatography into the aldrin and dieldrin (D) fractions. Each fraction was then subdivided into two equal aliquots. One aliquot was then treated with acetonitrile (*4*) to remove waxes. This portion was used for paper chromatography and bioassays, while the other was used for the colorimetric analyses as described by O'Donnell *et al.* (*11*, *12*). Known amounts of aldrin added to crop material were recovered to an extent of 82 to 96% and known amounts of dieldrin to an extent of 85 to 98% as determined by colorimetric analysis.

Fractions of extracts containing heptachlor or heptachlor epoxide (HO) were analyzed by the Polen-Silverman method (*13*, *14*). In this case, waxes were removed by an acetonitrile treatment (*4*) prior to analyses. Known amounts of heptachlor added to crop material were recovered to an extent of 84 to 98% and known amounts of heptachlor epoxide to an extent of 87 to 98% as determined by colorimetric analysis.

Whenever possible, analyses were run in duplicate, using a soil or a crop blank for the determination of apparent insecticide content. The analytical procedure for each analysis was checked by adding known amounts of insecticide to insecticide-free samples. The unknowns were then calculated on the basis of the values obtained from the known amounts after making suitable correction for apparent insecticide content. Results were expressed in parts per million based on the dry weight for soils and the fresh weight for plants.

In many cases colorimetric absorption curves were established for the recovered residue. In this way it could be checked if the absorption peak obtained from the unknown sample coincided with the peak obtained from a sample to which a known amount of an insecticide had been added.

In addition to the colorimetric analyses, fractions of extracts containing aldrin, dieldrin, heptachlor, or heptachlor epoxide were used for ascending paper chromatography as described by Mitchell (*10*). R_f values for the unknowns were established and compared to R_f values obtained from reference grade insecticides. Data were reported as zero residue when two or all of the methods used (colorimetric readings, absorption curves, paper chromatography) indicated the absence of insecticidal residues. When the results obtained by only one of the methods were negative, the data were reported as trace residues.

Bioassay. It was felt that an analysis of the insecticidal residues by a biological method which assays the toxicity of a chemical would be extremely important. Therefore, some of the crops grown in 1958 and all of the crops grown in 1959 were bioassayed. *Drosophila melanogaster* Meig. was used as the test insect (*2*). The flies were exposed to residues obtained from aliquots of the same extracts used for the colorimetric analyses. Usually three different amounts of the same extract were tested by pipetting a given amount into each of two jars ($2^3/_4$ inches in diameter and 3 inches deep) containing 0.5 ml. of a corn oil solution (1 ml. of corn oil in 200 ml. of hexane). The solvent was then evaporated by placing the jars at the opening of a fume hood. After a fine screen had been inserted into the screw top, 50 flies were introduced through a hole in the screen of each of the six jars (three different amounts of extracts in duplicate). Mortality counts were made 21 to 24 hours after exposure of the flies. Mortalities obtained with unknown samples were compared to dosage-mortality curves obtained from serial dilutions of reference grade insecticide. Results were reported as traces when mortalities obtained were below 15%.

Table I. Recoveries of Aldrin (A), Dieldrin (D), Heptachlor (H), and Heptachlor Epoxide (HO) Residues

[From a Carrington silt loam and crops grown in May 1958 on aldrin- and heptachlor-treated plots (colorimetric analyses)]

		Insecticides Applied to Soil, Lb./5-Inch Acre				
		Aldrin			Heptachlor	
		5	25		5	25
		Recovered from Soils, July 21, 1958, P.P.M.				
	A	1.21	9.77	H	2.17	10.78
	D	0.36	1.31	HO	0.21	0.63
	T[a]	1.57	11.08	T	2.38	11.41
		Recovered from Crops at Harvest, P.P.M.				
Radishes	A	0.03	0.28	H	0.00	0.14
	D	0.06	0.24	HO	0.13	0.45
	T	0.09	0.52	T	0.13	0.59
Beets	A	0.00	0.07	H	0.00	0.13
	D	0.07	0.18	HO	0.08	0.29
	T	0.07	0.25	T	0.08	0.42
Potatoes	A	0.05	0.53	H	Traces	0.66
	D	0.09	0.67	HO	0.14	0.56
	T	0.14	1.20	T	0.14	1.22
Onions	A	0.00	Traces	H	Traces[b]	Traces[b]
	D	0.00	0.05	HO	Traces[b]	0.02[b]
	T	0.00	0.05	T	Traces	0.02
Carrots	A	0.15	0.94	H	0.47	3.55
	D	0.09	0.32	HO	0.08[b]	0.43[b]
	T	0.24	1.26	T	0.55	3.98
Cucumbers	A	0.00	0.00	H	0.02	0.04
	D	0.07	0.07	HO	0.08	0.11
	T	0.07	0.07	T	0.10	0.15
Lettuce	A	0.04	0.15	H	0.05[b]	0.27
	D	0.12	0.26	HO	0.05[b]	0.29
	T	0.16	0.41	T	0.10	0.56
Beans (seeds)	A	0.00	0.00	H	0.00	0.00
	D	0.00	0.00	HO	0.00	0.00
	T	0.00	0.00	T	0.00	0.00

[a] Sum of aldrin and dieldrin or heptachlor and heptachlorepoxide, respectively.
[b] Bioassay.

The bioassay method was five to 10 times more sensitive than the available chemical methods. Therefore, the bioassay was the more dependable analysis when low residue levels were present.

Results and Discussion

Data obtained from analyses of soils and crops are presented in Tables I, II, and III, where the figures represent averages of analytical results obtained from soils and from crops grown on two replicated plots. The standard deviation obtained for data (Tables I, II, III) was:

Range, P.P.M.	Standard Deviation (±) Chem.	Bioassay
0.01–0.1	0.009	0.007
0.11–0.20	0.014	0.017
0.21–0.50	0.038	0.049
0.51–1.0	0.08	0.07
1.1–5.0	0.14	0.23

Soils on which vegetables were grown and which had been treated in two consecutive years at a rate of 5 pounds per 5-inch acre showed a slight increase in the total amounts of residues recovered in the fall of 1959 (aldrin + dieldrin = 1.88 p.p.m., heptachlor + heptachlor epoxide = 2.20 p.p.m.) as compared to the amounts found within the soils in the fall of 1958 (1.28 and 1.88 p.p.m., respectively). However, in the fall of 1959 soils which had been treated 17 months previously at 25 pounds per 5-inch acre contained about half the amount of residues obtained in the previous year (fall 1958). The amount of epoxides in per cent of the total residues recovered from each treated plot was larger in 1959 than in 1958. Although aldrin is more readily converted in soils to dieldrin than is heptachlor to its epoxide, the total amounts of residues recovered from aldrin- and heptachlor-treated soils were very similar, because in soils aldrin is somewhat less persistent than heptachlor (*9*).

However, residue data obtained from crop analyses seem to indicate that the rate of epoxidation of aldrin and heptachlor within the plant tissue was in many cases very similar. Moreover, it is dependent on the particular crop. This could be due to the presence of certain biological systems which might effect the epoxidation of aldrin and heptachlor or to different absorption rates for the parent compounds and their epoxides from the soils.

Of all crops tested, carrots were outstanding, in that they contained the largest amounts of insecticidal residues. Those grown in plots treated at 5 pounds per 5-inch acre in 1958 and 1959 contained more insecticidal residues in the second than in the first year of the experiment. However, the opposite was true of carrots grown in soils treated at 25 pounds per 5-inch acre in 1958 only.

The proportion of dieldrin found in carrots ranged from 25 to 60% of the total residue (aldrin and dieldrin) recovered from the plant tissue. The respective figures for heptachlor epoxide were 11 to 21%.

Unpeeled potatoes contained more

Table II. Recoveries of Aldrin (A), Dieldrin (D), Heptachlor (H), and Heptachlor Epoxide (HO) Residues

(From a Carrington silt loam and crops grown in 1959 on aldrin- and heptachlor-treated plots)

		Insecticides Applied to Soil, Lb./5-Inch Acre			
		Aldrin		Heptachlor	
		5[a]	25[b]	5[a]	25[b]
		Recovered from Soils,[c] P.P.M.			
	A	1.78	3.10	H 2.00	4.22
	D	0.66	1.83	HO 0.39	0.78
	T[d]	2.44	4.93	T 2.39	5.00

		Recovered from Crops at Harvest, P.P.M.							
		Chemical anal.	Bioassay	Chemical anal.	Bioassay		Chemical anal.	Bioassay	Chemical anal.	Bioassay
Radishes	A	Traces	0.03	0.05	0.05	H	Traces	0.04	Traces	0.05
	D	0.06	0.07	0.13	0.15	HO	0.12	0.12	0.12	0.13
	T	0.06	0.10	0.18	0.20	T	0.12	0.16	0.12	0.18
Beets	A	Traces	0.01	Traces	0.01	H	0.03	0.05	0.03	0.04
	D	0.09	0.06	0.17	0.14	HO	0.10	0.10	0.12	0.13
	T	0.09	0.07	0.17	0.15	T	0.13	0.15	0.15	0.17
Potatoes (whole)	A	0.05	0.03	0.11	0.11	H	0.09	0.14	0.29	0.33
	D	0.15	0.18	0.31	0.30	HO	0.26	0.31	0.49	0.53
	T	0.20	0.21	0.42	0.41	T	0.35	0.45	0.78	0.86
Potatoes (peels)	A	0.27	0.26	0.63	0.65	H	0.93	0.96	3.03	3.16
	D	0.91	0.91	1.82	2.10	HO	1.20	1.52	2.35	2.90
	T	1.18	1.17	2.45	2.75	T	2.13	2.48	5.38	6.06
Potatoes (pulp)	A	Traces	Traces	Traces	Traces	H	0.00	0.00	Traces	0.04
	D	0.06	0.06	0.16	0.14	HO	0.13	0.14	0.24	0.24
	T	0.06	0.06	0.16	0.14	T	0.13	0.14	0.24	0.28
Onions	A	Traces	0.00	Traces	0.00	H	[e]	0.00	[e]	0.00
	D	0.03	0.02	0.05	0.05	HO	[e]	0.03	[e]	0.04
	T	0.03	0.02	0.05	0.05	T		0.03		0.04
Carrots	A	0.25	0.30	0.36	0.43	H	0.97	1.26	1.34	1.51
	D	0.23	0.23	0.55	0.59	HO	[e]	0.25	[e]	0.40
	T	0.48	0.53	0.91	1.02	T		1.51		1.91
Cucumbers	A	Traces	Traces	Traces	0.01	H	0.03	0.04	0.04	0.06
	D	0.17	0.14	0.17	0.16	HO	0.10	0.12	0.13	0.15
	T	0.17	0.14	0.17	0.17	T	0.13	0.16	0.17	0.21
Lettuce	A	Traces	Traces	0.03	0.02	H	Traces	0.03	Traces	0.03
	D	0.07	0.06	0.17	0.18	HO	0.03	0.03	0.05	0.05
	T	0.07	0.06	0.20	0.20	T	0.03	0.06	0.05	0.08

Peas (seeds and pods) (*6*).

[a] Treated at 5 lb./5-inch acre in May 1958 and May 1959.
[b] Treated 25 lb./5-inch acre in May 1958 only.
[c] Averages of spring and fall analyses as obtained by chemical analyses.
[d] Sum of aldrin and dieldrin or heptachlor and heptachlor epoxide, respectively.
[e] Analytical data not available.

Table III. Recoveries of Aldrin (A), Dieldrin (D), Heptachlor (H), and Heptachlor Epoxide (HO)

[From a Carrington silt loam and from alfalfa grown in 1959 on plots treated with aldrin or heptachlor one year previously (colorimetric analyses)]

	Insecticides Applied to Soil in May 1958, Lb./5-Inch Acre				
	Aldrin			Heptachlor	
	5	25		5	25
	Recovered from Soils,[a] P.P.M.				
A	0.22	4.04	H	0.60	5.98
D	0.98	4.18	HO	0.74	2.53
T[b]	1.20	8.22	T	1.34	8.51
	Recovered from alfalfa leaves and stems,[c] P.P.M.				
A	0.00	0.00	H	Traces	0.02
D	0.06	0.08	HO	0.10	0.09
T	0.06	0.08	T	0.10	0.11

[a] Fall sample.
[b] Sum of aldrin and dieldrin or heptachlor and heptachlor epoxide, respectively.
[c] Bioassay results for alfalfa grown on aldrin-treated soils not available. Bioassays on alfalfa grown on heptachlor-treated soils resulted in a total of 0.13 p.p.m. (5 lb./acre treatment) and 0.12 p.p.m. (25 lb./acre treatment), respectively.

insecticidal residues than any other crop, with the exception of carrots. Slightly more toxicants were recovered in 1959 than in 1958 in unpeeled potatoes grown in the plots treated with 5 pounds per acre and considerably less were recovered in 1959 than in 1958 from potatoes grown in the 25 pounds per acre treated plots. The amounts of dieldrin in unpeeled potatoes ranged from 56 to 75% of the total residue recovered from the whole potatoes. The respective figures for heptachlor epoxide ranged from 46 to 100%.

When potato peels and pulp were analyzed separately, it was found that 70 to 75% of the total residue in potatoes grown in aldrin-treated soil was located within the peels. Potatoes grown in heptachlor-treated soils contained 71 to 78% of their total residues within the peels. The amounts of toxicants recovered from potato pulp were similar to those found in radishes and beets. In 1959 these latter two crops contained residues which ranged from 0.06 to 0.13 p.p.m. in plants obtained from the 5 pounds per acre treated soils and from 0.12 to 0.18 p.p.m. in plants obtained from the 25 pounds per acre treated soils.

Onions were unique among the root crops because they either did not contain any insecticidal residues or the amounts found were below 0.05 p.p.m.

Among the nonroot crops tested, lettuce contained the highest amounts of insecticidal residues, which ranged from 0.10 to 0.56 p.p.m. in 1958 and from 0.03 to 0.20 p.p.m. in 1959.

The major part of the toxicants recovered from lettuce, cucumbers, or alfalfa was in the form of dieldrin or heptachlor epoxide.

Cucumbers and alfalfa were the only plants in which the absolute amounts of residues recovered from either crop were similar on both the 5 and the 25 pounds per acre insecticide-treated soils. Therefore, the highest amounts of toxicants in the plant relative to the soil were found in those plants which had been grown in the less contaminated soils.

Seeds of Lima beans or peas (*6*) did not contain any residues.

From the data presented it is obvious that insecticidal residues were found within the edible parts of crops grown in insecticide-treated loam soils. The amounts recovered varied for each crop investigated. In most cases the absolute amounts of residues recovered from crop tissues were higher in those plants which were grown in the most contaminated soils.

A more valuable figure for the understanding of the problem of insecticidal translocation from soils into crops might be the relative amount of toxicants recovered. This is the total amount of toxicants recovered from crop tissues in percent of the total amounts present in the soil (average of spring and fall soil samples) during the growing season.

Table IV. Probable Insecticidal Residues (Aldrin Plus Dieldrin or Heptachlor Plus Heptachlor Epoxide)

[Crops grown on a Carrington silt loam containing 2 pounds/6-inch acre (1 p.p.m.) of toxicants]

	Crop Residue, % of Soil Residues[a]		Probable Crop Residues,[b] P.P.M.	
	A[c]	H[d]	A[c]	H[d]
Carrots	15.9 ± 3.42	31.7 ± 6.18	0.16	0.32
Potatoes (unpeeled)	8.9 ± 0.74	11.8 ± 3.75	0.09	0.12
Potatoes (pulp)	2.8 ± 0.40	5.1 ± 0.40	0.03	0.05
Radishes	4.2 ± 1.23	4.6 ± 1.33	0.04	0.04
Beets	3.5 ± 0.79	3.9 ± 0.93	0.04	0.04
Onions	<1.0	<1.0	<0.01	<0.01
Lettuce	5.2 ± 2.82	2.9 ± 1.77	0.05	0.03
Cucumbers	3.8 ± 2.27	3.6 ± 1.46	0.04	0.04
Peas (seeds)	0.0	0.0	0.00	0.00
Beans (seeds)	0.0	0.0	0.00	0.00
Alfalfa	3.0 ± 2.00	4.4 ± 3.10	0.03	0.04

[a] Based on field experiments conducted during 1958 and 1959.
[b] Calculated from experimental data as presented in left column of this table, assuming that the proportion of insecticide translocating from a soil into the crops is independent of the concentration of the insecticide within the soil.
[c] Grown in aldrin-treated soils.
[d] Grown in heptachlor-treated soils.

These relative figures obtained from one plant variety were in most cases similar on both the 5- and 25-pounds per acre treated plots and during both of the two years of the experiment. Therefore, it might be possible to approximate the amounts of insecticidal residues in a particular crop, once the amount of aldrin or heptachlor residues in the soil during the growing season as well as the crop residue in per cent of the soil residue was known. Such an attempt has been made, assuming that a soil treated at a rate of 2 pounds per 6-inch acre has an insecticide concentration of 1 p.p.m. Under more normal conditions the total amount of residues in all crops, with the exception of carrots and unpeeled potatoes, would range from 0.00 to 0.05 p.p.m. (Table IV). These theoretical recoveries are maximum figures, since a 2 pounds per 6-inch acre treatment at the beginning of the growing season will result in much smaller soil residues with time. In the case of radishes, which grew during the first month after soil treatment, the insecticidal residues in the soil were larger than toward the end of the growing season. Therefore the relative amounts of insecticides found in radishes would be somewhat smaller than presented in Table IV. In the case of potatoes, which were harvested in September, the relative amounts of residues within the tubers might be slightly higher than presented in Table IV. Field studies similar to those described in this paper are under way, using normal dosages of aldrin and heptachlor.

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Translocation of Some Chlorinated Hydrocarbon Insecticides into the Aerial Parts of Pea Plants

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The increased use of chlorinated hydrocarbon insecticides in soil has often raised the question of whether or not these insecticides might translocate into the aerial parts of plants from the soil in spite of their extremely low solubility in water. This was tested by resorting to very high concentrations of insecticide and using a soil of minimum sorptivity and complexity. Plants were also grown in insecticide-free sand within a glass container surrounded by lindane-treated sand. Lindane is translocated through the root system into the aerial parts of the plant. No evidence of absorption through the leaf cuticle of lindane vapors released from the soil surface was obtained. Plants grown in covered pots containing aldrin- or heptachlor-treated sand contained only the epoxides within the leaves and stems. Epoxidation of aldrin to dieldrin and of heptachlor to heptachlor epoxide apparently took place within the plant tissue, after aldrin or heptachlor had been absorbed through the root system. Plants grown in dieldrin- or heptachlor epoxide–treated sand contained those insecticides within their leaves and stems. No evidence of DDT translocation was obtained. Under field conditions, seeds from pea plants grown in a Carrington silt loam soil treated with 5 and 25 pounds per acre of aldrin or heptachlor contained no measurable insecticidal residues.

During recent years, increased attention has been given to the insecticide residue problem. Positive evidence of the translocation of benzene hexachloride from treated soils into the aerial portions of plants was reported by Starnes (*18*), who found that both aqueous suspensions and benzene extracts of potato foliage from plants grown in soil treated with 10 pounds of γ-benzene hexachloride per acre were highly toxic to *Aedes* mosquito larvae. Haines (*3*) reported "the presence of a toxic material in both guttated liquid and tissues of corn plants grown in lindane-treated nutrient solution." Paraoxon was found (*2*) in the fluid guttated from leaves of cabbage and wheat plants, grown in parathion-treated soil. Crops grown on lindane-, DDT-, and aldrin-treated soils contained from zero to various amounts of insecticides (*5*), depending on the insecticide, its concentration in the soil, the crop itself, and the soil type in which the crops were grown.

Mechanisms by which an insecticide applied to a soil might contaminate the aerial parts of plants grown in that soil are: rain or other physical factors resulting in the direct deposition of small amounts of treated soil on the foliage; volatilization of the insecticide from the soil and subsequent penetration through the leaf cuticle; and potential penetration of the compound through the roots and then translocation into the aerial parts of the plant.

To obtain more data on insecticidal residues in the aerial parts of the crops, peas were grown in an insecticide-treated quartz sand under greenhouse conditions, using extremely high concentrations of insecticide, and in a treated loam soil under field conditions. The object was to find out if insecticidal residues could be detected in stems and leaves at all, and if so, by what pathways they would find their way into the plant tissue. In addition, an attempt was made to determine the site of epoxidation of aldrin and heptachlor under such conditions.

Procedure

Laboratory Experiments. Peas (Wilt Resistant Alaska) were used throughout. They were grown in quartz sand treated at 30 p.p.m. with analytical grade lindane, aldrin, dieldrin, heptachlor, heptachlor epoxide, or *p,p'*-DDT. These conditions were extreme ones and not representative of agricultural practice. The quartz sand possesses neither the insecticide-retention qualities of any agricultural soil nor its microbial contents. Moreover, 30 p.p.m. (approximately 60 pounds per 6-inch acre) is far above what normally might be expected as an insecticidal residue content in agricultural soils. These extreme experimental conditions were selected to determine whether insecticides of extremely low water solubility could be translocated into plants through the root system.

The quartz sand was treated by pipetting measured amounts of an insecticide in acetone solution onto the sand contained in a wide-mouthed 1-gallon jar. The sand was mixed by rolling horizontally for 20 minutes on a specially designed apparatus (*8*). The acetone evaporation was aided by a very mild air stream.

Lindane was used for the first three sets of experiments because of its slight water solubility (to 10 p.p.m. at 20° C.) (*12*) and its relatively high vapor pressure, factors presumably favoring absorption by either roots or possibly the leaf cuticle.

1. Seventeen nonglazed clay pots (6 inches wide) were filled with lindane-treated sand. Twenty-four germinated pea seeds with a root length of $^3/_4$ to 1 inch were planted in each pot. In addition, 17 pots containing untreated sand were prepared in the same way.
2. Seventeen clay pots filled with lindane-treated sand were covered with extra-heavy aluminum foil. Twenty-four holes ($^1/_{16}$ to $^1/_8$ inch wide) were made in the aluminum foil of each pot. Peas were planted through those holes, the seed itself being left on top of the aluminum foil. Seventeen aluminum foil–covered pots containing untreated sand were prepared in the same way.

All pots stood in saucers and nutrient solution was added to the saucers as necessary (Figure 1). In this way water splashing was prevented. The aluminum foil also prevented excessive water loss and the possibility of codistillation of insecticidal vapors.

3. Ten peas were planted in untreated quartz sand within glass containers 2.5 inches wide. These jars were placed within clay pots 9 inches wide and surrounded by lindane-treated (30 p.p.m.) quartz sand (Figure 2). Nutrient solution was added as necessary to the surface of the untreated sand and distilled water to the surface of the treated sand.

4. Tests were conducted with aldrin, dieldrin, heptachlor, and heptachlor epoxide. For each insecticide used, 17 clay pots were filled with treated sand and covered with aluminum foil. Planting and watering were done as above. Seventeen control pots were similarly prepared.

5. Carbon-14–labeled *p,p'*-DDT was used, which had an activity of 79 counts per μg. per minute after subtraction of background. Because a relatively small amount of DDT was available, only one aluminum foil–covered pot containing DDT-treated quartz sand (30 p.p.m.) was planted with peas.

Twenty-one days after planting, when flowers started to develop, the plants were cut 1 inch above the sand surface. All plants from similarly treated sand were pooled and used for analyses. In addition, the sand was thoroughly mixed and aliquots were taken for analyses.

Field Experiments. In May 1958, duplicate Carrington silt loam plots (30 × 40 feet) were treated with aldrin and heptachlor at 5 and 25 pounds per acre and then rototilled to a depth of 4 to 5 inches. Treatment was done as described by Lichtenstein and Schulz (*9*). One year later the plots previously treated at the lower application rate were retreated with the same insecticides at 5 pounds per 5-inch acre. Two days after application in 1959, one 30-foot row was seeded with peas (Wilt Resistant Alaska) on both treated and control plots. At the end of July pea pods were harvested from the plants of each experimental plot. The seeds were separated from the pods. All seeds or all pods obtained from one particular plot were pooled for extraction and analyses.

Soils were sampled in May and September of 1959 as described by Lichtenstein (*5*).

Analytical Methods

Bioassays. Preliminary screening tests were conducted with two bioassay procedures.

Though chlorinated hydrocarbon insecticides have been reported (*10*) as inefficient aphicides, the pea aphid (*Macrosiphum pisi* Harris) was used. The bottom of a Petri dish (3.5 inches in diameter) was covered with moistened filter paper. Eight leaves taken at random from plants of one pot were placed at the periphery of the paper. In addition four stem pieces each 1 inch long were used. Twenty-five adult aphids were then introduced onto the center of the filter paper. Those aphids which did not start feeding after 15 minutes were replaced until all aphids had moved onto the plants. Four replicated Petri dishes containing plant material from one pot were set up. All together eight (2 × 4) tests were conducted with plants grown in two pots which contained sand treated with one particular insecticide. Mortality counts were made 29 hours after the pea aphids had been introduced. Corrections for check mortalities were made according to Abbott's formula (*1*).

In the second bioassay procedure *Drosophila melanogaster* Meig. was used as a test insect. Plants from two replicated pots were finely ground, dried with anhydrous sodium sulfate, and subsequently extracted with a mixture of hexane–isopropyl alcohol (2 to 1 by volume) by tumbling. After that the isopropyl alcohol was removed by washing with a saturated solution of sodium chloride. The hexane extract containing all the plant extractives ("crude") was then added quantitatively to small jars ($2^3/_4$ inches in diameter and 3 inches deep). After the solvent had been evaporated in a hood, 50 three-day-old flies were introduced to each jar. Mortality counts were made after 4 and 24 hours.

To minimize any possible masking effect from plant extractives, the residue in the jar was re-extracted after the first test with *Drosophila*. Hexane was used for re-extracting residues from plants grown in lindane-, aldrin-, or dieldrin-treated sand and pentane for the others. Waxes were removed by using acetonitrile (*4*). Hexane extracts were then added to a column (8 × 1 inch) of magnesium oxide–Celite 503 (1 to 1) and eluted with 600 ml. of redistilled hexane. The pentane extracts were cleaned up by using carbon as an absorbent, followed by a Florisil column (*7*) from which the residues were eluted with 200 ml. of a mixture of pentane-ether (94 to 6 by volume).

After the purified extracts had been concentrated, they were transferred quantitatively to the bioassay jars and the solvents were evaporated in a hood. Fifty 3-day-old flies were then introduced into each jar. Mortality counts were made at 24 and 48 hours.

Colorimetric Analyses. For extraction of pea vines the total amount of fresh plant material was passed through a food grinder. The macerated material weighing from 226 to 372 grams was then mixed with a double amount of anhydrous sodium sulfate and kept overnight in a refrigerator. The crop–sodium sulfate mixture was placed in 2-quart wide-mouthed Mason jars for extraction.

When pea pods or pea seeds were extracted, the total amount obtained from one field plot was ground and mixed and 300-gram aliquots were prepared for extraction as described above.

Sand and loam soils were screened and mixed prior to extraction. Four hundred grams of moist soil and 200 grams of anhydrous sodium sulfate were then placed in 2-quart wide-mouthed Mason jars. An additional portion of 100 grams of soil was dried for 24 hours at 50° C. to determine the dry weight of the soil.

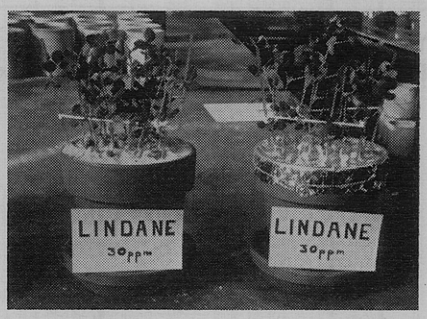

Figure 1. Peas growing in noncovered and covered pots containing lindane-treated sand

Figure 2. Peas growing in glass jars containing untreated sand

Surrounded by lindane-treated (30 p.p.m.) sand within clay pot

The extraction solvents used were a mixture of redistilled hexane and isopropyl alcohol (2 to 1 by volume) for aldrin, dieldrin, and DDT and a mixture of colorimetric pentane and acetone (4 to 1 by volume) for heptachlor and heptachlor epoxide. Two milliliters of solvent were used per gram of wet soil and 3 ml. of solvent per gram of fresh plant material.

After 1 hour of head-to-end tumbling, the jars and their contents were chilled to minimize evaporation of solvents during filtering. The supernatant liquid was then decanted through filter paper and the recovery volume was recorded to be used for calculating the results.

The isopropyl alcohol or the acetone was removed from the extracts by washing once with water and three times with a saturated solution of sodium

Table I. Mortalities of *Drosophila melanogaster* Meig. after Exposure to Extracts of Pea Vines Grown in Insecticide-Treated Quartz Sand (30 P.M.)

Insecticide Used	Plant Material Extracted, Grams	Hours of Exposure: 4	24	24	48
		Mortality from Plant Extract, %			
		Crude[a]		Purified[b]	
None	16.3	0	0	0	6
Lindane[c]	14.9	100	..	..	..
Lindane surrounded[d]	5.0	0	0	0	0
Aldrin	9.9	0	0	2	28
Dieldrin	10.4	0	0	0	36
Aldrin + dieldrin[e]	17.5	0	0	0	52
Heptachlor	17.5	0	0	80	..
Heptachlor epoxide	16.2	0	0	88	..

[a] Containing hexane-isopropyl alcohol–soluble materials.
[b] Purified by acetonitrile treatment and column chromatography.
[c] From covered and noncovered pots.
[d] Plants grown in untreated sand, but surrounded by lindane-treated sand.
[e] Plant material obtained from peas grown in two different pots, one containing aldrin-treated sand, the other dieldrin-treated sand.

Table II. Insecticidal Residues Recovered, P.P.M., from Sand and Pea Vines Grown in Treated Quartz Sand (30 P.P.M.)

Insecticides: Treated	Insecticides: Recovered	Sand, Chem. Anal.	Pea Vines: Chem. anal.	Pea Vines: Bioassay
Lindane	Lindane			
Open[a]		20.2 ± 0.2	18.0 ± 0.7	
Covered[b]		23.8 ± 0.3	21.9 ± 5.8	
Surrounded[c]		0.0	0.0	0.0
Aldrin[b]	Aldrin	24.85 ± 1.25	0.0	Traces
	Dieldrin	0.11 ± 0.07	0.34 ± 0.01	0.34 ± 0.02
Heptachlor[b]	Heptachlor	20.60 ± 1.30	Traces	Traces
	Heptachlor epoxide	Traces	0.17[d]	0.18 ± 0.06
Dieldrin[b]	Aldrin	0.0	0.0	
	Dieldrin	24.45 ± 0.9	1.08[e]	0.87 ± 0.09
Heptachlor epoxide[b]	Heptachlor	0.0	0.0	
	Heptachlor epoxide	29.30 ± 0.4	0.19[d]	0.22 ± 0.05
		Radioassay	Radioassay	
DDT (C^{-14})[b]	DDT (C^{-14})	26.70 ± 0.6	0.01	0.0

[a] Grown in noncovered pots.
[b] Grown in aluminum foil–covered pots.
[c] Grown in untreated sand but surrounded by lindane-treated sand.
[d] Absorption peak at 415 mμ. R_f value same as obtained from reference grade heptachlor epoxide.
[e] *Rf* value same as obtained from reference grade dieldrin.

chloride. The alcohol- or acetone-free phase was then dried over anhydrous sodium sulfate.

Extracts containing aldrin and dieldrin were cleaned up by using a column (8 × 1 inch) of magnesium oxide–Celite 503 (1 to 1). After the extract had been added to the column, aldrin was eluted with 100 ml. of redistilled hexane. Dieldrin could then be washed from the column with an additional 600 ml. of the same solvent. Aldrin and dieldrin fractions were analyzed according to the method of O'Donnell *et al.* (*13*, *14*). Known amounts of aldrin added to soils were recovered to an extent of 93 to 97%, and known amounts of dieldrin to an extent of 90 to 94%. The respective figures obtained when aldrin or dieldrin was added to crop material were 88 to 92%.

Extracts containing heptachlor or heptachlor epoxide were cleaned up as described by Lichtenstein and Polivka (*7*). Waxes contained in extracts of pea seeds or pea pods were removed by using acetonitrile (*4*). The two insecticides were analyzed according to the Polen-Silverman method (*15*, *16*). Known amounts of heptachlor or heptachlor epoxide added to soil were separately recovered to an extent of 90 to 96%. The figures obtained when crop material was used were 85 to 91% for heptachlor and 90 to 92% for heptachlor epoxide.

DDT extracts were cleaned up by passing through a column (6 × 1 inch) of aluminum oxide (reagent, Baker and Adamson). Analysis was done by radioassay using a Geiger-Muller counter, after the extract had been evaporated to dryness in planchettes. Results were corrected for self-absorption.

Lindane was analyzed according to the Schechter-Hornstein method (*17*). A special extraction procedure was eliminated (*6*), which permitted the determination of lindane directly in soils and crops. Known amounts of lindane added to soils and crops were recovered at approximately 100%.

In some cases colorimetric absorption curves were established for the recovered residue to check if the peak obtained from the unknown sample coincided with the peak obtained from a sample to which a known amount of an insecticide had been added. Where the amount of available material permitted, analyses were run in duplicate, using a soil or a crop blank for the determination of apparent insecticide content. In all cases known amounts of insecticides were added to insecticide-free samples. This permitted a check of the analytical procedure for each analysis done. The unknowns, after the value for apparent insecticide had been subtracted, were calculated on the basis of the values obtained from the known amounts. Results were expressed in parts per million, based on the dry weight for soils and the fresh weight for plants.

Fractions of extracts containing aldrin, dieldrin, heptachlor, or heptachlor epoxide were used for ascending paper chromatography as described by Mitchell (*11*). R_f values for the unknowns were established and compared to R_f values obtained from reference grade insecticides. In addition, aliquots of the extracts were used for a *Drosophila* bioassay. Mortalities obtained with unknown samples were compared to dosage-mortality standard curves obtained from serial dilutions of reference grade insecticide.

Data were reported as zero when results obtained by colorimetric analyses as well as paper chromatography were negative. Where the colorimetric analysis indicated the absence of insecticidal residues but R_f values obtained from the unknowns were the same as those obtained from reference grade insecticide, data were reported as "traces." When bioassay procedures were employed, results were reported as traces when mortalities obtained were below 15% (corrected for check mortalities).

Results and Discussion

Laboratory Experiments. Screening Tests by Bioassay. None of the plant material from pea plants grown in insecticide-treated quartz sand was toxic to the pea aphids, except that derived from the lindane experiment. Exposure to plant material obtained from plants grown in noncovered pots containing lindane-treated sand yielded 26± 9% mortality of the pea aphids. When plant material from covered pots was used, 63.5± 15% of the pea aphids died 29 hours after exposure. No insect mortality was observed on leaves

and stems obtained from plants grown in untreated sand surrounded by lindane-contaminated sand.

These results indicate that toxic material was present in the aerial parts of pea plants grown in lindane-treated sand. Moreover, more toxic material was present in plants grown in lindane-treated sand covered with aluminum foil.

The screening results with *Drosophila* are summarized in Table I. Crude extracts from plant material grown in lindane-treated sand were the only ones that caused any fly mortality. Purified plant extracts from peas grown in heptachlor- or heptachlor epoxide–treated sand killed nearly all the flies within 24 hours. Forty-eight hours' exposure of the flies yielded 24 to 49% mortality [corrected for check mortalities according to Abbott's formula (*1*)] with purified extracts of plants grown in aldrin- or dieldrin-treated sand. However, in the latter case, the amount of plant material available was only half of that used when flies were exposed to plant extracts obtained from peas grown in heptachlor- or heptachlor-epoxide–treated sand.

Chemical Analyses and Quantitative Bioassays. Insecticidal residues recovered from the aerial parts of pea plants grown in insecticide-treated sand are summarized in Table II.

If lindane vapors had been absorbed through the leaf cuticle, most of the insecticide would have been found within the plants grown in uncovered pots and possibly some in those grown in untreated sand surrounded by lindane-contaminated sand. The amount of lindane residues in leaves and stems of peas was greater than that recovered when the other insecticides were used. Plants grown in covered pots contained 22 p.p.m. of lindane, which was similar to that recovered from the sand. Volatilization of lindane and subsequent absorption of the insecticide through the leaves were considerably reduced by the aluminum foil cover. Thus the insecticide apparently entered the leaves and stems through the roots.

More lindane was lost by the sand in the noncovered than in the covered pots, probably because of a greater volatilization of the lindane from the sand surface. Plants grown in noncovered and covered pots contained similar amounts of lindane.

Under the described conditions no lindane residue was found in the plants growing in untreated sand surrounded by lindane-treated sand (Figure 2). Therefore, it seems rather unlikely that an aerial translocation of lindane took place.

Plants grown in aldrin- or heptachlor-treated sand contained only the respective epoxides: dieldrin (1.4% of aldrin residue in the sand) or heptachlor epoxide (0.83% of heptachlor residue in the sand). However, the amount of dieldrin in the sand was only 0.5% of the amount of aldrin recovered, and only traces of heptachlor epoxide were detected in the heptachlor-treated sand.

The epoxidation of heptachlor and especially aldrin is dependent on biological factors (*9*). Since quartz sand exhibits negligible biological activities, it was not surprising that the epoxidation of aldrin to dieldrin in this substrate was very small as compared to various soil types. It seems, therefore, that aldrin and heptachlor penetrated the root system and epoxidation took place within the plant tissue.

When plants were grown in dieldrin- or heptachlor epoxide–treated sand, only dieldrin (4% of dieldrin residue recovered from the sand) or heptachlor epoxide (0.7% of heptachlor epoxide residue recovered from the sand) was found in the aerial parts of the plants. Aldrin or heptachlor residues could not be detected in either the sand or the plant tissue.

Pea plants grown in sand treated with carbon-14–labeled DDT did not show any translocation of this insecticide. On the basis of extraction of plant material and radioassay, less than 0.01 p.p.m. of carbon-14 derived from DDT was found.

The availability of insecticides in soils to plants decreases with the increase of organic matter in soils (*5*). Therefore, repetition of the described experiments with agricultural soils would probably result in much lower residues or none at all within the plant tissue. Using economic dosages of insecticide would undoubtedly further reduce the residue content.

Table III. Recoveries of Aldrin (A), Dieldrin (D), Heptachlor (H), and Heptachlor Epoxide (HO) Residues

(From a Carrington silt loam and peas grown in 1959 on aldrin- or heptachlor-treated soils)

	Insecticides Applied to Soil, Lb. per 5-Inch Acre							
	Aldrin				Heptachlor			
	5[a]		25[b]		5[a]		25[b]	
	Recovered from Soils,[c] P.P.M.							
A	1.78		3.10		H	2.00		4.22
D	0.66		1.83		HO	0.39		0.78
T[d]	2.44		4.93		T	2.39		5.00
	Recovered from Seeds, P.P.M.							
	Chem. anal.	Bio-assay	Chem. anal.	Bio-assay		Chem. anal.	Bio-assay	Chem. anal.	Bio-assay
A	0.0	0.0	0.0	0.0	H	0.0	0.0	0.0	0.0
D	0.0	0.0	0.0	0.0	HO	0.0	0.0	0.0	<0.01
T	0.0	0.0	0.0	0.0	T	0.0	0.0	0.0	<0.01
	Recovered from Pods, P.P.M.								
A	0.0	0.0	0.0	0.0	H	0.0	0.0	0.0	0.0
D	0.0	<0.02	0.0	<0.02	HO	0.03[e]	0.03	0.04[e]	0.05
T	0.0	<0.02	0.0	<0.02	T	0.03	0.03	0.04	0.05

[a] Treated at 5 lb. per 5-inch acre in May 1958 and May 1959.
[b] Treated at 25 lb. per 5-inch acre in May 1958 only.
[c] Average of spring and fall analyses (chemical).
[d] Sum of aldrin and dieldrin or heptachlor and heptachlor epoxide, respectively.
[e] R_f value same as obtained from reference grade heptachlor epoxide.

Field Experiments

Results obtained from seeds and pods from pea plants grown in either aldrin- or heptachlor-treated Carrington silt loam are summarized in Table III. The total amounts of aldrin and dieldrin or heptachlor and heptachlor epoxide recovered from the soil during the summer of 1959 were considerably larger than would be present normally. Seeds from pea plants grown in these soils did not contain measurable amounts of insecticidal residues; neither did pods from plants grown on aldrin-treated soil. However, in pods from plants grown on heptachlor-treated soils, 0.03 to 0.04 p.p.m. of heptachlor epoxide was found as determined by chemical analysis, bioassay, and paper chromatography.

Therefore, under normal conditions no aldrin or heptachlor or their epoxides would be expected in pea seeds or pods from aldrin- or heptachlor-treated loam soils.

Acknowledgment

The authors thank M. S. Schechter, Entomology Research Division, U.S. Department of Agriculture, for supplying radioactive DDT and Ursula Anderson and Mike LaBreche for their assistance in the analytical work.

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ISOTOPE-LABELED INSECTICIDES

Synthesis of Carbon-14-Labeled Aldrin and Dieldrin

ROGER M. McKINNEY and GEORGE W. PEARCE

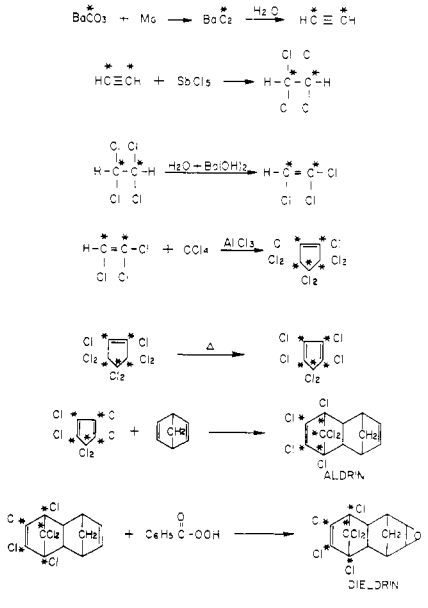
Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Savannah, Ga.

The insecticides 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo*-exo-5,8-dimethanonaphthalene (aldrin) and 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,-8a-octahydro-1,4-*endo*-exo-5,8-dimethanonaphthalene (dieldrin) were prepared labeled with carbon-14. They were synthesized by labeling hexachlorocyclopentadiene and subsequent reaction with 2,5-norbornadiene. Starting with $BaC^{14}O_3$, a 28% yield of aldrin (melting point 102.5° C.) and a 22% yield of dieldrin (melting point 181° C.) were obtained. The specific activities were 3.6 and 3.5 ± 0.1 mc. per gram for aldrin and dieldrin, respectively.

DURING STUDIES at this laboratory on the fate of aldrin and dieldrin in susceptible and resistant strains of insects and in higher animals it became evident that the chemical methods now available for these compounds are not adequate, especially when an attempt is made to account fully for the dosage applied. It was obvious that C^{14}-labeled aldrin and dieldrin would be a great aid in our studies and, accordingly, their synthesis was undertaken. The present paper is a report on this work.

Aldrin is ordinarily prepared by the Diels-Alder reaction of hexachlorocyclopentadiene with 2,5-norbornadiene (bicyclo[2,2,1]hepta - 2,5 - diene), and dieldrin is prepared by the peracid oxidation of aldrin. The problem was to prepare either hexachlorocyclopentadiene or 2,5-norbornadiene with a C^{14} label. Synthesis of C^{14}-labeled hexachlorocyclopentadiene appeared to be the most promising approach.

The following six- and seven-step reaction scheme was used in the synthesis of C^{14}-labeled aldrin and dieldrin:



Carbon-14 labeled acetylene was prepared by a modification of the method of Cramer and Kistiakowski (*2*). The yields were approximately 70%, as determined by gas volumetric measurements with inactive runs.

The labeled acetylene was converted to tetrachloroethane by treatment with a mixture of antimony pentachloride and trichloride. This reaction as given by Krall (*3*) was found to give very low yields. After the acetylene had been complexed with the antimony pentachloride, it was necessary to decompose the complex by heating for a much longer period of time than that used by Krall to obtain satisfactory yields. With inactive acetylene, 85% yields were consistently obtained in this reaction.

Trichloroethylene was prepared by refluxing the tetrachloroethane with barium hydroxide and steam-distilling the product. Yields of 85 to 90% were obtained with inactive trichloroethylene.

Octachlorocyclopentene was prepared from the trichloroethylene by the method of Prins (*4*). This reaction gave a 70% yield.

The dechlorination of octachlorocyclopentene to hexachlorocyclopentadiene and the subsequent conversion to aldrin and dieldrin were based on

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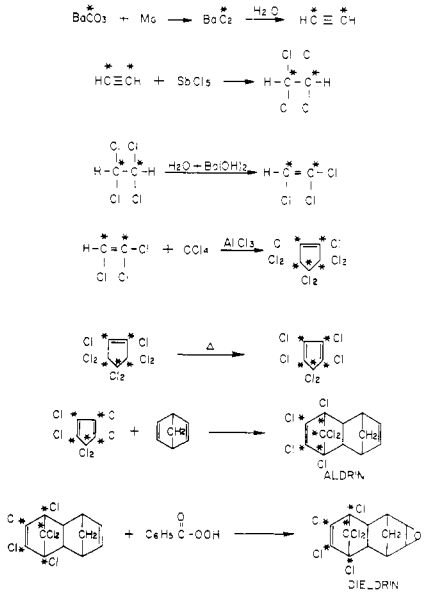

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The dechlorination of octachlorocyclopentene to hexachlorocyclopentadiene and the subsequent conversion to aldrin and dieldrin were based on

methods described by Burton, Potter, and Silverman (*1*). The dechlorination was found to be essentially quantitative and the Diels-Alder reaction gave 70 to 75% yields. Oxidation of aldrin to dieldrin gave a 63% yield after two crystallizations.

Experimental

Tetrachloroethane, $C^{14}HCl_2C^{14}HCl_2$. A mixture of 0.59 gram of barium carbonate containing 39 mc. of activity and 19.5 grams of inactive barium carbonate was mixed with 48.6 grams of 70- to 80-mesh magnesium. This was placed in a stainless steel reaction bomb, which later was used as the acetylene generator (*A*, Figure 1). The bomb was sealed with a copper gasket and screw cap fitted with inlet and outlet tubes. The reaction was carried out under a helium atmosphere, the bomb being heated at 725° C. for 15 minutes.

After cooling to room temperature the screw cap of the bomb was replaced by another screw cap with a male 40/50 standard-taper joint and rubber O-ring. This was immediately put into position as the acetylene generator (*A*, Figure 1). The system (Figure 1) was purged with dry nitrogen to remove moisture and oxygen before the liquid nitrogen Dewar flasks were put in place. After the flasks were put in position, the nitrogen flow was reduced to a rate that was just perceptible, as evidenced by the bubbles produced in the oil (*B*, Figure 1) at the end of the train.

A total of 200 ml. of water was very carefully added through the dropping funnel (*C*, Figure 1) over a 2-hour period, followed by the careful addition of 500 ml. of concentrated hydrochloric acid over another 2-hour period. The acetylene generator was kept cold throughout the addition of water and acid with an ice water bath. The contents were finally refluxed for 15 minutes to drive over the last traces of acetylene. The acetylene was condensed in the liquid nitrogen–cooled traps (*D*, *E*, Figure 1), while any moisture not condensed in the water-cooled condenser was retained by the ice water–cooled trap or the silica gel drying tube (Figure 1).

For chlorinating the acetylene, a solution of 12.0 grams of antimony trichloride and 89.7 grams of antimony pentachloride was placed in the reaction flask (*F*, Figure 2). Traps *D* and *E* with the acetylene were attached to the vacuum train as shown in Figure 2. With *D*, *E*, and *F* immersed in liquid nitrogen, the entire system (Figure 2) was evacuated to 30-micron pressure.

The acetylene was then vacuum-transferred to the chlorinating flask, *F*. With stopcocks *A'* and *B'* closed and *C'*, *D'*, and *E'* open, the acetylene–antimony chloride mixture was allowed to warm up to room temperature. The

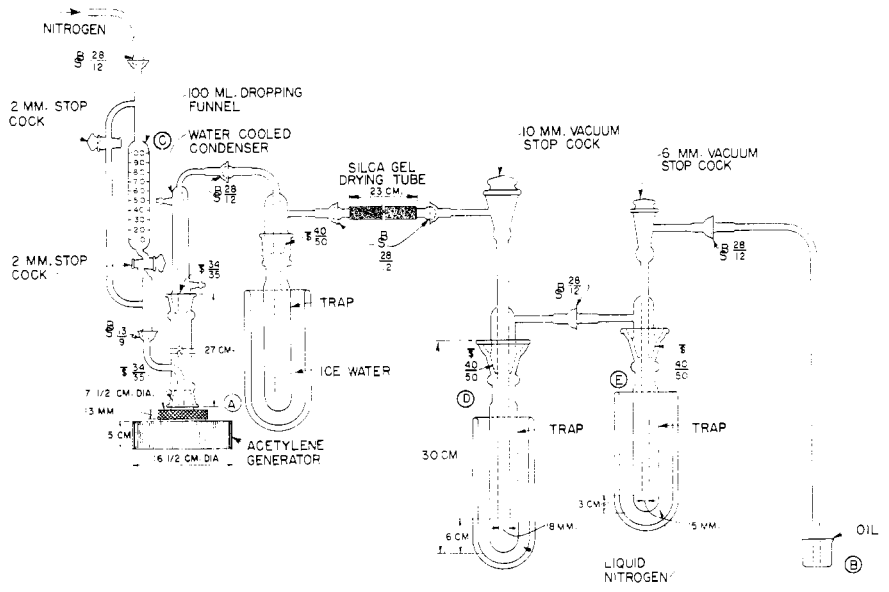

Figure 1. Acetylene generation apparatus

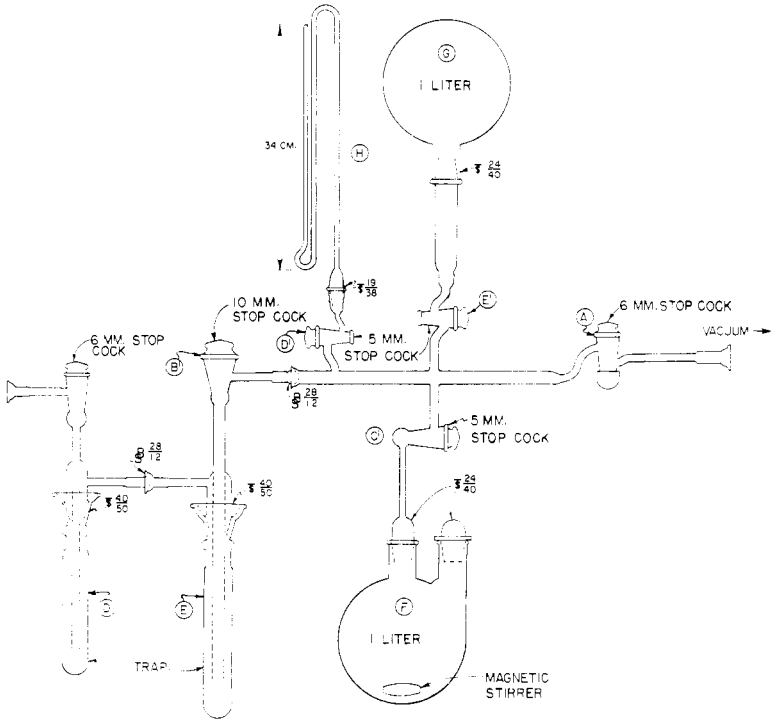

Figure 2. Chlorination apparatus

purpose of flask *G* is to add sufficient volume to the system to keep it well below atmospheric pressure. The pressure indicated by manometer *H* at this point was 28 cm., so it was safe to confine the gas in a smaller volume.

The liquid nitrogen bath was replaced on flask *F*, and the acetylene was frozen onto the antimony chloride mixture. With stopcocks *C'* and *D'* open and all other stopcocks closed, the liquid nitrogen bath was removed from flask *F* and replaced by an ice water bath. When the mixture became liquid, it was stirred magnetically and the progress of the absorption of acetylene was followed by the pressure readings. A minimum pressure of 4.4 cm. was reached after 1 hour and the absorption was assumed to be complete.

The liquid nitrogen was again replaced on flask *F* and the remaining gas was frozen. Stopcock *C'* was closed and the reaction mixture was held at 100° C. on a steam bath for 16 hours to bring about complete decomposition of the acetylene–antimony pentachloride complex to produce tetrachloroethane.

The assembly (Figure 2) was modified by removing the 1-liter flask, *G*.

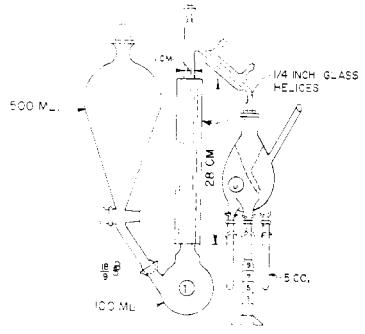

Figure 3. Distillation apparatus

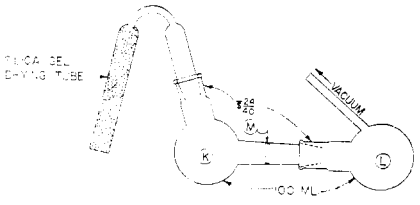

Figure 4. Recrystallization apparatus

Traps *D* and *E* were replaced by a ball joint stopper and the stopper in flask *F* was replaced by a 2-necked adaptor with standard-taper joints. A tube containing Ascarite was placed in one neck of the adaptor to serve as a vent, and an Erlenmeyer flask containing 70 grams of calcium hydroxide was attached to the other neck by means of a flexible rubber tube.

An ice bath was placed around flask *F*, and 200 ml. of water were slowly added to the reaction mixture with stirring. This was followed by the careful addition of 70 grams of calcium hydroxide to hydrolyze the antimony chlorides. The tetrachloroethane and some water were then separated from the reaction mixture by direct steam distillation. About 30 ml. of distillate were collected.

Trichloroethylene. Tetrachloroethane was dehydrochlorinated by refluxing with water and barium hydroxide. The receiving flask for the steam distillation served as the reaction flask. An additional 40 ml. of water and 10.0 grams of barium hydroxide octahydrate were added to the reaction flask.

The reaction mixture was refluxed for 5 hours and the trichloroethylene and some water were then distilled and collected in a receiver. The yield was 2.5 ml. or 3.7 grams. This is about 56% of the theoretical yield based on barium carbonate.

Octachlorocyclopentene. The synthesis of C^{14}-labeled trichloroethylene, starting from barium carbonate, was repeated on the same scale and the two batches were combined for a total of 7.4 grams. The trichloroethylene was dried over 6 grams of silica gel and then transferred with the aid of carbon tetrachloride to a 250-ml. dropping funnel. Carbon tetrachloride was added to give a total volume of 170 ml. Two grams of freshly sublimed aluminum chloride and 30 ml. of carbon tetrachloride were placed in a 500-ml., two-necked, round-bottomed flask. The 250-ml. dropping funnel was attached to one neck and a reflux condenser with a drying tube was attached to the other neck.

The reaction mixture was refluxed gently and stirred with a magnetic stirrer throughout the reaction. The trichloroethylene in carbon tetrachloride was added dropwise over a 9-hour period. This was followed by the dropwise addition, over a 2.5-hour period, of 1.74 grams of inactive trichloroethylene in 50 ml. of carbon tetrachloride. The reaction mixture was refluxed for one additional hour and, after cooling, it was filtered with suction through a bed of Celite on a Büchner funnel and received directly in a 500-ml. dropping funnel. The solution was washed three times with 30-ml. portions of water and then mounted in position on the distilling flask, *I*, as shown in Figure 3.

The carbon tetrachloride was removed by flash distillation at atmospheric pressure. When all of the solvent was removed, the fraction cutting adaptor, *J*, was put in position and the product was distilled under reduced pressure. A low-boiling forerun was collected below 75° C. at 0.75-mm. pressure. The fraction distilling above 75° C. at 0.75 mm. was collected as octachlorocyclopentene. A small amount of tarry residue remained in the distilling flask, *I*, at the end of the distillation.

The yield of crude octachlorocyclopentene was 5.4 ml. or 9.89 grams. This was vacuum-transferred to flask *K* of the recrystallizing apparatus shown in Figure 4. Methanol (10 ml.) was added and the product was solidified by cooling in a dry ice–acetone bath and broken up into a fine powder with the use of a glass rod. The methanol was removed while still cold by tilting the apparatus so that the receiver *L* was in an upright position and applying a vacuum. The octachlorocyclopentene was retained on the sintered-glass disk, *M*. The yield of octachlorocyclopentene was 8.68 grams or 72% based on trichloroethylene. This is 44.5% based on barium carbonate.

Hexachlorocyclopentadiene. The octachlorocyclopentene was thermally dechlorinated to hexachlorocyclopentadiene by the procedure described by Burton, Potter, and Silverman (*1*). The yield was 6.75 grams. This is 98% based on octachlorocyclopentene and 43.6% based on barium carbonate.

Aldrin. Aldrin was prepared by the reaction of hexachlorocyclopentadiene with 2,5-norbornadiene (*1*). The product was distilled at a pressure of 30 microns, giving a yield of 8.48 grams of crude aldrin. The infrared spectrum of this product indicated that it was contaminated with a small amount of isodrin, a geometrical isomer of aldrin. The presence of isodrin was detected by an absorption band at 13.58 microns which is not present in pure aldrin.

To remove the isodrin from the aldrin, the crude product was chromatographed on an alumina column which was 4 cm. in diameter and 115 cm. long. The alumina was Fisher 80- to 200-mesh adsorption grade which was activated by heating at 200° C. *n*-Hexane was used as the eluting solvent. The aldrin was eluted first, 7.3 grams of isodrin-free aldrin being collected. This is 81.0% based on hexachlorocyclopentadiene or 35.3% based on barium carbonate. The succeeding fractions contained a mixture of radioactive isodrin and aldrin. They were combined and reserved for further separation.

The aldrin at this point was almost pure. The infrared spectrum was identical with that of a pure authentic sample and the melting point was 98° C. as compared with 102.5° C. for the authentic sample. A 3.86-gram portion was recrystallized from 11 ml. of isopropyl alcohol with the use of the recrystallizing apparatus shown in Figure 4. Recovery was 3.1 grams of pure radioactive aldrin, melting point 102.5° C. This represents a 65% yield from hexachlorocyclopentadiene and a 28.3% yield from barium carbonate. The specific activity was 3.6 mc. per gram.

Dieldrin. Using the method of Burton *et al.* (*1*) as a guide, the following procedure was used to convert aldrin to dieldrin: A 3.96-gram portion of chromatographed, but not recrystallized, radioactive aldrin was placed in a 125-ml. glass-stoppered Erlenmeyer flask. To this were added 70 ml. of 0.5*M* perbenzoic acid in chloroform. The reaction mixture was allowed to stand for 17 hours at 6° C. It was then transferred to a 250-ml. separatory funnel and washed once with 50 ml. of a 10% sodium bisulfite solution, twice with 50 ml. of saturated sodium carbonate solution, and three times with 50 ml. of water. The chloroform solution was transferred to a 300-ml. flask and the solvent was removed with a rotary evaporator. The crude product was vacuum-transferred to the recrystallizing apparatus shown in Figure 4. The transfer was effected by heating the dieldrin to 180° C. at 60-micron pressure.

The product was recrystallized twice from 10 ml. of isopropyl alcohol to give a yield of 2.58 grams of pure dieldrin or 62.6% based on aldrin. The infrared spectrum was identical with that of an authentic sample of pure dieldrin and the melting point, 181° C., was the same as that of the authentic sample. The over-all yield of dieldrin based on barium carbonate was 22%. The specific activity was 1.32 mc. per mmole or 3.5 mc. per gram.

Because 79 mc. of C^{14} in 200 moles of barium carbonate were used, dieldrin with a specific activity of 4.2 mc. per gram would be expected. However, the 19% dilution of radioactive trichloroethylene with inert trichloroethylene in the preparation of octachlorocyclopentene results in a net expected activity of 3.4 mc. per gram. This is in remarkably good agreement with values obtained and indicates that no exchange of C^{14} occurred in any of the reactions involved.

Acknowledgment

The authors express their appreciation to the Agricultural Research Division, Shell Development Co., Modesto, Calif., and particularly to John C. Potter and William B. Burton of that company for valuable information and suggestions during the course of this work.

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FUNGICIDE DETERMINATION

Rapid Polarographic Determination of Pentachloronitrobenzene on Forage and Comparison with Spectrophotometric Method

C. A. BACHE and D. J. LISK

Pesticide Residue Laboratory, Department of Entomology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

A method is described for the polarographic determination of the fungicide pentachloronitrobenzene on forage. The chemical is extracted by tumbling with Skellysolve B. The extract is filtered and dried, and part of the extractives are removed by freezing and absorption on Attaclay. Chromatography of the concentrated extract using Florisil removes the remainder of the interfering substances. After chromatography the solvent is evaporated and the residue dissolved in isopropyl alcohol. Sodium acetate and acetic acid are added as a supporting electrolyte and after the solution has been deoxygenated, the polarogram is recorded from 0.00 to −1.15 volts against a saturated calomel electrode. Comparison data are presented from analysis of samples by this procedure and a spectrophotometric method.

PENTACHLORONITROBENZENE (PCNB) will control fungus diseases such as Sclerotinea crown rot and clubroot in forage. The method of Ackermann *et al.* (*1*) has been used here for the colorimetric determination of residues of the fungicide on red clover–timothy forage. Because of difficulties in the chromatographic cleanup, more than one analysis was required with some samples to obtain reliable results.

Webster and Dawson (*4*) developed a polarographic method for the determination of tetrachloronitrobenzene residues on potatoes. In the work reported, PCNB is determined polarographically by an adaptation of the Webster and Dawson procedure, after interfering substances have been removed by freezing and adsorption on Attaclay and Florisil.

Analysis of Forage

Using the method of Ackermann *et al.* (*1*), extract 150 grams of finely ground forage material with 900 ml. of Skellysolve B by tumbling for 45 minutes. Filter the extract through a double thickness of cheesecloth into a separatory funnel. Dry the extract, using sodium sulfate. Add 5 grams of an equal-volume mixture of Celite 545–Attaclay to the separatory funnel and shake for about 1 minute. Allow the solid material to settle and drain off the filtrate through the sodium sulfate layer. Discard the first 50 ml. Take an aliquot of the extract up to 300 ml. and concentrate it to 20 ml. by distillation through a Snyder column. While warm, transfer the solution quantitatively to a 16 × 50 mm. test tube. Remove plant waxes by immersing the tube in a dry ice–isopropyl alcohol bath (about −50° C.) for 10 minutes according to the method of Ordas, Smith, and Meyer (*3*). The addition of a small amount of Celite will keep the precipitate dispersed and facilitate washing. Filter the cold extract through S. & S. 595 paper and wash the precipitated waxes with three 5-ml. portions of *n*-hexane previously cooled to −50° C. Combine the filtrate and washings, and concentrate the solution to 10 ml. by distillation through a Snyder column.

Pack a 15 × 300 mm. chromatographic column with 15 grams of unactivated Florisil. Wet the column with about 25 ml. of *n*-hexane and add the extract to the column. Elute the PCNB with 100 ml. of *n*-hexane and collect the eluate in a 125-ml. acetylization flask. Distill off the hexane through a Snyder column and remove the last traces with a gentle stream of air. Add 2.5 ml. of 0.04*N* acetic acid, 2.5 ml. of 0.04*N* sodium acetate, and 5 ml. of C.P. isopropyl alcohol. Reflux the mixture for 5 minutes, using a boiling water bath and an Allihn condenser. Remove the water bath and immerse the flask, with condenser attached, in cold water. After the solution has cooled, filter it through S. & S. 595 paper into the polarographic cell.

The polarographic determination was carried out in an H-cell similar to that of Lingane and Laitinen (*2*), except

The product was recrystallized twice from 10 ml. of isopropyl alcohol to give a yield of 2.58 grams of pure dieldrin or 62.6% based on aldrin. The infrared spectrum was identical with that of an authentic sample of pure dieldrin and the melting point, 181° C., was the same as that of the authentic sample. The over-all yield of dieldrin based on barium carbonate was 22%. The specific activity was 1.32 mc. per mmole or 3.5 mc. per gram.

Because 79 mc. of C^{14} in 200 moles of barium carbonate were used, dieldrin with a specific activity of 4.2 mc. per gram would be expected. However, the 19% dilution of radioactive trichloroethylene with inert trichloroethylene in the preparation of octachlorocyclopentene results in a net expected activity of 3.4 mc. per gram. This is in remarkably good agreement with values obtained and indicates that no exchange of C^{14} occurred in any of the reactions involved.

Acknowledgment

The authors express their appreciation to the Agricultural Research Division, Shell Development Co., Modesto, Calif., and particularly to John C. Potter and William B. Burton of that company for valuable information and suggestions during the course of this work.

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FUNGICIDE DETERMINATION

Rapid Polarographic Determination of Pentachloronitrobenzene on Forage and Comparison with Spectrophotometric Method

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A method is described for the polarographic determination of the fungicide pentachloronitrobenzene on forage. The chemical is extracted by tumbling with Skellysolve B. The extract is filtered and dried, and part of the extractives are removed by freezing and absorption on Attaclay. Chromatography of the concentrated extract using Florisil removes the remainder of the interfering substances. After chromatography the solvent is evaporated and the residue dissolved in isopropyl alcohol. Sodium acetate and acetic acid are added as a supporting electrolyte and after the solution has been deoxygenated, the polarogram is recorded from 0.00 to −1.15 volts against a saturated calomel electrode. Comparison data are presented from analysis of samples by this procedure and a spectrophotometric method.

PENTACHLORONITROBENZENE (PCNB) will control fungus diseases such as Sclerotinea crown rot and clubroot in forage. The method of Ackermann *et al.* (*1*) has been used here for the colorimetric determination of residues of the fungicide on red clover–timothy forage. Because of difficulties in the chromatographic cleanup, more than one analysis was required with some samples to obtain reliable results.

Webster and Dawson (*4*) developed a polarographic method for the determination of tetrachloronitrobenzene residues on potatoes. In the work reported, PCNB is determined polarographically by an adaptation of the Webster and Dawson procedure, after interfering substances have been removed by freezing and adsorption on Attaclay and Florisil.

Analysis of Forage

Using the method of Ackermann *et al.* (*1*), extract 150 grams of finely ground forage material with 900 ml. of Skellysolve B by tumbling for 45 minutes. Filter the extract through a double thickness of cheesecloth into a separatory funnel. Dry the extract, using sodium sulfate. Add 5 grams of an equal-volume mixture of Celite 545–Attaclay to the separatory funnel and shake for about 1 minute. Allow the solid material to settle and drain off the filtrate through the sodium sulfate layer. Discard the first 50 ml. Take an aliquot of the extract up to 300 ml. and concentrate it to 20 ml. by distillation through a Snyder column. While warm, transfer the solution quantitatively to a 16 × 50 mm. test tube. Remove plant waxes by immersing the tube in a dry ice–isopropyl alcohol bath (about −50° C.) for 10 minutes according to the method of Ordas, Smith, and Meyer (*3*). The addition of a small amount of Celite will keep the precipitate dispersed and facilitate washing. Filter the cold extract through S. & S. 595 paper and wash the precipitated waxes with three 5-ml. portions of *n*-hexane previously cooled to −50° C. Combine the filtrate and washings, and concentrate the solution to 10 ml. by distillation through a Snyder column.

Pack a 15 × 300 mm. chromatographic column with 15 grams of unactivated Florisil. Wet the column with about 25 ml. of *n*-hexane and add the extract to the column. Elute the PCNB with 100 ml. of *n*-hexane and collect the eluate in a 125-ml. acetylization flask. Distill off the hexane through a Snyder column and remove the last traces with a gentle stream of air. Add 2.5 ml. of 0.04*N* acetic acid, 2.5 ml. of 0.04*N* sodium acetate, and 5 ml. of C.P. isopropyl alcohol. Reflux the mixture for 5 minutes, using a boiling water bath and an Allihn condenser. Remove the water bath and immerse the flask, with condenser attached, in cold water. After the solution has cooled, filter it through S. & S. 595 paper into the polarographic cell.

The polarographic determination was carried out in an H-cell similar to that of Lingane and Laitinen (*2*), except

Table I. Recovery of PCNB from Alfalfa–Clover–Bird's-foot Trefoil Forage

Added, P.P.M.	Recovery, %
0.2	130
0.2	120
0.2	110
0.5	98
1.0	83
2.0	100
2.0	76
2.0	78.5

Check (average of 4 analyses), 0.34 p.p.m.

Table II. Determination of PCNB on Forage[a]

Days after Application	Residue, P.P.M. Spectrophotometric	Polarographic
7	32.0	30.0
	2290.0	1950.0
	725.0	744.0
	416.7	522.0
21	15.6	12.8, 13.2
	62.0	69.6
	3.6	7.7
	31.3	70.0, 73.5
	2.4	4.6, 3.8
	48.8	79.8, 78.0
35	1.8	3.3, 3.3
	35.6	35.2, 38.0
	18.8	17.4, 17.4
	14.3	11.9
	28.8	18.0, 18.6
	49.0	43.5

[a] Dry subsamples of same field sample.

that the outside diameter of the sample portion of the cell was reduced to 18 mm. to accommodate a smaller sample. A stopcock was attached to the base of the sample holder to facilitate cleaning. The entire sample was added to the H-cell and deoxygenated for 10 minutes with nitrogen. The polarogram was determined over the range of 0.00 to −1.15 volts against a saturated calomel electrode. The diffusion current was measured manually with a Fisher Elecdropode, which has a sensitivity range of 0.01 to 0.00001 equivalent per liter. The half-wave potential is −0.47 volt.

Standard Curve. Prepare a standard curve by adding 2-, 3-, 10-, 20-, and 30-ml. portions of a hexane solution of PCNB (5 μg. per ml.) to a series of 125-ml. acetylization flasks. Distill off the hexane through a Snyder column and remove the last traces with a gentle stream of air. Add 2.5 ml. each of the sodium acetate and acetic acid solutions and 5 ml. of isopropyl alcohol. Reflux for 5 minutes, cool, filter, and record the polarogram as in the analysis of forage.

Results and Discussion

The method was used to recover PCNB added to forage before extraction. Table I shows the recoveries obtained.

On May 6, 1957, PCNB was applied to clover-timothy forage plots for control of Sclerotinea crown rot. The rate of application was 75 pounds per acre, which was about 10 times the recommended rate. The PCNB was applied as a 75% wettable powder in 400 gallons of water per acre from a hand sprayer. The object was to study the disappearance of the fungicide with time after a very high rate of application. Samples were taken for residue analysis 7, 21, and 35 days after application. Table II shows PCNB found by the method of Ackermann *et al.* (*1*) and by the polarographic procedure.

Although very good agreement was obtained between the two methods, large differences can be observed among samples from the same field treatment. This variability might be due to errors in calibration of the hand sprayer, lack of agitation of the spray formulation during application, and variations in plant composition (differences in the percentage of weeds present) at harvest. Unfortunately, a satisfactory sample of check material from this experiment could not be obtained.

The half-wave potential shifted to a limit of about −0.53 volt during analysis of forage material. The magnitude of this shift was roughly proportional to the size of the sample. This did not affect interpretation of the polarogram, as no other waves occur in this region.

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FUNGICIDE RESIDUES

Colorimetric Estimation of Dodecylguanidine Acetate Residues

W. A. STELLER, K. KLOTSAS, E. J. KUCHAR,[1] and M. V. NORRIS

Central Research Division, American Cyanamid Co., Stamford, Conn.

THE FUNGICIDE *n*-dodecylguanidine acetate, Cyprex 65W [American Cyanamid's 65% dodine (coined name) formulated as a wettable powder], has been proved useful for control of specific plant diseases. It is especially effective against apple scab [*Venturia inaequalis* (Cke.) Wint.], pear scab (*Venturia pirina* Aderh.), and cherry leaf spot (*Coccomyces hiemalis* Hig.) Additional uses are being developed.

[1] Present address, Olin Mathieson Chemical Corp., New Haven, Conn.

In conjunction with the field research which established its utility, a satisfactory analytical method was required to obtain data on the rate of disappearance of *n*-dodecylguanidine acetate (dodine) residues and to establish that its use would not result in hazardous residues at harvest.

A method was developed for determining dodine residues on apples using a surface extraction procedure to remove the chemical from the fruit. Subsequently, Hamilton and Szkolnik (*7*) reported local penetrant properties for the fungicide into apple leaves. When this property of dodine was first recognized, a macerate extraction procedure for total residues was developed to evaluate the efficacy of the surface extraction technique for determining total dodine residues.

Many high molecular weight nitrogenous bases have been shown to form salt-like addition products with acidic dyes in buffered aqueous solution (*1, 4, 12*). These addition products partition favorably into water-immiscible organic solvents, such as chloroform and benzene.

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Many high molecular weight nitrogenous bases have been shown to form salt-like addition products with acidic dyes in buffered aqueous solution (*1, 4, 12*). These addition products partition favorably into water-immiscible organic solvents, such as chloroform and benzene.

A colorimetric method has been developed for the determination of *n*-dodecylguanidine acetate (dodine) residues in plant materials. A long-chain base cation, dodecylguanidine, is made to complex with an anionic dye, bromocresol purple, in a buffered aqueous alcohol solution. This complex is then extracted into chloroform. The bromocresol purple, proportional in amount to the DDG, is next extracted from the chloroform into aqueous alkali. The absorbance of this alkaline phase is measured spectrophotometrically at 590 mμ and is used to calculate the quantity of dodine present. The method is applicable to determining residues of dodine varying from 0.2 to 2.6 p.p.m. in a 50-gram sample. Residues from apples, apple leaves, pears, and sour cherries have been measured.

The dye equivalent of the nitrogenous base can then be extracted into aqueous alkali and its absorbance read at the proper wave length. Dodecylguanidine (DDG) was found to form with bromocresol purple a complex which could be extracted satisfactorily into chloroform. The analytical procedure which has been developed for determining residues of dodecylguanidine utilizes this principle.

Surface residues are extracted by tumbling a representative fruit sample with methanol; total residues, by macerating the fruit sample with a 2 to 1 (v./v.) mixture of methanol and chloroform. After a preliminary cleanup of the extract, the DDG is complexed with bromocresol purple in a buffered water-alcohol solution and the complex is then extracted into chloroform. The chloroform solution is shaken with aqueous alkali which allows the bromocresol purple equivalent to the DDG to be extracted completely into the aqueous alkaline phase. The absorbance of this phase at 590 mμ is a measure of the dodine present. Calibration curves prepared by the procedure obey Beer's law between 10 and 130 γ of dodine. The method (except for the leaf modification) is applicable to determining concentrations of dodine from 0.2 to 2.6 p.p.m. in a 50-gram sample. In some instances, the range has been extended by varying the sample size or by diluting the final alkaline indicator phase with 0.05*N* sodium hydroxide before measuring the color intensity.

The applicability of the method was tested by analyzing samples fortified with known amounts of the fungicide. These results are shown in Tables I through III. To create surface residue, a methanolic solution of dodine was dripped on the sample and the solvent was allowed to evaporate overnight before analysis. For total residue, a methanolic solution of dodine was added during maceration and the macerate was allowed to stand at least 30 minutes before analysis.

Table I. Recovery of Dodine from Apples

Sample Wt., Grams	Dodine, P.P.M. Added	Dodine, P.P.M. Found	Recovery, %
	Surface Residues		
33	0	0.14	...
67	0	0.07	...
100	0	0.09	...
33	0.67	0.64	96
	0.67	0.64	96
	1.0	0.80	80
	1.0	0.80	80
	1.0	0.80	80
	1.0	0.81	81
	6.55	5.80	89
67	0.39	0.34	87
100	0.13	0.11	85
	Total Residues		
50	0	0.1[a]	...
100	0	0.06[b]	...
50	0.24	0.21	87
	0.32	0.23	72
	0.54	0.38	70
	0.59	0.62	105
	0.76	0.55	72
	1.18	1.13	96
	2.50	2.10	84
100	0.12	0.14	117
	0.10	0.07	70
	0.20	0.18	90
	0.30	0.23	77

[a] Average of 5 analyses with standard deviation of 0.023.
[b] Average of 5 analyses with standard deviation of 0.013.

Table II. Recovery of Dodine from Apple Leaves and Cherries

Sample Wt., Grams	Dodine, P.P.M. Added	Dodine, P.P.M. Found	Recovery, %
	Apple Leaves		
10	0	0.66	...
	0	0.47	
20	0	0.37	...
30	0	0.32	...
20	0.5	0.60	120
30	0.5	0.43	86
10	1.0	1.8	180
20	1.3	1.2	92
10	2.6	2.6	100
20	2.6	1.9	73
	3.0	2.7	90
10	3.9	3.4	87
	3.9	3.4	87
	5.2	4.4	85
	5.2	4.9	94
	6.5	5.2	80
	Cherries		
50	0	0.07[a]	...
	0.13	0.16	123
	0.13	0.16	123
	0.26	0.21	81
	0.26	0.21	81
	0.26	0.18	69
	0.52	0.37	71
	0.52	0.39	75
	0.52	0.32	62
	0.52	0.32	62
	0.78	0.48	62
	0.78	0.48	62
	0.78	0.55	71
	0.78	0.58	74
	1.04	0.66	63
	1.04	0.80	77
	1.04	0.76	73
	1.04	0.75	72
	1.04	0.70	67
	1.04	0.65	63
	1.04	0.71	68
	1.30	0.86	66
	1.30	0.90	69
	1.30	0.85	65

[a] Average of 11 analyses with standard deviation of 0.025.

Procedure

Reagents. Dodecylguanidine Acetate (Dodine), purified sample, obtainable from Products Laboratory, Agricultural Division, American Cyanamid Co., Box 672, Princeton, N. J.

Bromocresol Purple Reagent (high purity material is essential). Three lots purchased thus far from Hartman and Leddon Co., Philadelphia, Pa., have been satisfactory without purification. Different lots from other suppliers often produced calibration curves with varying slopes. Related impurities, such as cresol red and bromocresol green, are believed responsible for these effects. Reproducible calibration curves could not be prepared from samples in which cresol red was detected. A new calibration curve should be prepared when-

Table III. Recovery of Dodecylguanidine Acid Phthalate from Apples

Sample Wt., Grams	Dodecylguanidine Acid Phthalate, P.P.M. Added	Dodecylguanidine Acid Phthalate, P.P.M. Found	Recovery, %
50	0	0.23	...
	0	0.31	...
	0	0.27	...
	0	0.27	...
	0.26	0.19	73
	0.26	0.24	92
	0.52	0.44	85
	0.52	0.50	96
	0.78	0.79	101
	0.78	0.59	76
	1.04	1.00	96
	1.30	1.29	99
	1.30	1.27	98
	1.30	1.11	85

ever a different lot of bromocresol purple is put into use.

A completely reliable purification technique has not been developed but the following recrystallization procedure (*8*) has proved suitable in most cases: Add 15 grams of bromocresol purple to 500 ml. of toluene and heat to 85° C. while stirring constantly; filter off the insoluble material and allow the toluene solution to cool slowly to room temperature while stirring constantly; filter off the bromocresol purple crystals and allow them to air-dry.

The following screening test was found useful for detecting the presence of cresol red in bromocresol purple: Dissolve 50 mg. of sample in toluene and pass the solution through a 1 × 15 cm. column containing activated Florisil (magnesium silicate gel from Floridin Co., Tallahassee, Fla., 60- to 100-mesh; activate by slurrying with 3*N* hydrochloric acid, wash free of chloride with water, and heat at 790° C. for 4 hours). Elute the column with 5% butanol in toluene and extract the eluate with aqueous alkali (10 ml.). A red-colored aqueous solution having an absorption peak at 572 mμ indicates the presence of cresol red (*6*). If the absorbance exceeds 0.100 at 572 mμ, the material should be recrystallized.

Bromocresol Purple Solution. Dissolve 0.4 gram of bromocresol purple in 75 ml. of 0.01*N* sodium hydroxide, adjust to pH 6.0 to 6.1 with sodium hydroxide or hydrochloric acid, and dilute to 500 ml. with distilled carbon dioxide–free water. Store in a stoppered brown bottle and allow to age at least 24 hours before use. This solution is generally stable for at least 1 month.

Buffer Solution, pH 5.5. Dissolve 15.2 grams of disodium hydrogen phosphate ($Na_2HPO_4 . 7H_2O$) and 74.0 grams of sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$) in distilled carbon dioxide–free water and dilute to 1 liter with water.

Special Apparatus. Beckman Model DU Spectrophotometer (or equivalent) with 1-cm. cells.

Waring Blendor (or equivalent), 1-quart size.

Tumbling Machine, motor-driven rollers geared to produce agitation at about 40 r.p.m.

Surface Residues on Apples

Calibration Curve. Dissolve approximately 32.5 mg. of purified dodine in 250 ml. of synthetic grade methanol (99.85% minimum) in a volumetric flask. Transfer a 25-ml. aliquot to another 250-ml. volumetric flask and dilute to volume with methanol (1 ml. is equivalent to about 13 γ of dodine). Using this standard solution, take aliquots of 0, 1, 2, 3, 4, and 5 ml. through the following procedure.

Place each aliquot in a 400-ml. beaker, dilute to 100 ml. with methanol, and add 50 ml. of water, 30 ml. of 30% sodium chloride solution, and 1 ml. of concentrated hydrochloric acid. Adjust the pH to about 5.5 with 4*N* sodium hydroxide. Add 20 ml. of buffer and 20 ml. of bromocresol purple solution and readjust the pH to 5.5 by means of a pH meter (the pH rises to 6.0 to 6.5 probably because of the change in the ionization of the phosphate salts by the alcohol present).

Transfer the solution to a 500-ml. separatory funnel and extract the DDG–bromocresol purple complex into chloroform (U.S.P. grade) by shaking for 2 minutes with each of two 50-ml. portions of this solvent. Shake the combined chloroform layers with 20 ml. of 0.05*N* sodium hydroxide for 1 minute to remove all free and combined bromocresol purple. Recomplex the DDG which remains in the chloroform as the free base by shaking this phase for 3 minutes with 5 ml. of the bromocresol purple solution and 20 ml. of the buffer solution. Extract any excess free bromocresol purple from the chloroform by shaking with 15 ml. of buffer for 1 minute. Transfer the chloroform layer to a clean, dry 250-ml. separatory funnel and shake for 2 minutes with 20 ml. of 0.05*N* sodium hydroxide (measured by pipet).

Read the absorbance of the aqueous layer at 590 mμ in a 1-cm. cell using 0.05*N* sodium hydroxide as a reference. Plot micrograms of dodine *vs.* corresponding absorbance (corrected for a reagent blank). Twenty micrograms of dodine produces a color having an absorbance of about 0.170. The calibration curve between 10 and 65 γ is essentially a straight line with a slope of 10.2 (absorbance *vs.* milligrams of dodine).

Determination. Extract a representative sample of apples with a suitable measured volume of methanol by rolling in a sealed bottle for 1 hour. Filter the extract through filter paper and wash the paper with 10 ml. of methanol. For analysis, take a measured portion of the methanol extract which will contain 20 to 60 γ of dodine. Dilute to 100 ml. with methanol and add 1 ml. of concentrated hydrochloric acid. (If an aliquot larger than 100 ml. is taken, add 1 ml. of concentrated hydrochloric acid and concentrate to 100 ml. on a steam bath.) Add 80 ml. of 10% sodium chloride and extract the aqueous-alcohol phase twice with 50-ml. portions of carbon tetrachloride, shaking each time for 1 minute. Discard the carbon tetrachloride layers.

These extractions remove plant material which would cause considerable emulsion difficulty later in the procedure. If the second portion of carbon tetrachloride is not clear or if an interface persists between the two phases, extract the aqueous-alcohol phase a third time with carbon tetrachloride. Continue from that point in the calibration curve procedure beginning, "Adjust the pH of the aqueous-alcohol solution to about 5.5 with 4*N* sodium hydroxide," etc. Measure the absorbance of the alkaline indicator phase, and obtain the dodine equivalent from the calibration curve prepared in a similar manner.

Total Residues in Apples and Cherries

Calibration Curve. Follow the procedure described for the preparation of the calibration curve for surface residues on apples through the point where the DDG–bromocresol purple complex is extracted into chloroform from 220 ml. of the methanol-water phase. Then shake the combined chloroform layers twice with 25-ml. portions of the 5.5 buffer solution, the first for 30 seconds and the second for 1 minute. The buffer washes at this point are necessary to lower the control value (apparent micrograms of dodine in untreated samples) to an acceptable level.

Transfer the chloroform layer to another separatory funnel and shake for 2 minutes with 20 ml. of 0.05*N* sodium hydroxide. Recomplex the DDG in the chlorofrom layer by shaking for 3 minutes with 5 ml. of the bromocresol purple solution, 20 ml. of the buffer solution, 25 ml. of methanol, and 10 ml. of 30% sodium chloride. Transfer the chloroform to another separatory funnel, and shake for 1 minute each with 3 succesive 15-ml. portions of the buffer solution. Transfer the chloroform layer to a clean, dry separatory funnel, and shake for 2 minutes with 20 ml. of 0.05*N* sodium hydroxide (measured by pipet).

Read the absorbance of the aqueous alkali at 590 mμ in a 1-cm. cell using 0.05*N* sodium hydroxide as a reference. Prepare a calibration curve by plotting the absorbance *vs.* the micrograms of dodine. The reagent blank is negligible in this procedure. Twenty micrograms of dodine produces a color having an absorbance of about 0.150. The calibration curve between 10 and 65 γ is essentially a straight line with a slope of 9.4 (absorbance *vs.* milligrams of dodine).

Determination. Macerate a representative sample of apples or pitted cherries in a Waring Blendor with 2-to-1(v./v.) methanol-chloroform using the ratio of 400 ml. of solvent per 100-gram sample. Filter the pulp from the extract by suction using two pieces of No. 1 Whatman filter paper. Wash the pulp by slurrying it with 2-to-1 methanol chloroform using the ratio of 100 ml. of solvent per 100-gram sample. (Wash cherry pulp a second time with an additional 100 ml. of methanol per 100-gram sample.)

Measure the volume of the extract and transfer a portion equivalent to a 50-gram sample to a 400-ml. beaker. Add 1 ml. of concentrated hydrochloric acid and several small glass beads, and evaporate to 50 ml. on a steam bath. Stir occasionally until boiling begins to avoid loss due to superheating. Add 30 ml. of aqueous 30% sodium chloride and 100 ml. of methanol. Allow the solu-

tion to cool and transfer it to a 500-ml. separatory funnel.

Extract gently with 50 ml. of carbon tetrachloride by inverting the separatory funnel and returning it to the upright position six to eight times. Discard the carbon tetrachloride layer and repeat the extraction with another 50 ml. of carbon tetrachloride. Allow the phases to separate and discard the carbon tetrachloride. Gentle agitation with carbon tetrachloride is essential at this point to prevent the formation of a stable emulsion. Two extractions usually remove sufficient emulsifying materials so that vigorous shaking is possible for further extractions. Continue to extract with 50-ml. portions of carbon tetrachloride until the carbon tetrachloride extract is clear and colorless. Do not draw off the interphase until after the final extraction.

Transfer the aqueous methanolic extract to a 400-ml. beaker, and adjust the pH to 5.5 with 4*N* sodium hydroxide. Add 20 ml. of buffer and 20 ml. of bromocresol purple solution and readjust the pH to 5.5 by means of a pH meter. Transfer the solution to a 500-ml. separatory funnel. Extract with an additional 50-ml. portion of carbon tetrachloride to remove completely the last trace of that solvent which separates as an oily phase when the water concentration in the aqueous phase is increased. Discard the carbon tetrachloride. Extract the DDG–bromocresol purple complex into chloroform by shaking with each of two 50-ml. portions of this solvent for 2 minutes. Continue from that point in the calibration curve procedure which reads, "Then shake the combined chloroform layers with 25 ml. of the 5.5 buffer solution," etc. Measure the absorbance of the alkaline indicator phase and obtain the dodine equivalent from the calibration curve prepared by a similar procedure.

Total Residues in Apple Leaves

Calibration Curve. Carry aliquots of 0, 1, 2, 3, 4, and 5 ml. of the diluted standard solution (13 γ per ml.) through the following procedure: Dilute the aliquot in a 500-ml. separatory funnel with 100 ml. of methanol, 100 ml. of sodium chloride solution, and 5 ml. of 10*N* sodium hydroxide solution. Extract the DDG from this solution by shaking for 1 minute with each of six 50-ml. portions of technical grade carbon tetrachloride (because of an unfavorable distribution, approximately 10 to 15% of the DDG is lost at this point as it still remains in the aqueous phase after six extractions).

To the combined carbon tetrachloride layers, add 50 ml. of methanol and 1 ml. of concentrated hydrochloric acid, and mix well. Then add 50 ml. of aqueous 20% sodium chloride solution and shake for 2 minutes. Transfer the carbon tetrachloride layer to another separatory funnel, add methanol, hydrochloric acid, and aqueous sodium chloride, and extract as before. Combine the two aqueous-alcohol phases containing the DDG; shake for 30 seconds with each of two 50-ml. portions of carbon tetrachloride and discard the carbon tetrachloride. Add 5 ml. of 10*N* sodium hydroxide and 50 ml. of chloroform to the aqueous-alcohol phase. Shake for 1 minute and transfer the chloroform to a clean 250-ml. separatory funnel. Repeat the extraction of the aqueous-alcohol phase with another 50-ml. portion of chloroform. Combine the two chloroform extracts; add 20 ml. of 0.05*N* sodium hydroxide and shake for 1 minute.

Transfer the chloroform layer to another 250-ml. separatory funnel. Add 20 ml. of the buffer and 5 ml. of the bromocresol purple solution, and shake for 3 minutes. Transfer the chloroform layer to another 250-ml. separatory funnel, and wash it with two 15-ml. portions of the buffer solution by shaking with each for 1 minute. Then transfer the chloroform phase to a clean, dry 250-ml. separatory funnel. Add 20 ml. of 0.05*N* sodium hydroxide (measured by pipet) and shake for 2 minutes. Read the absorbance of the aqueous phase at 590 mμ in a 1-cm. cell using 0.05*N* sodium hydroxide as a reference. Prepare a calibration curve by plotting the absorbance *vs.* micrograms of dodine.

A working calibration curve can also be prepared by the following condensed procedure: Dilute the standard solution aliquot with methanol, aqueous sodium chloride solution, and alkali, as described in the above procedure, and then shake with each of two 50-ml. portions of chloroform for 1 minute. Continue as described above beginning with, "Combine the two chloroform extracts; add 20 ml. of 0.05*N* sodium hydroxide and shake for 1 minute."

The longer procedure is more representative of the one actually used for analysis of plant extracts. Results based upon a calibration curve prepared by the shorter procedure will be approximately 20% lower, because the curve does not compensate for successive small losses of DDG which occur when the samples are carried through the longer multiextraction procedure.

Determination. Disintegrate 50 grams of apple leaves (without solvent) in about 10-gram portions in a Waring Blendor. Extract the leaves by tumbling mechanically for 1 hour in a 1-quart jar with 500 ml. of methanol. Separate the extract from the pulp by filtering through paper. Transfer an aliquot of the extract which will contain 10 to 60 γ of dodine to a 500-ml. separatory funnel, dilute to 100 ml. with methanol (if an aliquot of more than 100 ml. is taken for analysis, evaporate to 100 ml.), and add 100 ml. of 20% aqueous sodium chloride solution and 5 ml. of 10*N* sodium hydroxide solution. Continue as described for preparation of the calibration curve beginning, "Extract the DDG from this solution by shaking," etc. Measure the absorbance of the alkaline indicator phase at 590 mμ and obtain the dodine equivalent from the calibration curve prepared by a similar procedure.

Results and Discussion

All recovery and field sample data reported in this paper have been corrected for control values—i.e., the amount of "apparent" dodine found when untreated samples are carried through the analytical procedure. Appropriate controls should be run for each type of material being analyzed.

The data reported in Table III were obtained using the acid phthalate rather than the acetate salt of dodecylguanidine. As expected, the procedure proved equally applicable for both salts, and the calibration curves were identical when equimolar concentrations were used as a basis for comparison.

The recovery data shown in Tables I (total residues) and III were obtained by a slight modification of the procedure recommended. Methanol and sodium chloride were not added at the point in the procedure which reads, "Recomplex the DDG in the chloroform layer by shaking for 3 minutes with 5 ml. of the bromocresol purple solution, 20 ml. of the buffer solution, 25 ml. of methanol, and 10 ml. of 30% sodium chloride." During subsequent work addition of methanol and sodium chloride helped to eliminate an occasional erratic value obtained during preparation of the calibration curve. These erratic values were a result of the dodecylguanidine–bromocresol purple complex forming an insoluble film between the aqueous and chloroform layers which could not be extracted quantitatively into the chloroform layer. This difficulty was not encountered during analysis of apples, and either procedure was found applicable.

When residues are quite low it may occasionally be desirable to analyze extracts representing 100 grams of sample. To do this, the aliquot is acidified with 2 ml. of concentrated hydrochloric and evaporated to 100 ml. on a steam bath. Nine grams of sodium chloride and 100 ml. of methanol are added, and the solution is extracted with carbon tetrachloride and analyzed as described for total residues. Control values for samples of 100 grams were generally of the same magnitude (parts per million) as those for 50 grams.

A comparison of surface *vs.* total residues found on field sprayed apples (Table IV) indicates that the more simplified surface residue method may be adequate for measuring total residue levels at harvest.

Typical rate of disappearance data is shown in Table V. Surface and total residues were determined on McIntosh apples sampled from trees receiving no dodine after second, third, fourth, and fifth cover sprays, respectively. In general, the data indicate a fair degree of uniformity in the rate of disappearance of residues. The value of 0.9 p.p.m. of total residue found on the one harvest sample (Tables IV and V) taken 56 days after the last spray is believed due to inadequate sampling, and this value therefore is not considered typical. Rate of disappearance studies made on McIntosh, Cortland, and Red Delicious apples have indicated no significant differences among varieties.

The recoveries obtained from cherry samples fortified with dodine are lower than those from apples. Some material appears to be present in cherries which has a slightly adverse effect on the partitioning of the DDG–bromocresol purple complex between the solvents involved. The recommended calibration curve procedure for total residues in both apples and cherries does not include the series of lengthy preliminary cleanup extractions required when sample extracts are analyzed. Incorporation of these preliminary extractions in the calibration curve procedure would automatically produce recoveries 15 to 25% higher than those reported. A similar situation has been mentioned in connection with residues on apple leaves. The residue data shown in Table II for apple leaves, however, were obtained by use of a calibration curve which included these cleanup extractions.

The methods described have been applied successfully by several independent investigators to the determination of residues of dodine in apples (*2, 3, 5, 9, 10*) and pears (*11, 13*).

Table IV. Comparison of Surface vs. Total Residues of Dodine on Field-Sprayed Apples

(Number of sprays, 11)

Days since Last Spray	Dodine Found, P.P.M.	
	Surface	Total
3	1.3	0.9
14	0.7, 0.7	0.5, 0.5
35	0.3, 0.3	0.3, 0.3
56	1.0, 1.0	0.9

Table V. Residues of Dodine on Apples from Trees in Field Spray Program

Rate of Application[a]	Last Spray Applied	No. of Sprays	Days since Last Spray	Dodine Found, P.P.M.
	SURFACE RESIDUES			
0.75	2nd cover 6/7/57	7	13	3.7
			22	0.3
			33	0.6
	Harvest		91	0
	3rd cover 6/18	8	12	1.2
			23	1.2
	Harvest		81	0
	4th cover 6/28	9	12	0.7
	Harvest		70	0.1
	5th cover 7/9	10	1	2.5
	Harvest		59	0.1
2	2nd cover 6/7/57	7	13	20.8
			22	2.6
			33	3.8
	Harvest		91	1.0
	3rd cover 6/18	8	12	5.0
			23	4.5
	Harvest		81	0.4
	4th cover 6/28	9	12	4.0
	Harvest		70	0.6
	5th cover	10	1	10.0
	Harvest		59	0.7
	TOTAL RESIDUES			
0.5	2nd cover 6/6/58	8	48	0.3
			69	0
			90	0
			111	0
	3rd cover 7/10	9	14	0.4
			35	0.4
			56	0.1
			77	0
	4th cover 7/21	10	66	0.2
	5th cover 7/31	11	3	0.4
			14	0.5
			35	0.2
			56	0.2
0.75	2nd cover 6/6/58	8	48	0.3
			69	0
			90	0
			111	0
	3rd cover 7/10	9	14	0.4
			35	0.4
			56	0.2
			77	0.1
	4th cover 7/21	10	66	0.2
	5th cover 7/31	11	3	0.9
			14	0.5
			35	0.3
			56	0.9

[a] Pounds of wettable powder (65% actual dodine) per 100 gallons.

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Dodecylguanidine Acetate (Dodine) Residues on Apples

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The colorimetric method of Steller *et al.* was successfully used to analyze 73 samples of apples for dodine residues. Samples receiving no dodine showed an average apparent dodine residue of 0.10 p.p.m. Fifty-six samples of apples sprayed with Cyprex 65-W at rates of from 0.5 to 1.75 pounds per 100 gallons of water contained residues at harvest up to 1.29 p.p.m., with an average residue of 0.28 p.p.m. Disappearance studies showed residues after 33 days of 0.2, 0.4, and 0.6 p.p.m., respectively, from applications of 0.25, 0.50, and 0.75 pound of 65% dodine wettable powder formulation.

A COLORIMETRIC METHOD for the determination of *n*-dodecylguanidine acetate (dodine) in plant materials has been described by Steller *et al.* (*1*). During the 1958 season, the authors analyzed a considerable number of apple samples for dodine residues by this method and felt that the experience with the method and the results obtained would be of interest to others.

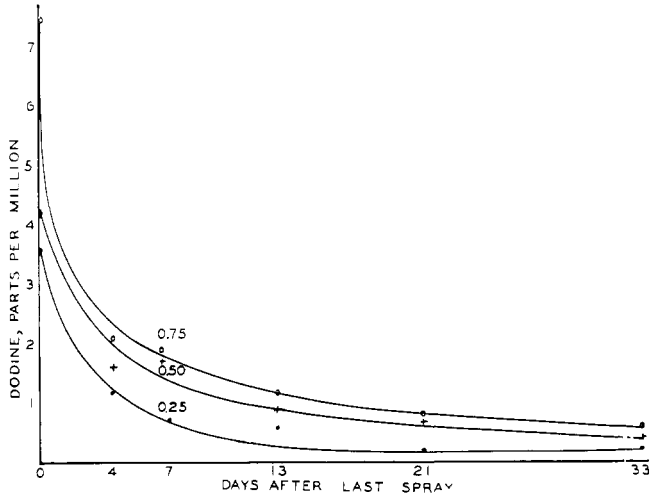
Results and Discussion

In general, the method has given excellent results when applied to apples. In all cases, 100-gram samples were macerated in a Waring Blendor; recoveries of dodine added to apple samples were from 70 to 115%, and were thus in the same range as reported by Steller *et al.* (*1*). Seventeen apple samples not sprayed with dodine were analyzed to establish blank values (Table I). These apples represented nine varieties from seven localities in the East. The maximum blank value was 0.20 p.p.m. and the minimum 0.05 p.p.m., with a mean of 0.10 p.p.m. There appeared to be no relation between variety and the apparent dodine found, and the earlier spray history of other fungicides (Captan, Ferbam, or Glyodin) did not affect the blank values.

As shown in Table II, 56 apple samples were analyzed for dodine residues at harvest. These were collected from experimentally sprayed orchards from Michigan to Maine and included 10 varieties. The complete spray history of every sample was known and is indicated in the table. Dodine was applied as Cyprex 65-W, a wettable powder formulation containing 65% dodine (American Cyanamid Co.). Rates of application varied from 0.5 to 1.75 pounds of Cyprex

Table I. Apparent Dodecylguanidine Acetate Residues on Harvest Samples of Apples Not Sprayed with Dodine

Variety	Location	Treatment	Apparent Dodine, P.P.M.
Cortland	Wooster, Ohio	No fungicide	0.07
	E. Lansing, Mich.	Captan, 2 lb.	0.05
	Geneva, N. Y.	Captan, 2 lb.	0.19
Golden Delicious	Urbana, Ill.	No fungicide	0.08
	Newark, Del.	No fungicide	0.15
Jonathan	Newark, Del.	No fungicide	0.20
	Urbana, Ill.	No fungicide	0.09
	Mich.	No fungicide	0.08
McIntosh	E. Lansing, Mich.	Captan, 2 lb.	0.12
	Geneva, N. Y.	Ferbam, 1.5 lb.	0.14
	Geneva, N. Y.	Ferbam, 1.5 lb.	0.15
Red Delicious	Newark, Del.	No fungicide	0.13
	Monroeville, N. J.	Unknown	0.08
	Staunton, Va.	Glyodin and Ferbam	0.04
Rome Beauty	Wooster, Ohio	No fungicide	0.05
Stayman	Mich.	No fungicide	0.05
York Imperial	Staunton, Va.	Glyodin and Ferbam	0.07
		Mean	0.102



Cyprex 65-W (containing 65% dodine) applied at 0.25, 0.50, and 0.75 pounds per 100 gallons. Date of last spray July 31, 1958

Figure 1. Disappearance curves for dodine on Golden Delicious apples in North Carolina

Table II. Residues of *n*-Dodecylguanidine Acetate (Dodine) on Apples at Harvest

Variety	Location	Rate[a]	No. of Sprays	Insecticides	Interval[b]	Dodine,[c] P.P.M.
Grimes Golden	Delaware	0.5	13	Dieldrin	32	0.01
	Delaware	0.5	13	Systox, lead arsenate	32	0
Jonathan	Delaware	0.5	13	Dieldrin	32	0.08
	Delaware	0.5	13	Genite	32	0.08
	Delaware	0.5	13	Systox, lead arsenate	32	0.08
Red Delicious	Delaware	0.5	13	DDT	32	0.22
	Delaware	0.5	13	TDE, Kelthane	32	0.23
Rome Beauty	Delaware	0.5	13	Aramite	32	0.06
	Delaware	0.5	13	Parathion	32	0.12
McIntosh	Maine	0.5	15	Lead arsenate	32	0.10
	Maine	0.5	15	DDT	32	0.08
Cortland	New York	0.5	11	Aramite,	69	0.04
	New York	0.5	11	TDE, DDT,	69	0.08
	New York	0.5	11	TEPP, du	69	0.03
McIntosh	New York	0.5	11	Pont S. S.	58	0.19
	New York	0.5	11		58	0.19
	New York	0.5	11		58	0.12
Jonathan	Michigan	0.75	7	DDT, malathion	20	0.36
	Michigan	0.75	7	DDT, malathion	20	0.28
Stayman	Michigan	0.75	7	Systox, TDE	71	0.03
	Michigan	0.75	7	Systox, TDE	71	0.05
	Michigan	0.75	7	Systox, TDE	71	0.07
Rome Beauty	Virginia	0.75	9	Aramite,	42	0.52
	Virginia	0.75	9	TDE,	42	0.51
	Virginia	0.75	9	DDT,	42	0.55
	Virginia	0.75	9	dieldrin,	42	0.30
	Virginia	0.75	9	malathion	42	0.31
	Virginia	0.75	9		42	0.26
Red Delicious	New Jersey	0.75	[d]	Parathion, oil	12	0.46
	New Jersey	0.75	[d]	Parathion, oil	12	0.54
	New Jersey	0.75	[d]	Parathion, oil	12	0.55
	New Jersey	0.75	[d]	Parathion, oil	12	0.69
Cortland	Ohio	0.75	10	TDE, DDT,	55	1.23
	Ohio	0.75	10	lead arse-	55	1.29
	Ohio	0.75	10	nate	55	1.28
Rome Beauty	Virginia	1.0	9	Aramite,	42	0.52
	Virginia	1.0	9	TDE,	42	0.51
Stayman	Virginia	1.0	9[e]	DDT, dieldrin, malathion	43	1.08
Cortland	Michigan	1.0	12	Methoxy-	55	0.09
	Michigan	1.0	12	chlor, lead	55	0.10
McIntosh	Michigan	1.0	12	arsenate,	55	0.25
	Michigan	1.0	12	parathion	55	0.16
	Michigan	1.0	12		55	0.13
Golden Delicious	Illinois	1.0	9	Malathion	72	0
	Illinois	1.0	9	DDT	72	0.02
Jonathan	Illinois	1.0	9	Malathion	72	0.05
	Illinois	1.0	9	DDT	72	0.07
Rome Beauty	Ohio	1.5	9	Lead arsenate	55	0.09
	Ohio	1.5	9	TDE, DDT	55	0.05
Lowery	Virginia	1.75	10	TDE, EPN,	53	0.26
	Virginia	1.75	10	lead arse-	53	0.32
	Virginia	1.75	10	nate	53	0.27
Red Delicious	Virginia	1.75	10	Aramite,	53	0.18
	Virginia	1.75	10	DDT,	53	0.13
York Imperial	Virginia	1.75	10	Ferbam,	53	0.17
	Virginia	1.75	10	parathion	53	0.14

[a] In pounds of Cyprex 65-W (65% dodine) per 100 gallons.
[b] Days between last spray application and harvest.
[c] Corrected for blank values.
[d] Only final spray contained Cyprex.
[e] Plus methoxychlor.

65-W per 100 gallons, and the number and composition of spray mixtures applied followed closely the local recommendations.

Because of the rather wide variation in intervals between the final spray application and harvest, it was impossible to obtain a correlation between the amount of dodine applied and the residues at harvest. The residues varied from 0 to 1.29 p.p.m., with only four out of 56 samples higher than 1.0 p.p.m. The average of all 56 samples was 0.28 p.p.m.

Apple trees of the Golden Delicious variety in North Carolina were sprayed 10 times with mixtures containing 4 pounds of malathion wettable powder per 100 gallons plus Cyprex 65-W at 0.25, 0.50, and 0.75 pound per 100 gallons. The dates of spray applications were April 9, 18, and 30; May 13 and 23; June 4 and 18; and July 2, 16, and 31. Samples were collected at 0, 4, 7, 13, 21, and 33 days after the last application. During this 33-day period, 3.75 inches of rain fell. The samples were analyzed by the method of Steller *et al.*, and the results, corrected for appropriate blanks, are given in Figure 1.

Figure 1 shows that dodine residues of 7.5, 4.2, and 3.6 p.p.m. immediately after the last spray application were reduced to 0.6, 0.4, and 0.2 p.p.m., respectively, 33 days later. It should be noted that the slopes of the individual curves in Figure 1 are not as steep as those for certain other pesticides, indicating a good degree of retention.

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FERTILIZER TECHNOLOGY

Prereactor for Liquid Raw Materials in Mixed Fertilizer Manufacture

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A continuous prereactor, consisting of a compact concentric pipe arrangement, has been developed in which all the liquid raw materials required for granulated mixed fertilizers are premixed and prereacted before introduction into the solid raw materials. The prereactor has been successfully operated in full scale production plants. Its advantages are substantial elimination of ammonium chloride fume and loss of free ammonia, simplicity of construction, flexibility of operation, and absence of toxic gas evolution.

Most conventional processes for the manufacture of granulated mixed fertilizer used in the United States consist of introduction of solid raw materials and recycle into an ammoniator into which the liquid raw materials are introduced separately underneath the bed of solids, which is kept in agitation by a tumbling or milling action (*3, 4*). The usual solid raw materials are one or more of the following: normal superphosphate, triple superphosphate, ammonium sulfate, potassium chloride, and potassium sulfate. The usual liquid raw materials are one or more of the following: water, sulfuric acid, phosphoric acid, anhydrous ammonia, aqueous ammonia, and nitrogen solution (an aqueous solution of ammonia, ammonium nitrate, and/or urea).

In the ammoniator liquids and solids are uniformly blended and certain desirable chemical reactions take place, primarily neutralization of sulfuric and phosphoric acids to form ammonium sulfate and ammonium phosphates, and reaction of ammonia with superphosphates to form ammonium phosphates and dicalcium phosphate.

Certain undesirable reactions may also take place. Some of the acids usually react with potassium chloride to form hydrochloric acid, which in turn reacts with ammonia to form ammonium chloride in the form of a dense white fume constituting an atmospheric pollutant. Removal of this fume from the mixer exhaust stack is difficult and requires highly efficient fume removal equipment. Other undesirable chemical reactions are decomposition of nitrogen compounds to nitrogen oxides and elemental nitrogen. This may result in significant loss of nitrogen and constitute a personnel hazard due to the toxic nature of some of the gases (*2*). Furthermore, under unusual circumstances concentrations of the decomposition gases may become great enough to cause flash fires in the ammoniator.

A further problem in conventional processes is incomplete absorption of ammonia by the superphosphates, which also results in loss of nitrogen and may cause unpleasant and unsafe working conditions around the ammoniator.

These problems can be overcome, or minimized, if the sulfuric and phosphoric acids are neutralized before introduction into the ammoniator. If this is done, there can be no reaction between potassium chloride and acid, and thus no ammonium chloride fume formation. Furthermore, under the conditions of uniform and intense mixing in a prereactor, there should be little or no decomposition of the nitrogen compounds, as this is believed due to the localized action of strong acids in excessive proportions on ammonium nitrate and urea in conventional ammoniators. Also, absorption of ammonia by the superphosphates should be improved, because there is no competition for the ammonia by acid. For some ratios—for example, 1–4–4—acids should be preneutralized in a way to preserve the heat of the neutralization reactions, as in such grades one of the principal functions of acid is to generate heat as an aid to granulation.

In recent years a number of preneutralization processes have been developed (*1, 5*). Most of these operate on the principle of neutralizing the acids in an open tank with substantially the stoichiometric proportion of ammonia or nitrogen solution and separate introduction of the additional ammonia or nitrogen solution required for ammoniation of the superphosphates. These processes are particularly advantageous for very high nitrogen ratios such as 2–1–1, where external removal of water and heat is helpful, and they have to a large extent overcome the above problems. However, they have certain disadvantages. Additional metering and control equipment is required, because the nitrogen solution or ammonia is introduced into the process in two streams rather than one. A fair-sized neutralization tank and exhaust equipment are required, which constitutes a space problem in many plants. When nitrogen solutions containing both ammonium nitrate and urea are used, a toxic gas may be formed which is dangerous to operators (*5*).

The present work was undertaken to develop a prereactor in which all of the liquid raw materials required for mixed fertilizer manufacture could be combined, reacted, and introduced into the ammoniator in a single stream. The advantages of such a prereactor were expected to be simplicity and compactness of construction, elimination of ammonium chloride fume, improvement of ammonia absorption by superphosphates, elimination of undesirable decomposition reactions by carrying out the preneutralization in the presence of a substantial excess of ammonia, and wide flexibility in grades that can be produced, including both low and high nitrogen ratios.

This paper is a progress report of the present state of development of a prereactor which has been shown in plant operation to have the above advantages. Further experience will undoubtedly result in improvements and may also reveal limitations not now recognized.

Laboratory Work

Laboratory tests were conducted with a continuous-flow concentric-pipe prereactor made from stainless steel tubing. Several different combinations of the liquids water, anhydrous ammonia, nitrogen solution (37% N, 66.8% NH_4NO_3, 16.6% free NH_3, and 16.6% H_2O), sulfuric acid (60° and 66° Bé.), and phosphoric acid (75% H_3PO_4) were preblended and prereacted, and then discharged into a stainless steel beaker.

In one series of tests sufficient sulfuric acid (60° Bé.) was prereacted with nitrogen solution to neutralize the free ammonia. No additional water was used and the products were discharged from the reactor pipe in the form of a thin boiling paste at temperatures as high as 250° F. Approximately 3 feet of reaction tube was needed to complete the neutralization and no clogging occurred.

To determine the most advantageous arrangement for blending water, anhydrous ammonia, and nitrogen solution, water and nitrogen solution were mixed satisfactorily in a simple tee. However,

the flow of both developed pulsations when anhydrous ammonia was added through a second tee. The pulsations were eliminated by replacing the second tee with a concentric pipe arrangement. The plant scale blending pipe was patterned after the laboratory device.

Phosphoric acid (75% H_3PO_4, wet process) and anhydrous ammonia were prereacted at a calculated NH_3–H_3PO_4 mole ratio of 1.5 in laboratory tests of limited scope. The purpose was to find a method for eliminating internal clogging of the reactor discharge tube section while producing concentrated ammonium phosphates. Clogging was avoided by blending approximately 2 pounds of water into each pound of ammonia previous to its introduction into the reactor section and feeding the acid through the inner pipe. To prevent clogging while starting the liquid flows, compressed air was fed through each liquid supply tube for a few minutes before and during each start-up.

Description of Prereactor

The general arrangement required to blend and react the liquids in simple concentric pipes was adequately established using laboratory apparatus. However, problems as to materials of construction, method of discharging the products into the ammoniator-granulator, and sizing of the components for efficient performance were necessarily solved in plant scale development work. The Davison Trenton process plant at Curtis Bay, Md., was used in the initial plant tests. An inexpensive mild steel prototype with very thick reactor and discharge pipe walls ($^3/_4$-inch wall thickness) was installed and used in normal granulation operations. The sections of the expendable test unit were joined with standard pipe flanges and thus could be removed and altered or replaced with a minimum of time and expense. If malfunction or poor performance of any one component was observed, an improved replacement for the component was fabricated, installed, and observed in operation. In this manner, the final prereactor design evolved.

Figure 1 shows the details of the prereactor now being used in Trenton process granulation operations. Each liquid raw material is metered and sup-

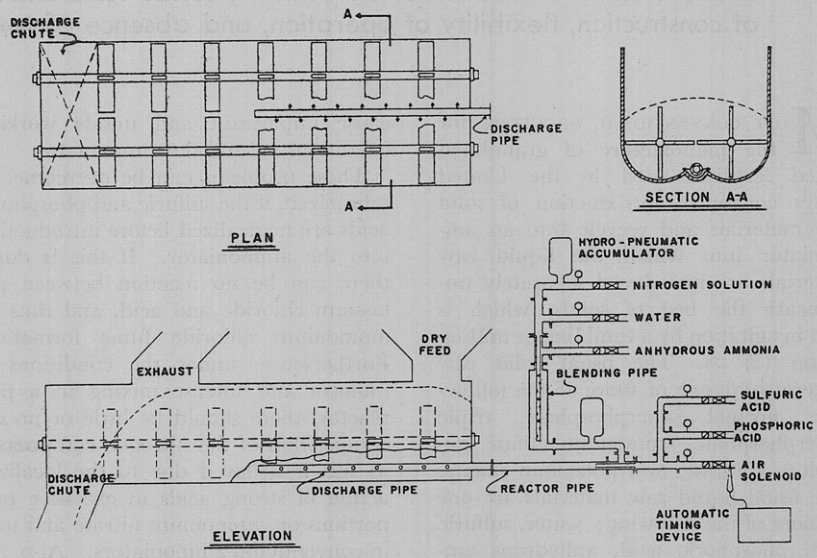

Figure 1. Arrangement of prereactor in granulation pug mixer

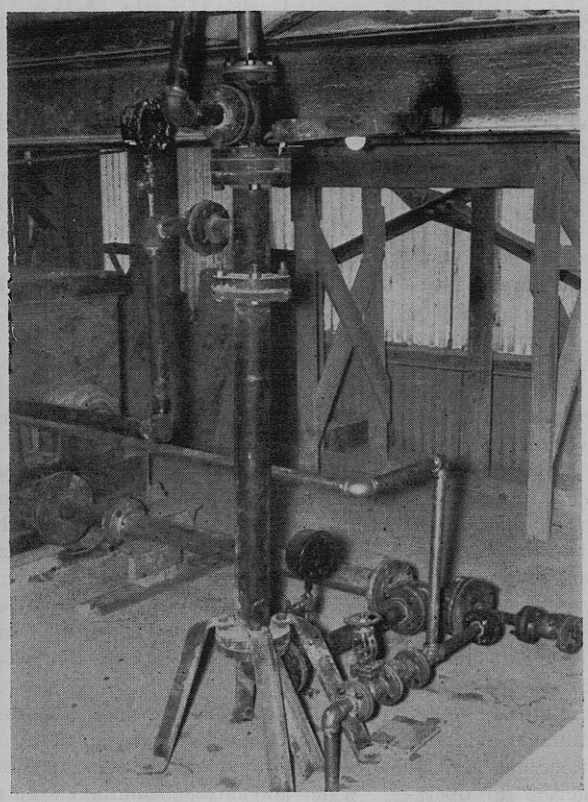

Figure 2. Prereactor in early stage of development

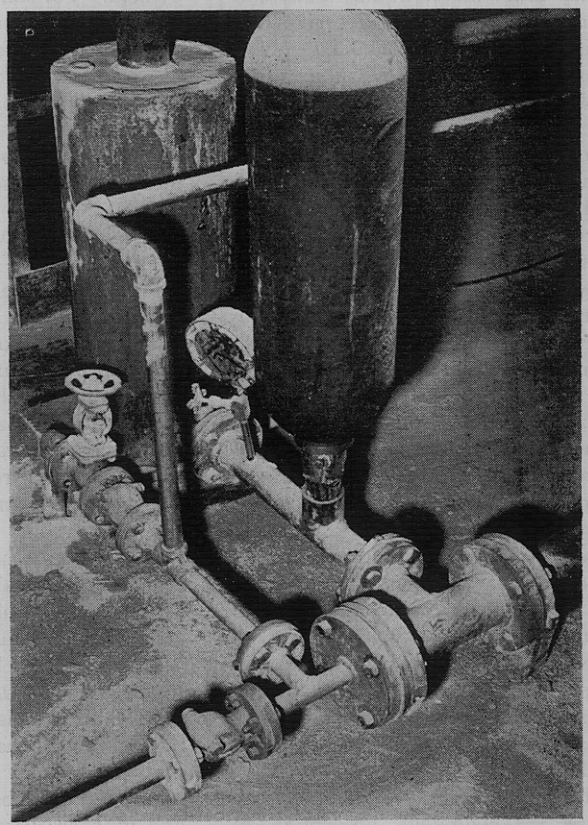

Figure 3. Reactor tee and other components of prereactor

plied to the prereactor through conventional instruments and piping (not shown). Also not shown is a surge tank arrangement in the water supply system, which may be necessary when water is used from city supply systems. Each supply line is provided as shown with a gate valve, a check valve, and a pressure gage immediately upstream from the prereactor.

The water and nitrogen solution, supplied through $1^1/_2$-inch pipes, blend in a $1^1/_2$-inch pipe tee and subsequently discharge from a perforated $1^1/_2$-inch pipe into a 6-inch pipe section 42 inches long. Anhydrous ammonia is supplied through a $1^1/_2$-inch pipe, then flows through a 3-inch pipe tee, and enters the 6-inch pipe section, where it blends with the nitrogen solution and water. The preblended liquids then flow through a 3-inch pipe section into a 3-inch pipe tee (the reactor tee). Sulfuric and phosphoric acids, supplied through 1-inch lines, preblend in a 1-inch pipe tee and subsequently flow through a 1-inch acid discharge pipe which passes concentrically through the reactor tee and terminates in a simple shower head arrangement 3 inches inside the reactor pipe section. The shower head is $1^3/_4$ inches in outside diameter and has 25 drilled discharge holes, each $^3/_{16}$ inch in diameter. Its purpose is to break up the acid flow into many small streams, thus assuring that neutralization by ammonia is largely completed in the reactor pipe section, 3 inches in diameter and 4 feet long.

The discharge pipe is 3 inches in diameter and 10 feet long and has 18 discharge holes $^1/_2$ inch in diameter, which are located along each side at 6-inch intervals. The preblended and prereacted liquids discharge laterally into the lower region of the pug mixer approximately 2 feet beneath the surface of the bed of dry materials. The discharge pipe was designed and located with due regard for two important aspects of mixed fertilizer granulation—efficient ammoniation of the superphosphates and uniform wetting of the dry materials.

A 1-inch compressed air supply line enters the blended acid line in a simple pipe tee. The air line is equipped with a solenoid valve which is arranged with an automatic timing device, so that on a preset timing cycle (usually 5 minutes) a 2- or 3-second blast of compressed air passes through the reactor and discharge pipe sections and assures that the discharge orifices remain open.

Hydropneumatic accumulators (Greer Hydraulics, Inc., Brooklyn, N. Y.) are located in the nitrogen solution supply line and in the preblended nitrogen line for the purpose of dampening out pressure pulsations caused by the blending and reaction actions. Each is a 5-gallon steel vessel equipped with a spring-loaded feed valve and containing a rubber bladder which is inflated to 5 p.s.i.g. (about one half of the normal line pressure).

Several corrosion-resistant materials of construction are used for the prereactor components. The preblending section is constructed of Type 304 stainless steel. The reactor tee is Type 316 stainless steel and the acid discharge pipe is Hastelloy C. The reactor pipe section is Teflon-lined mild steel and the discharge pipe is Teflon-lined Type 316 stainless steel. The Teflon lining has shown excellent resistance to the severe neutralization reaction environment. After 10 months of plant service, the original reactor section shows negligible deterioration.

Figure 2 shows a prereactor at an early stage of development, installed for use in a typical Trenton process pug mixer. The vertical section in the foreground is the nitrogen solution–water-ammonia blending pipe. The reactor tee in the lower background receives liquids from the blender pipe and contains the concentrically located acid discharge nozzle. The horizontal Teflon-lined reactor pipe extends to the left of the reactor tee, flanging to the discharge pipe seen entering the bottom of the pug mixer in the midline a few inches below the paddle shafts. The air supply check valve is on the right.

Figure 3 shows a portion of the reactor pipe section, the reactor tee, liquid feed lines, and the hydropneumatic accumulator located in the blended nitrogen line. Figure 4 shows a portion of the reactor pipe flanged to the discharge pipe which enters the pug mixer through a flanged access fitting. Figure 5 shows the discharge pipe located in the mixer immediately below the arc formed when the paddles rotate. The bar seen at the termination of the pipe is an anchor post.

Operating Results

Previous to the development of the prereactor and the design of the location of the discharge section beneath the arc of mixer paddle rotation, introduction of liquid raw materials into pug mixers presented difficulties (*4*). Various approaches were used, all of which involved multiple pipes and spargers for distributing the liquids. The mineral acids discharged from one set of pipes or spargers, ammonia and water through a second set, and nitrogen solution through a third set. Anhydrous ammonia and nitrogen solution were not preblended because of resultant flow pulsations. The spargers were usually arranged so that the mineral acids entered the mixer in close proximity to the other liquids. Although the better designs helped to assure rapid neutralization of the mineral acids by ammonia, the reactions occurred in the mass being granulated and undesirable side reactions were unavoidable. Separate liquid flows into the mass were also undesirable because they complicated the problem of uniformly distributing the liquids into the dry materials.

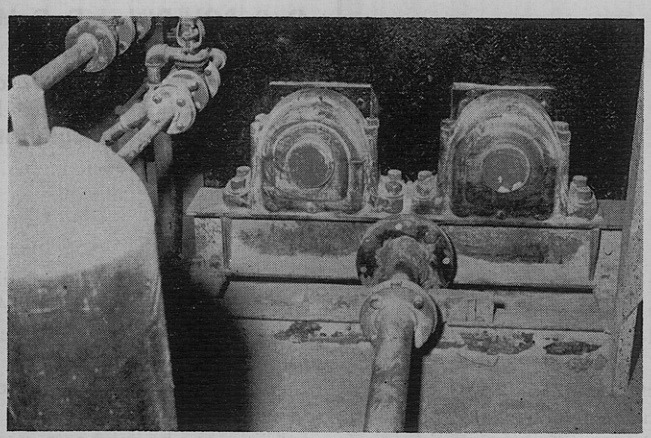

Figure 4. Prereactor distribution pipe entering pug mixer

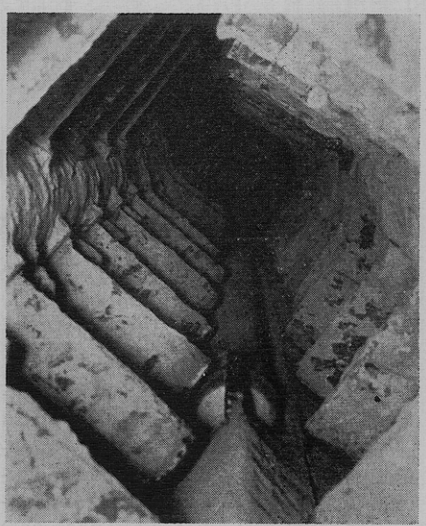

Figure 5. Prereactor distribution pipe inside pug mixer

Table I. Typical Proportions of Liquids Prereacted

Grade	Lb./Ton of Fertilizer Product: Anhydrous ammonia	Nitrogen solution[a]	Sulfuric acid (66° Bé. H_2SO_4)	Phosphoric acid (75% H_3PO_4)	Additional water
3–12–12	77 (3)[b]		105		210
5–20–20 (Formula A)	125 (5)[b]		125		350
5–20–20 (Formula B)	98 (4)[b]	50 (1)[b]	105		300
5–20–20 (Formula C)	77 (3)[b]	96 (2)[b]	55	130 (3-1/2)[c]	275
10–20–20	48 (2)[b]	410 (8-1/2)[b]	80	150 (4)[c]	None
12–12–12		510 (10-1/2)[b]	115		None
15–15–15		750 (15-1/2)[b]	225	150 (4)[c]	None

[a] 41.4% N, 6% urea, 19% free NH_3, 65.6% NH_4NO_3, 9.4% H_2O.
[b] Units of N shown in parentheses.
[c] Units of P_2O_5.

Table II. Granulation Plant Operating Data with Prereactor

	3–12–12	5–20–20 (Formula A)	12–12–12	15–15–15
Production rate, tons/hour	36	36	20	15
Recycle rate, tons/hour	25	25	25	40
Water usage, lb./ton produced	210	350	None	None
Material temperatures, ° F.				
Recycle entering mixer	125	115	120	120
Material leaving mixer	205	200	170	175

Performance of the prereactor in plant granulation operations has been satisfactory, and its use has resulted in important improvements. The heavy ammonium chloride fume from the mixer is eliminated by neutralizing the mineral acids inside the pipe. After start-up operations have provided a bed of material above the discharge pipe, a section of the mixer top cover is often removed by the control operator to permit unimpaired observation of the granulating mass. In previous systems, the dense fumes rising from the mass interfered with this practice.

Other advantages to the granulation operation which have resulted from the prereactor include unobstructed mixer paddle action, more uniformly regulated wetting of the materials being granulated, and automatic means for clearing partially clogged discharge orifices (which could also be used on other feed systems).

Table I shows the range of liquids that have been used through the prereactor in plant operations. Maximum and minimum limits on usage of the various liquid raw materials have not been established and performance thus far indicates considerable flexibility. Schedules have not yet permitted granulation of the high nitrogen 2–1–1 and X–O–X ratio grades; but it is believed that both types of grades can be successfully produced.

Table II presents typical prereactor granulation data. The water usages were determined by requirement for granule size control in the mixer. With well controlled operations, less water has been required than with previous sparger systems. It was not previously feasible to use five units of anhydrous ammonia in the formulations. Decreased water usage and improved ammonia neutralization have resulted in increased material temperatures in the mixer. Those shown for 3–12–12 and 5–20–20 grades were 5° to 10° F. above the usual previous figures for the grades. Only a few 12–12–12 and 15–15–15 grade production operations with the prereactor have been made. Consequently it is not known whether the temperatures and recycle rates shown for the grades are optimum. Limited experience has indicated that sufficient moisture can be evaporated from the mass in the mixer to accomplish reduced recycle requirements with high liquid nitrogen usages. However, further plant tests will be needed for verification.

There is no evidence of significant decomposition of nitrogen compounds in the prereactor, nor of the formation of toxic gases, as reported with other types of prereactors. A slight ammonia odor over the pug mill is usually detectable only during start-up operations, before a normal bed of material has accumulated over the discharge pipe and when formulations require high rates of ammoniation for the superphosphates.

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(1) Floyd, C. E., Proc. Fert. Ind. Round Table, National Plant Food Institute, Washington, D. C., p. 24, 1957.
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GRANULAR FERTILIZERS

Interaction between Dicalcium and Monoammonium Phosphates Granulated Together

D. R. BOULDIN, J. D. DeMENT, and E. C. SAMPLE

Soils and Fertilizer Research Branch, Division of Agricultural Relations, Tennessee Valley Authority, Wilson Dam, Ala.

Commercial ammoniated superphosphates normally contain monocalcium phosphate, monoammonium phosphate (MAP), and diammonium phosphate singly or in various proportions; dicalcium phosphate dihydrate (DCPD) and anhydrous dicalcium phosphate (DCPA)—principally the latter—and perhaps more basic calcium phosphates such as hydroxyapatite and collophane (*3*). The qualitative and quantitative distribution of these compounds changes with methods and degree of ammoniation of superphosphate. The composition of superphosphates ammoniated to varying degrees is reflected partially by the Association of Official Agricultural Chemists (AOAC) procedures for determining water- and citrate-soluble phosphorus (*3*). Hence, for many practical purposes, AOAC water solubility may be used as a means of characterizing the chemical properties of

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With greenhouse cultures, decreasing granule size increased plant response to phosphorus in dicalcium phosphate but decreased plant response to phosphorus in monoammonium phosphate. Plant response to granulated mixtures of dicalcium phosphate and monoammonium phosphate was related to geometric surface area of the fertilizer and monoammonium phosphate content per granule.

ammoniated superphosphates, but as AOAC water solubility is changed the quantitative distribution of the several compounds in the fertilizer also is changed.

In terms of agronomic evaluations for mixed placement, differences in plant response to phosphorus fertilizers varying in AOAC water solubility may be fairly large with large granules, but are usually small with small granules (*4–6*). For a given granule size, plant response may depend upon the assemblages of compounds within the AOAC water- and citrate-soluble phosphorus fractions, as well as upon the ratio of water- to citrate-soluble phosphorus (*1*).

The purpose of the experiments reported here was to study the influence of granule size on plant response to fertilizers prepared from mixtures of dicalcium and monoammonium phosphates in various proportions. These two compounds, which occur commonly in ammoniated superphosphates, were used so as to vary the ratio of AOAC water- and citrate-soluble fractions, and yet keep constant the components in these fractions.

Table I. Fertilizers Used in Greenhouse Experiment

Fertilizer No.	Components	Granule Size (Tyler)	Granules/ 30 Mg. P	Geometric Surface Area, Sq. Mm./ 30 Mg. P	P, %	Ca, %	Mg. P as MAP/ Granule	% P as MAP
1	DCPA	−9+14	25.8	206	22.9	27.5	...	...
2	DCPA	−16+20	138	367	...	...	...	...
3	DCPA	−28+35	618	495	23.0	27.8	...	...
4	DCPA	−35+48	2224	896	...	...	...	...
5	DCPA	−48+60	4708	1106	23.0	27.4	...	...
6	DCPA + sand	−9+14	73.1	584	12.6	14.7	...	...
7	DCPA + sand	−16+20	306	814	13.0	15.0	...	...
8	DCPA + sand	−28+35	1357	1087	13.8	15.9	...	...
9	DCPA + sand	−35+48	4394	1771	13.8	15.8	...	...
10	DCPD	−9+14	63.8	510	17.9	23.1	...	...
11	DCPD	−28+35	1316	1054	...	...	...	...
12	MAP	−9+14	27.8	222	...	...	1.08	100
13	MAP	−16+20	113	300	...	...	0.266	100
14	MAP	−28+35	698	559	...	...	0.0430	100
15	MAP	−35+48	2177	877	...	...	0.0138	100
16	MAP	−48+60	5055	1188	...	...	0.00593	100
17	MAP + DCPA	−9+14	26.1	208	23.1	26.3	0.0559	4.9
18	MAP + DCPA	−28+35	704	564	23.0	25.1	0.00391	9.2
19	MAP + DCPA	−9+14	27.7	221	23.6	20.2	0.311	28.8
20	MAP + DCPA	−28+35	790	633	24.0	18.4	0.0137	36.1
21	MAP + DCPA	−9+14	25.8	206	24.9	12.7	0.668	57.6
22	MAP + DCPA	−28+35	867	694	24.8	12.4	0.0202	58.4
23	MAP + DCPA	−9+14	27.1	216	25.7	7.7	0.824	75.1
24	MAP + DCPA	−28+35	818	655	25.8	7.4	0.0278	76.2
25	MAP + DCPD	−9+14	42.6	340	19.7	19.0	0.136	19.3
26	MAP + DCPD	−28+35	1233	988	19.6	19.1	0.00457	18.8
27	MAP + sand	−9+24	44.6	356	18.2	...	0.672	100
28	MAP + sand	−28+35	1200	961	19.2	...	0.0250	100

Methods and Materials

Fertilizers. The properties of the fertilizers used are listed in Table I. The anhydrous dicalcium phosphate fertilizers were prepared by pelleting anhydrous dicalcium phosphate (−100 mesh), moistening the pellets with a solution prepared from monocalcium phosphate monohydrate, and then drying for 2 days at 110° C. The pellets were crushed and then soaked in water for 2 days to remove water-soluble phosphate. After drying, the fertilizer was screened into fractions of various sizes. The anhydrous dicalcium phosphate-plus-sand fertilizers were prepared as above by pelleting mixtures of anhydrous dicalcium phosphate and acid-washed quartz sand (−48 + 60 mesh). The water washing was limited to 2 hours to prevent disintegration of the granules. The dicalcium phosphate dihydrate fertilizers were prepared by placing a thin layer of fine dicalcium phosphate dihydrate (−100 mesh) in the bottom of a crystallizing dish, adding a thin layer of solution saturated with monocalcium phosphate monohydrate, and then placing in an atmosphere of 0.5*N* ammonium hydroxide for several days at 5° C. The resulting cake was broken up, washed with water, and screened.

The monoammonium phosphate fertilizers were screened from reagent grade monoammonium phosphate. The monoammonium phosphate-plus-sand fertilizers were prepared by pelleting a moist mixture of −48 + 60-mesh monoammonium phosphate and acid-washed quartz sand. The pellets were dried at room temperature over calcium chloride, crushed, and screened. The monoammonium phosphate and dicalcium phosphate (anhydrous or dihydrate) fertilizers were prepared by pelleting appropriate mixtures, moistening the pellets with saturated solutions of monoammonium phosphate, and drying over calcium chloride at room temperature. The pellets were crushed and screened.

Samples of the fertilizers were dissolved in 1.0*M* perchloric acid. An aliquot was analyzed for phosphorus, using the vanadate color reagent (*7*). In a second aliquot, calcium was separated from phosphorus by precipitation as calcium oxalate and determined by titration with (ethylenedinitrilo)-tetraacetic acid (EDTA) after destruction of the oxalate by heating to 500° C. The average calcium-phosphorus weight ratio for the anhydrous dicalcium phosphate fertilizers was 1.20. Using this ratio, the phosphorus content of the monoammonium phosphate–anhydrous dicalcium phosphate fertilizers in the form of anhydrous dicalcium phosphate was calculated from the analytical values for calcium. The remainder of the phosphorus was assumed to be present as monoammonium phosphate.

The average number of granules per unit weight of fertilizer was determined by counting the number of granules in several weighed samples. The geometric surface area was calculated assuming spherical granules with diameter equal to the average openings in the sizing screens.

Greenhouse Procedures. The fertilizers were mixed with 3-kg. samples of Hartsells fine sandy loam (pH 5.2) or Mountview silt loam (pH 6.5). All fertilizers were added in amounts to supply 30 and 60 mg. of phosphorus per culture. With fertilizers 3 and 14,

amounts to supply 90 and 120 mg. of phosphorus also were added. Three replications of each treatment were prepared.

Columbian oats were seeded December 16, 1958, and thinned to 30 plants per culture December 29. Fertilizer nitrogen and potassium levels were equalized at 100 mg. per culture on December 30 by addition of solutions of ammonium nitrate and potassium sulfate. On January 21, 100 mg. of nitrogen were added to each culture as ammonium nitrate. Above-ground portions of the plants were harvested February 5, 1959.

The surfaces of the pots were cultivated and oats replanted February 12, 1959. On February 19, 200 mg. of nitrogen as ammonium nitrate and 100 mg. of potassium as potassium sulfate were added in solution. On March 4, 100 mg. of nitrogen as ammonium nitrate were added in solution. Above-ground portions of the plants were harvested March 30. After drying at 80° C., the above-ground portions of the plants were weighed and analyzed for phosphorus.

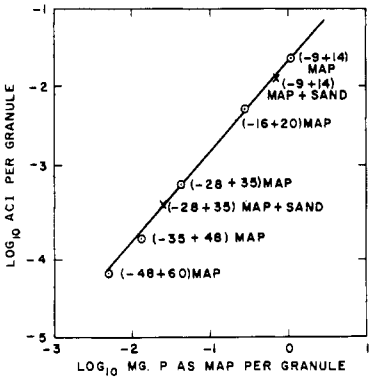

Figure 1. Availability coefficient index per granule plotted against monoammonium phosphate content per granule

Results and Discussion

Yields of dry matter were plotted against yields of phosphorus for each crop and each soil. In all cases, visual examination of the resulting graphs indicated that yields of dry matter were the same function of phosphorus uptake with all sources of phosphorus. Mitscherlich equations were fitted to the yields of dry matter and availability coefficient indexes were calculated for both crops on both soils (*2*). Because all sources behaved in a similar manner in both soils, only the average availability coefficient indexes are listed in Table II. The significance of availability coefficient indexes in relation to fertilizer evaluation is discussed by Bouldin and Sample (*2*).

When monoammonium phosphate is placed in moist soil, dissolution and movement into the surrounding soil are complete within about 2 weeks. The distribution and solubility of soil–fertilizer phosphorus reaction products in this volume of soil about each granule determine plant response to phosphorus. If monoammonium phosphate is mixed with an equal volume of sand or some other inert material, but the quantity of monoammonium phosphate per granule remains constant, the distribution and solubility of soil-fertilizer reaction products about each granule should not be changed appreciably. Hence, plant response is expected to depend upon monoammonium phosphate per granule and not granule size as such. The data obtained with monoammonium phosphate and monoammonium phosphate-plus-sand fertilizers are consistent with this hypothesis. In Figure 1, the availability coefficient index (ACI) per granule (crop 1) is plotted against the monoammonium phosphate content per granule (on a log-log scale). This relationship indicates that the availability of granular monoammonium phosphate depends primarily on monoammonium phosphate per granule and not granule size as such.

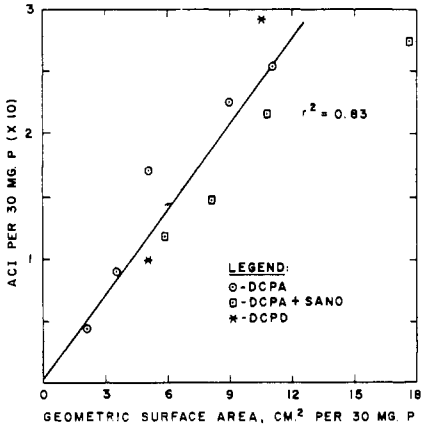

Figure 2. Availability coefficient index per 30 mg. of phosphorus plotted against geometric surface areas of fertilizers

When the dicalcium phosphate fertilizers are considered, somewhat different processes are involved. Previous work (*2*) indicated that geometric surface area was the important variable in comparisons of different granule sizes of the same fertilizer.

According to the hypothesis presented earlier (*2*), reaction of dicalcium phosphate fertilizer with the soil is a diffusion-controlled process. If a portion of the surface of a dicalcium phosphate granule is replaced with small pores or sand grains, diffusion of phosphorus from the granule into the soil will be rather complicated geometrically. Diffusion zones surrounding each particle of dicalcium phosphate in the surface of the granule will move outward with components both tangential and normal to the surface of the granule. If the size of the pores (or sand) is relatively small in relation to the distance of diffusion normal to the surface, then sooner or later the region of soil immediately surrounding a granule of dicalcium phosphate with many pores (or sand grains) should be very similar in nature to the

Table II. Average Availability Coefficient Index (×10³) per 30 Mg. of Phosphorus for Different Mesh Sizes of Fertilizers

(Based on dry matter yields of oats grown on Hartsells fine sandy loam and Mountview silt loam)

	−9 + 14			−16 + 20			−28 + 35			−35 + 48			−48 + 60		
Components	Crop I	Crop II	Av.	Crop I	Crop II	Av.	Crop I	Crop II	Av.	Crop I	Crop II	Av.	Crop I	Crop II	Av.
DCPA	30	57	43	50	133	92	90	253	172	119	320	220	136	376	256
DCPA + sand	107	120	114	100	197	148	107	323	215	129	422	276			
DCPD	54	146	100				152	434	293						
MAP	608	211	410	601	168	384	423	246	334	387	220	303	331	217	274
MAP + sand	551	205	378				405	252	328						
MAP + DCPA															
10%[a]	86	64	75				107	246	176						
25%[a]	235	85	160				236	250	243						
50%[a]	382	146	264				300	234	267						
75%[a]	504	162	333				381	225	303						
MAP + DCPD															
50%[b]							261	184	222						
25%[a]	196	100	148				217	425	321						

[a] Approximate % of total P supplied as MAP. [b] 50% of P applied as −28 + 35-mesh DCPA and 50% as −28 + 35-mesh MAP.

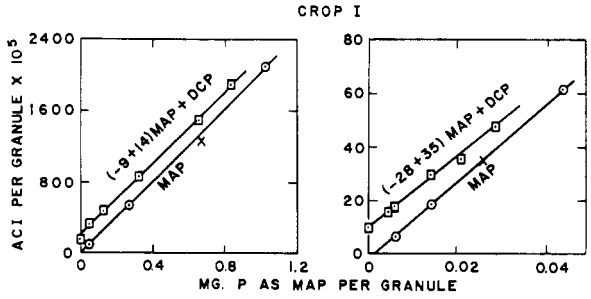

Figure 3. Availability coefficient index per granule for crop I plotted against monoammonium phosphate content per granule

⊡ MAP + DCP ⊙ MAP ×. MAP + sand

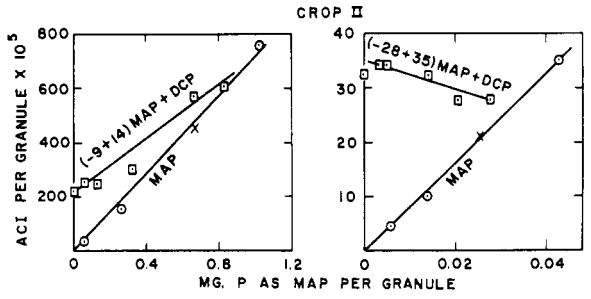

Figure 4. Availability coefficient index per granule for crop II plotted against monoammonium phosphate content per granule

⊡ MAP + DCP ⊙ MAP ×. MAP + sand

region of soil immediately adjacent to a nonporous granule of dicalcium phosphate of the same size. Hence, increasing the porosity of granules of dicalcium phosphate or granulation of it with sand, keeping granule size constant, should increase plant response to unit weight of dicalcium phosphate.

Consistent with the foregoing hypothesis, all of the anhydrous dicalcium phosphate-plus-sand fertilizers listed in Table II were more available per unit weight of phosphorus than the same granule size of anhydrous dicalcium phosphate alone. In Figure 2, the average availability coefficient indexes (over both crops) per 30 mg. of phosphorus are plotted against the geometric surface areas. Generally, on a unit geometric surface area basis, the anhydrous dicalcium phosphate-plus-sand fertilizers were less available than the ones without sand, which, of course, indicates that the reaction zone adjacent to an anhydrous dicalcium phosphate-plus-sand granule was somewhat less effective in promoting plant response than the zones adjacent to the same size granule of anhydrous dicalcium phosphate. This is particularly true with the −35 + 48 anhydrous dicalcium phosphate-plus-sand granules. Examination of these granules revealed that very often the individual granules were composed of a sand grain cemented to a granule of anhydrous dicalcium phosphate. With granules of this nature the reasoning discussed above does not apply, as the sand grains are essentially appendages on the surface rather than replacement of part of the surface. As an average of the four comparisons listed in Table II, one granule of anhydrous dicalcium phosphate-plus-sand was 72% as effective as a granule of anhydrous dicalcium phosphate of the same size. Thus, the results are intermediate between the expected extremes—that is, a granule of anhydrous dicalcium phosphate-plus-sand is less available than a similar granule without sand, but more than one half as available as a granule of anhydrous dicalcium phosphate, although approximately one half of the granule volume and surface consists of sand.

Considering all of the anhydrous dicalcium phosphate, dicalcium phosphate, and anhydrous dicalcium phosphate-plus-sand fertilizers, geometric surface area measurements explained 74% of the variation in availability coefficient indexes. When the −35 + 48-mesh anhydrous dicalcium phosphate-plus-sand fertilizer is omitted, the geometric surface area measurements explain 83% of the variation in availability coefficient indexes.

When mixtures of anhydrous dicalcium phosphate and monoammonium phosphate are placed in moist soil, the monoammonium phosphate will dissolve rapidly (in less than 2 weeks) and move into the surrounding soil. The anhydrous dicalcium phosphate is relatively insoluble in the monoammonium phosphate solutions (pH 3.4, $3M$ phosphorus) and will remain at the granule site as a porous residue. If anhydrous dicalcium phosphate is viewed as a diluent in much the same sense as sand, then any additional plant response not attributable to monoammonium phosphate may be regarded as being due to the presence of dicalcium phosphate (anhydrous or dihydrate) in the granules.

In Figure 3, the availability coefficient index per granule for crop I is plotted against milligrams of phosphorus as monoammonium phosphate per granule. The lower line in each graph includes the results with monoammonium phosphate and monoammonium phosphate-plus-sand. The upper line in the graph on the left represents the results with −9 + 14-mesh mixtures of anhydrous and dihydrated dicalcium phosphate-plus-monoammonium phosphate and of anhydrous dicalcium phosphate alone. The presence of dicalcium phosphate (anhydrous or dihydrate) has displaced the line upward from the monoammonium phosphate line. This difference represents the influence of the dicalcium salts on plant response. The upward displacement of the line is essentially equal to the availability coefficient index of one −9 + 14-mesh granule of anhydrous dicalcium phosphate. As the monoammonium phosphate content per granule increased, the anhydrous and dihydrated forms of dicalcium phosphate content per granule decreased. Hence, for all practical purposes the availability coefficient of the −9 + 14-mesh mixtures of monoammonium phosphate-plus-anhydrous or dihydrated dicalcium phosphate can be represented as a function of the geometric surface area and monoammonium phosphate content per granule. The same results were obtained with the −28 + 35-mesh granules (graph on right of Figure 3).

Crop II results are presented in the same manner in Figure 4. With crop II the availability coefficient indexes are somewhat less than would be expected on the basis of geometric surface area and monoammonium phosphate content per granule, particularly with the granules containing a large portion of phosphorus in the form of monoammonium phosphate. This may be the result of several factors. One factor is that the dicalcium phosphate in the granules composed largely of monoammonium phosphate may have dissolved completely during the cropping interval. A second factor may be that the very porous granules remaining after dissolution of the monoammonium phosphate have collapsed with a consequent decrease in geometric surface area.

The results indicate that the major differences in availability coefficient indexes can be explained on the basis of geometric surface area and monoammonium phosphate content per granule. The following equations approximately apply.

$$A_t = A_m + A_d$$

where A_t = availability coefficient index per unit of phosphorus

$$A_m = N(a + bX)$$

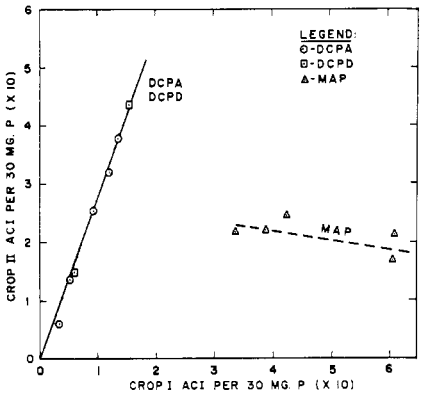

Figure 5. Availability coefficient index per 30 mg. of phosphorus

Crop I *vs.* crop II

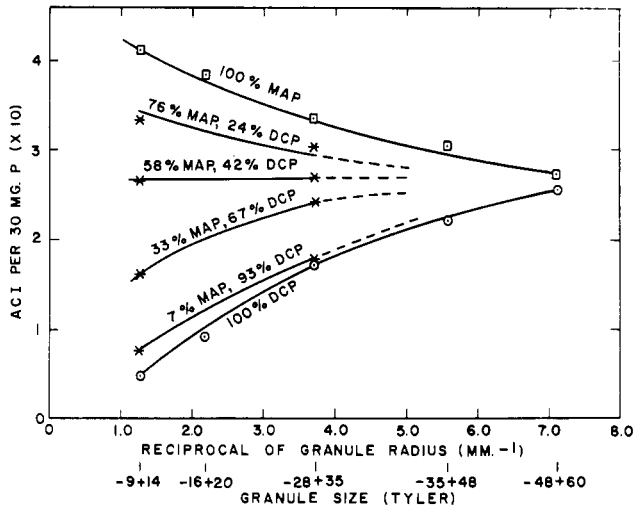

Figure 6. Availability coefficient index per 30 mg. of phosphorus (average of crops I and II) plotted against reciprocal of granule radius

where N = number of granules per unit weight of fertilizer phosphorus

$a + bX$ = availability of monoammonium phosphate per granule

when X = monoammonium phosphate per granule, and a and b are constants

$$A_d = c + gY$$

where $c + gY$ = availability of dicalcium phosphate with geometric surface area, Y, and c and g are constants

In the above equations, N is determined by counting the number of granules per unit weight of fertilizer. Geometric surface area of the fertilizer is calculated from the number of granules per unit weight of fertilizer and the average openings in the sizing screens. The monoammonium phosphate content per granule is calculated from analysis of the fertilizer and the number of granules per unit weight of fertilizer. Constants a, b, c, and g are calculated from plant response data obtained with the different granule sizes of monoammonium and dicalcium phosphates. Using these equations, the availability coefficient indexes of the mixtures of monoammonium phosphate and dicalcium phosphate (both forms) were calculated and compared with the measured availability coefficient indexes listed in Table II. When all the data (both crops) were used, the estimated availability coefficient indexes explained 75% of the variation in the measured availability coefficient indexes. In addition, within the limitation outlined above, the results of this experiment indicate that there is very little interaction between AOAC water- and citrate-soluble phosphorus when monoammonium phosphate and dicalcium phosphate (both forms) are granulated together. The availability of the two dicalcium phosphates depends upon geometric surface area and the availability of monoammonium phosphate depends upon monoammonium phosphate content per granule.

Some interesting comparisons between the results with crops I and II may be made. In Figure 5, the availability coefficient index for crop II is plotted against the availability coefficient index for crop I for the dicalcium (anhydrous and dihydrate) and monoammonium phosphate fertilizers. There is a very good correlation between crop I and crop II results with the anhydrous and dihydrated dicalcium phosphate fertilizers; the crop II availability coefficient index is about three times that of crop I. With the monoammonium phosphate, the reverse is true; crop I availability coefficient indexes are about two to three times larger than those with crop II and there is a tendency for the crop II availability coefficient indexes to be smaller the larger the values with crop I.

Furthermore, with crop I the monoammonium phosphate fertilizers were two to four times better than the smallest granule sizes of dicalcium phosphate, anhydrous and dihydrate (Table II). In crop II, however, the smallest granule sizes of anhydrous and dihydrated dicalcium phosphate were approximately two times better than the monoammonium phosphate fertilizers. Perhaps the most important conclusion to be drawn is that the dicalcium phosphate (anhydrous or dihydrate) fertilizers become more nearly like the monoammonium phosphate fertilizers as the cropping interval is increased. Thus, rather marked differences in response to anhydrous dicalcium phosphate and monoammonium phosphate would be expected with short season crops or differences in early growth with long season crops. However, with long season crops at harvest, the differences in response might become relatively small or disappear completely, particularly with small granules.

The data are effectively summarized in Figure 6, in which the availability coefficient index of both crops is plotted against the reciprocal of the radius of the granules. Several points are noteworthy about Figure 6: Decreasing granule size increased plant response with the anhydrous dicalcium phosphate fertilizers, but decreased plant response with the monoammonium phosphate fertilizers; the granule size effects with the two-component mixtures are intermediate between those for the single components, and when the two components are present in equal amounts in a mixture there is no appreciable effect of granule size; as granule size is reduced, all sources behave more nearly alike, and the two-component mixtures are all intermediate between the single component fertilizers applied alone.

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Thermal Decomposition of High Analysis Fertilizers

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High analysis fertilizers offer advantages to the manufacturer and the user, and each year many thousands of tons are made, shipped, and used, mostly without incident. There are occasional reports of fires, however, which show that these mixtures of chemicals must be handled properly. This paper shows some of the conditions that lead to fires, and how the products can be handled safely.

THIS STUDY of the thermal decomposition of high analysis fertilizers concerns only those that have been completely processed or have at least reached the drying stage. It does not deal with flash fires in ammoniators or fires due mainly to the action of nitric acid upon organic matter.

The type of decomposition dealt with here is due to inorganic reactions, though limited amounts of urea or other organic compounds do not necessarily prevent it. It may occur even in fertilizers that are well ammoniated. The reaction requires a high temperature for starting, but it is self-sustaining when once initiated, and will spread throughout the available mass of fertilizer. The heat of reaction may raise the temperature in the active zone to 732° F. (400° C.) or higher, and there is plentiful emission of fumes that smell strongly of chlorine. In this paper the onset of this sort of self-sustained decomposition is called "ignition."

Such decomposition destroys the fertilizer itself, and the heat is likely to damage buildings and equipment. Still more serious is the danger of toxic fumes, and protection of personnel should be the first consideration in any fire control effort. As the reaction does not require air, the liberal use of water is the only generally practical way of stopping it.

This type of decomposition was discussed by Drobot before the Division of Fertilizer and Soil Chemistry of the American Chemical Society in September 1957 (*1*).

The higher the temperature, the sooner ignition occurs. Considering the six fertilizers of principal interest here, spontaneous ignition occurred promptly (within about an hour) at temperatures from 410° to 482° F. (210° to 250° C.). At 338° F. (170° C.), ignition times of 96 to 600 hours were observed. At 302° F. (150° C.), only three of the six fertilizers ignited spontaneously, requiring 675 to 1700 hours. None ignited at 266° F. (130° C.), though ignition at this temperature has been observed occasionally in other fertilizers. Spontaneous ignition at 212° F. (100° C.) would not be expected, and it did not occur during this investigation.

Tests included analyses of the gases evolved before and during self-sustaining decomposition, a few analyses of the residual solids, and measurements of the rate at which gas was evolved. In most of the latter tests that were run at 302° F. (150° C.) or higher, heating was continued until self-sustaining decomposition set in. Other tests to show the conditions required for ignition were also made.

Although most of the work was done on fertilizers made in the pilot plant or produced commercially, other materials were tested, including mixtures of ammoniated superphosphate with fertilizer salts, and fertilizers with organic admixtures. The data on blends with ammoniated superphosphate showed that although both chloride and nitrate are needed for the type of decomposition under consideration, the concentration of one or the other can be rather low without preventing the reaction. The organic materials tend to inhibit the reaction.

Materials Tested

The most complete series of tests was run on a set of six fertilizers, each made on a pilot plant or commercial scale. Table I gives information about these products, including the process and the principal ingredients, the analyses, and the calculated compositions in terms of single compounds.

Three of the six were 12–12–12 fertilizers, made by different processes and differing widely in nitrate content. Two were of 16–8–8 grade, and differed mainly in that one contained urea, supplied as an ingredient of the ammoniating solution, while the other did not. The last fertilizer of the set was a 15–0–14, distinguished by its high dolomite content as well as its lack of phosphate.

The calculated compositions (Table I) are based upon the formulations, and are not entirely consistent with the analyses, though the differences are considered insignificant in the present connection. Reasonable assumptions were made concerning the compositions of the superphosphates and the reactions occurring during ammoniation. It was also assumed that enough liquid phase was present to permit interactions among the salts, and that solid salts would be formed in inverse order of solubility. The small amounts of sodium salts that are indicated would come from sodium chloride, assumed to be the principal impurity in the potassium chloride.

The dominant potassium salt appears to be the sulfate, except in the 12–12–12 nitraphosphate, which contains a large proportion of potassium nitrate. In all the other five cases the principal nitrate is the ammonium salt, and calculations indicate that in every case the chloride is present as ammonium chloride.

Other materials on which special tests were made are described in connection with the tests.

Test Procedures and Results

Large samples, 15 to 20 pounds, were held at elevated temperatures for a number of days or weeks, with periodic sampling of the gases evolved. The container that was used is a 19-inch length of 6-inch stainless steel pipe with ends welded on. A 1-inch connector holds a union that serves as a charging port and also holds a rupture disk and vent. There are a thermocouple well and another nipple that serves as a sampling tube and also provides a connection for a pressure gage. The sample container is submerged in an electrically heated, thermostatically controlled fused salt bath.

The valve of the sampling tube is normally left open so that the evolved gas can escape. When the first gas sample is to be taken from a fresh charge in the container, the gas is pumped from the container, and the valve is closed. The pressure gradually increases, and a

Table I. Ingredients, Analyses, and Compositions of Test Fertilizers

Grade	12-12-12[a]	12-12-12[a]	12-12-12[b]	16-8-8[a]	16-8-8[a]	15-0-14[a]
Process	Batch ammoniation	Prereactor	Nitraphosphate	Prereactor	Prereactor	Prereactor
Ammoniating agent	Nitrana[c,d]	Nitrana[c]	NH_3	Nitrana[c]	Urana[e]	Nitrana[c]
Acid in prereactor	...	H_2SO_4	...	H_2SO_4	H_2SO_4 and H_3PO_4	H_2SO_4
Principal sources of P_2O_5	Normal and concd. superphosphate	Normal and concd. superphosphate	Phosphate rock	Normal and concd. superphosphate	Normal superphosphate	...
Dolomite, lb. per ton	None	20	None	20	20	490
Analysis, wt. %						
Ammoniacal N	11.30	8.60	7.02	11.74	11.10	10.52
Nitrate N	1.45	3.90	5.32	4.26	4.42	4.61
Urea N	0	0	0	0	1.13	0
Total N	12.75	12.50	12.34	16.00	16.65	15.13
Water-soluble P_2O_5	7.47	5.59	2.23	4.35	2.98	0
Citrate-insoluble P_2O_5	0.17	0.12	0.0	0.35	0.05	0
Total P_2O_5	12.15	12.28	12.14	8.30	8.43	0
Water-soluble K_2O[f]	12.74	12.04	Not detd.	8.04	8.29	14.3
H_2O	2.4	0.9	1.1	0.4	0.8	1.0
Organic C	0.15	0.13	0.07	0.05	Not detd.	Not detd.
Cl	10.1	9.32	9.37	6.30	6.64	10.7
pH in water	5.0	5.1	Not detd.	5.4	5.5	6.1
Compn. calcd. from formulation, wt. %						
NH_4Cl	14.8	15.1	14.9	9.8	9.9	17.2
NH_4NO_3	7.9	19.2	11.1	29.0	27.5	25.1
$(NH_4)_2SO_4$	22.2	...	...	14.1	12.4	4.3
$NH_4H_2PO_4$	9.3	9.3	6.3	5.8	5.2	...
KNO_3	...	3.0	26.8	...	...	...
$NaNO_3$	...	...	1.2	...	...	...
Urea	...	...	...	...	2.5	...
K_2SO_4	22.8	20.5	...	15.2	15.0	26.3
Na_2SO_4	1.1	1.1	...	0.6	1.0	1.4
$CaHPO_4$	5.2	...	15.5	...	...	...
$Ca_3(PO_4)_2$	6.5	12.3	...	8.7	9.7	...
(Fe and Al)PO_4	1.7	1.8	1.6	1.3	1.2	...
$CaSO_4$ and $MgSO_4$	2.0	11.3	16.5	9.9	11.1	0.2
Dolomite or calcite	...	0.9	...	0.9	1.0	24.4
H_2O	2.4	0.9	1.1	0.4	0.8	1.0
Other (CaF_2, SiO_2, etc.)	4.1	4.6	5.0	4.3	2.2	0.1

[a] Pilot plant product.
[b] Commercial product.
[c] Nitrana. 19.0% NH_3; 72.5% NH_4NO_3; 8.5% H_2O.
[d] Urana. 19.5% NH_3; 66.3% NH_4NO_3; 6.0% urea; 8.2% H_2O.
[e] Sample also contained 766 pounds $(NH_4)_2SO_4$ per ton.
[f] In all six fertilizers, K_2O supplied as fertilizer grade KCl.

Table II. Analysis of Gases Evolved by Fertilizers Held at 338° F. (170° C.) Prior to Ignition of Fertilizer

Product	Time of Sampling from Start of Test, Hours	Weight %						
		H_2O	CO_2	NH_3	N_2O	N_2	NO[a]	Cl_2[b]
12-12-12								
By batch ammoniation	43	16.9	76.7	0	1.83	3.04	0.99	0.54
	67	17.8	11.8	0	4.87	64.6	0	0.92
By prereactor	99	14.44	13.98	0	4.27	66.93	0	0.38
Nitraphosphate	24	80.3	5.5	1.0	0.12	13.1	<0.1	0
	48	74.4	7.7	0.9	0.06	17.0	0	0
	72	71.5	7.8	0.7	0.06	20.0	0	0
	96	61.2	7.3	0.6	0.09	30.8	0	0
	168	65.2	2.3	0.2	1.5	30.8	0	0
16-8-8								
By prereactor (Nitrana)	8	3.84	44.56	0.05	0.22	51.33	0	0
	73	0.52	26.58	None	1.93	70.97	0	0
	174	4.48	3.69	0.16	5.00	86.67	0	0
By prereactor (Urana)	6	1.43	83.45	5.85	0.04	9.20	0.03	[c]
	124	0.91	37.19	1.34	0.37	60.19	0	[c]
	197	0.26	38.62	0.89	0.31	59.92	0	[c]
	289	17.94	42.47	0.29	0.17	39.09	0.04	[c]
	363	0.72	56.70	0.37	0.21	42.00	0	[c]
15-0-14 by prereactor	23	3.36	76.25	20.4	0	0	0	0
	66	5.68	67.00	27.48	0	0	0	0
	142	5.67	63.18	30.69	0	0	0	0

[a] May include NO, $NO_2(N_2O_4)$, NOCl, and HNO_3.
[b] May also include some NOCl and HCl.
[c] No analysis made for this component.

gas sample is taken when it reaches a convenient value, 10 p.s.i. or more. The valve is then left open until sampling is to be repeated.

Table II shows analyses of gases evolved by the six fertilizers at 338° F. (170° C.) at various times prior to ignition. Table III shows analyses of samples of 12–12–12 nitraphosphate held at 338° F. for two different periods short of ignition, and also shows very limited data on a 16–8–8 before and after heating.

Even larger samples of fertilizer are used when the gas evolved during self-sustained decomposition is to be analyzed. The container is a steel box, approximately a 1-foot cube with a vent, and an electrically heated sampling tube. The box is covered with magnesia insulation. The fertilizer is ignited by playing an oxyacetylene flame on the outside of the box, blocks of insulation being temporarily removed to permit this. During the active combustion a portion of the gas is run through an absorption train, or into a number of gas-sampling balloons. Table IV shows analyses of both gas samples and solid residues.

Because this work extended over more than a year, there was some variation in methods of analysis, which were adaptations of well known procedures. In brief, the procedures were as follows:

Water. When balloon samples were used, pyridine was admitted to one balloon, and the water in the pyridine was determined by a Karl Fischer titration. In an absorption train, the bulk of the water was caught in an ice-cooled trap, and the remainder was taken up by Dehydrite, which was weighed. In one run water was determined by difference.

Carbon Dioxide. The details depended upon the sampling procedure used, but in any event the carbon dioxide was finally weighed after absorption on Ascarite.

Chlorine. An aqueous solution obtained from the absorption train, or a sample from a balloon, was treated with 3% hydrogen peroxide, which reduced chlorine to chloride, and this was determined by a modified Volhard procedure. Though the results were calculated to elemental chlorine, it is likely that small amounts of nitrosyl chloride and hydrochloric acid were included.

Ammonia (Free or in Sublimed Salts). This was determined by Kjeldahl distillation of an aqueous solution obtained from the absorption train or a balloon sample.

Nitric Oxide and Nitrogen Dioxide. An aqueous solution of these acid-forming oxides of nitrogen was treated with hydrogen peroxide, and the nitrogen was determined by the Devarda procedure. Any nitric acid—from sublimed ammonium nitrate, for example—would be included, and so would any nitrogen evolved as nitrosyl chloride.

Nitrous Oxide. This was determined by infrared spectrophotometry, using either a balloon sample or the noncondensable gases from the absorption train.

Nitrogen was determined by difference.

Table III. Changes in Fertilizers Prior to Ignition When Held at Elevated Temperatures

	12–12–12 Nitraphosphate		16–8–8 by Prereactor (Nitrana)	
Hours in test	45	87	0	672
Temp., ° F. (° C.)	338 (170)	338 (170)	302 (150)	302 (150)
Total N, %	11.73	11.23	16.00	12.85
Ammoniacal N, %	6.63	6.29	...	...
Water-soluble P_2O_5, %	1.17	1.54	...	...
Citrate-insoluble P_2O_5, %	0.08	0.05	...	...
Total P_2O_5, %	13.02	13.18	...	...
Cl, %	9.82	9.94	...	...
H_2O, %	0.43	0.38	...	...
pH	4.05	3.85	5.4	3.4

Table IV. Products of Self-Sustained Decomposition

	12–12–12		16–8–8 by Prereactor	
	By prereactor	Nitraphosphate	Nitrana	Urana
Residue analysis, wt. %				
Initial sample	67.5	60.1	55.0	59.0
Ammoniacal N	2.59	0.01	4.57	2.70
Nitrate N	0.05	0.06	0.03	[a]
Urea N	0	0	0	[a]
Total N	2.64	0.07	4.60	3.13
Total P_2O_5	17.8	20.6	12.80	14.7
Water-soluble P_2O_5	1.53	0.29	6.95	3.58
Citrate-insoluble P_2O_5	2.22	10.4	1.88	1.82
K_2O	18.86	[a]	12.27	13.6
Cl	9.7	8.67	1.3	2.6
H_2O	0.22	0.12	0.27	0.25
pH	3.3	[a]	2.2	2.8
Analysis of gas during decompn., wt. %				
H_2O	62.0	56.0	30.3	68.4
CO_2	3.82	0.10	6.52	1.64
NH_3	2.57	0	3.02	3.82
N_2O	10.01	12.0	12.23	6.64
N_2	16.07	22.7	39.02	17.46
NO[b]	0.0	2.58	0.97	0.0
Cl_2[c]	5.56	6.63	7.94	2.08

[a] Not determined.
[b] Includes nitrogen of NO, NO_2, HNO_3, and NOCl, all calculated as NO.
[c] May also include some chlorine combined as NOCl and HCl.

Rate of Gas Evolution

The rates at which gas was evolved by the different fertilizers at elevated temperatures were measured. The procedure is simple, repeated measurements can be made on the same sample, and the rate gives a general idea of the activity of the decomposition. When the main gas-producing reaction is known to be exothermic, the results give a relative idea of the rate at which heat is being generated. Changes in the rate of gas evolution suggest changes in the nature or relative extents of the reactions.

Figure 1 is a schematic view of the apparatus.

The sample containers are 200-ml. round-bottomed flasks, with the single neck ending in the socket of a ball joint. Except during actual measurements, the flasks containing samples are kept immersed to their necks in an oil bath at

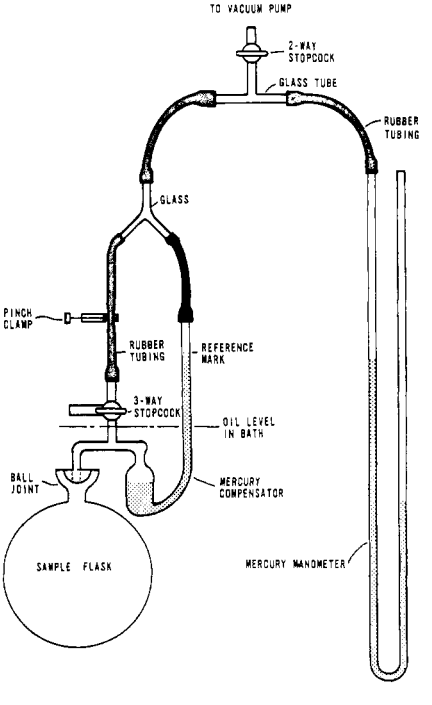

Figure 1. Apparatus for measuring rate of gas evolution

Table V. Gas Evolution and Spontaneous Ignition

Time Held at 338° F., Hr.	12-12-12 By batch ammoniation	12-12-12 By pre-reactor	12-12-12 Nitra-phosphate	16-8-8 by Prereactor Nitrana	16-8-8 by Prereactor Urana	15-0-14 by Pre-reactor
	Gas Evolution Rates, at 338° F., Mmoles/(Hr.)(100 G. Sample)					
25	1040	580	100	480	40	1330
50	1150	620	85	650	75	1210
75	1180	690	82	920	110	830
100	...	810	88	1430	155	560
150	...	1210	133	3330	250	300
200	...	...	268	...	375	220
250	...	...	...	...	510	160
300	...	...	...	...	675	130
350	...	...	...	...	860	...
400	...	...	...	...	1070	...
450	...	...	...	...	1300	...
500	...	...	...	...	1540	...
550	...	...	...	...	1810	...
Total gas evolved prior to ignition, g.-mole/100 g.	0.102	0.179	0.044	0.253	0.484	0.163
Time for spontaneous ignition at stated temp., hr.						
302° F. (150° C.)	800[a]	850	1700	675	2000[a]	2500[a]
338° F. (170° C.)	96	192	240	152	600	324
374° F. (190° C.)	22	15	25	20	57	134
Temp. for spontaneous ignition in 1 hr., ° F. (° C.)	437 (225)	437 (225)	410 (210)	450 (232)	457 (236)	482 (250)

[a] Failed to ignite in this period.

Table VI. Fertilizer Temperatures for Self-Sustained Decomposition

Grade	Nitrate N, %	Cl, %	Self-Sustained Decompn. after Ignition with Fertilizer 95° F.	176° F.
12-12-12				
Batch ammoniation	1.45	10.1	No	No
Prereactor	3.90	9.32	Yes	Yes
Nitraphosphate	5.32	9.37	Yes	Yes
16-8-8				
Prereactor (Nitrana)	4.26	6.30	Yes	Yes
Prereactor (Urana)	4.42	6.64	Yes	Yes
15-0-14, prereactor	4.61	10.7	No	Yes
18-9-9 with K_2SO_4	9.98	0.48	No	No
10-10-10 with N from urea and NH_3	0	7.8	No	No
16-8-8, commercial	1.77	6.58	No	No

the test temperature, completely unstoppered. The volume of each flask is determined, as is the volume of the compensator assembly up to the three-way stopcock on one side and the reference mark on the other. The working volume comprises the sum of the volumes of the flask and compensator, diminished by the volume of the mercury and the sample. The true density of the fertilizer, needed for computing the volume, is determined by displacement of acetone in a graduated cylinder. With the stopcocks and pinchclamp closed, the pressure of the gas can be computed from the barometric pressure, the manometer reading, and the previously determined pressure required to force the mercury in the compensator up to the reference mark.

At the start of a measurement, the absolute pressure in the sample container is reduced to about 50 mm. of mercury below atmospheric pressure, and it is usually allowed to rise to about 50 mm. above atmospheric pressure. Each measurement is made with the mercury adjusted to the reference mark, and the elapsed time is recorded. Results are expressed as micromoles of gas per hour per 100 grams of fertilizer.

Measurements on separate samples were made at 374° F. (190° C.), 338° F. (170° C.), 302° F. (150° C.), 226° F. (130° C.), and 212° F. (100° C.). When practical, they were continued until the samples had ignited. Ignition that happened to occur during a measurement was obvious, for the rapid flow of gas forced the mercury out the tubes. In the intervals between measurements, the temperatures of the samples were monitored with thermocouples connected to a recorder, and ignition of a sample appeared as a sudden rise in temperature. The occurrence of ignition could also be told by a change in the appearance of the sample, and in doubtful cases a portion was analyzed to show whether a large loss of nitrogen had occurred.

The gas evolution data from tests at 338° F. (170° C.) are representative, and are shown in Table V, the rates at round numbers of hours having been taken from plots of the rates of gas evolution against time. In these plots, the areas under the curves, up to the times of ignition, represent the total numbers of moles of gas evolved in the preignition periods, as given in the table.

The effect of temperature upon the rate of gas evolution is of great interest, but it is clear from inspection of Table V that no single rate of gas evolution can be stated for a given fertilizer at a given temperature. However, the effect of temperature is shown well by special calculations based on data for one of the fertilizers, the 16–8–8 by prereactor, made with Nitrana solution. For this fertilizer, the numbers of hours required for evolution of 10,000 micromoles of gas were calculated to be:

Temp., ° F.	Temp., ° C.	Hours
374	190	2
338	170	21
302	150	68
266	130	812
212	100	Over 2400

In fact, the total evolution of gas in 2400 hours (100 days) at 212°F. was calculated to be only 2800 μmoles.

Table V also gives the ignition times of the six fertilizers at various test temperatures. The temperatures for short-time ignition, approximately 1 hour, were determined by heating samples of the fertilizers in test tubes immersed in an oil bath, the temperature of which was raised at about 3° to 5° F. per minute. Ignition was shown by a sudden rise in the temperature of the sample, so that it was higher than the bath temperature, and by fuming.

Minimum Pile Temperature for Self-Sustained Decomposition. When the reaction provides ample heat for its own propagation, the initial temperature of the fertilizer makes little difference, but if the reaction generates barely enough heat, self-sustained combustion will occur only if the pile of fertilizer is somewhat hot to begin with. Just how hot it must be will depend upon a number of things, including the quantity of fertilizer, the nature of the container, the rate at which heat is applied, and the intensity and size of the source of heat. Under fixed test conditions, the required pile temperature should be fairly reproducible.

Figure 2 shows the apparatus used for the measurements reported here. The pipe is filled with the fertilizer, and three thermocouples are placed with their junctions 3, 9, and 15 inches, respectively, from the metal plate. The pipe was kept horizontal for the measurements shown here.

At the beginning of a test, the charge is brought to the desired temperature,

and an oxyacetylene flame is played upon the steel plate until its center is red hot, 5 minutes or less if ignition is evident sooner. If self-sustaining decomposition occurs, the progress of the reaction is shown by successive large increases in temperature at the three thermocouples. There is also heavy fuming.

Table VI shows the results of tests at two initial temperatures, 176° F. (80° C.) and 95° F. (35° C.). In a rough way, these represent the material entering the pile and the final pile temperature in summer storage. Four of the six fertilizers continued to burn when ignited by the hot metal, even with the initial temperature as low as 95° F., the 15–0–14 ignited only with the mass temperature 176° F., and the 12–12–12 made by batch ammoniation would not ignite with the mass at either temperature. No heat source of relatively short life, such as a burning brand or fertilizer bag, would be likely to ignite a pile of this 12–12–12.

Table VI shows results of tests on three other fertilizers as well, a low-chloride 18–9–9, a 10–10–10 with all nitrogen supplied as either ammonia or urea, and a commercial 16–8–8 of moderate nitrate content. None of these three could be ignited with the mass at either temperature.

Additional tests, not reported in detail here, showed that the range of pile temperatures needed to permit ignition by a flame extends at least from 40° F. (5° C.) to 284° F. (140° C.). Presumably there is no real upper limit, as some fertilizers should not undergo self-sustaining decomposition at any temperature.

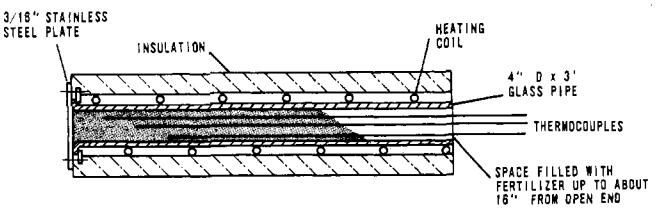

Figure 2. Apparatus for determining fertilizer temperature necessary for self-sustained combustion

Discussion

There is a tendency to treat the reactions of high analysis fertilizers such as these as being due solely to the ammonium nitrate that they contain, but the facts do not bear this out. The presence of chloride is evidently important.

Two important decomposition reactions of ammonium nitrate are:

$$NH_4NO_3 \rightarrow N_2O + 2\,H_2O \quad (1)$$

$$\Delta H_{410^\circ F.} = -51{,}900 \text{ B.t.u.}$$

$$\Delta H_{662^\circ F.} = -62{,}100 \text{ B.t.u.}$$

$$NH_4NO_3 \rightarrow N_2 + {}^1/_2O_2 + 2H_2O \quad (2)$$

$$\Delta H_{410^\circ F.} = -86{,}900 \text{ B.t.u.}$$

$$\Delta H_{662^\circ F.} = -96{,}900 \text{ B.t.u.}$$

These reactions are highly exothermic, but there is one that absorbs heat:

$$NH_4NO_{3(liquid)} \rightarrow NH_{3(gas)} + HNO_{3(gas)} \quad (3)$$

$$\Delta H_{410^\circ F.} = +39{,}900 \text{ B.t.u.}$$

The rate of this reation is such that when ammonium nitrate is freely exposed, so that the gases can escape, its decomposition is not self-sustaining, but ceases when the external supply of heat is cut off.

A mixture of ammonium nitrate and ammonium chloride can react as follows:

$$4NH_4NO_3 + 2NH_4Cl \rightarrow 5N_2 + Cl_2 + 12H_2O_{(gas)} \quad (4)$$

$$\Delta H_{410^\circ F.} = -508{,}000 \text{ B.t.u.}$$

$$\Delta H_{662^\circ F.} = -554{,}000 \text{ B.t.u.}$$

The chloride ion has an additional effect, in that it is known to catalyze the decomposition of ammonium nitrate according to Equation 2, without accelerating the dissociation into ammonia and nitric acid. As most fertilizer mixtures are distinctly acidic, there is also a chance that the following reaction will take place:

$$HNO_3 + 3HCl \rightarrow Cl_2 + NOCl + 2H_2O \quad (5)$$

In at least one case the presence of nitrosyl chloride was shown by its infrared absorption spectrum, but it is believed that Reaction 4 is generally of greater importance in the decomposition of fertilizers such as these.

There may be some loss of ammonia from ammonium sulfate:

$$(NH_4)_2SO_4 \rightarrow NH_3 + NH_4HSO_4 \quad (6)$$

However, the data in Table II indicate that this reaction is unimportant, at least before ignition.

The following reaction no doubt occurs in all of the fertilizers containing both ammonium nitrate and dolomite, especially the 15–0–14:

$$4NH_4NO_3 + CaCO_3.MgCO_3 \rightarrow Ca(NO_3)_2 + Mg(NO_3)_2 + 2CO_2 + 4NH_3 + 2H_2O \quad (7)$$

Part of the ammonia may be oxidized before it escapes. This reaction removes ammonium nitrate from the mixture, thus hindering Reactions 1 to 4. It also tends to make the mixture somewhat less acidic, by replacing part of the acid-reacting ammonium nitrate with calcium and magnesium nitrates. Because dolomite reacts so readily with acidic materials, no fertilizer mixture that contains a substantial proportion of dolomite can remain more than mildly acidic.

The only one of these fertilizers that contained urea was the 16–8–8 madewith Urana, and this contained only 2.5%. No definite equation can be written to show the fate of urea in such a mixture, but it is assumed that the carbon finally appears as carbon dioxide and the nitrogen as ammonia or its oxidation products.

Some carbon dioxide was evolved by each of the fertilizers, even the 12–12–12 made by batch ammoniation and the 12–12–12 nitraphosphate, though these fertilizers were made without urea or added dolomite. It is assumed that this came partly from calcium carbonate originally in the rock and not completely destroyed during acidulation, and partly from the oxidation of the small quantity of organic compounds originally in the phosphate rock and only partly oxidized during processing.

There are other possible reactions, such as those of the phosphates and fluorides with other constituents of the fertilizers. Some of these may be important—for example, in causing the reversion of phosphates to citrate-insoluble forms. Some of the reversion reactions probably involve disproportionation into a more basic, and hence more insoluble, residue, and a more acidic soluble portion. These will increase the apparent acidity of the mass.

In light of the discussion above, the following additional comments may be made.

None of the fertilizers gave important amounts of chlorine or of acid-forming nitrogen oxides in the preignition gases.

Relatively little gas was evolved by the 12–12–12 nitraphosphate, and this was especially low in carbon dioxide. This is presumably because conditions in the nitraphosphate process are especially favorable for decomposing carbonates and oxidizing organic matter.

The 16–8–8 that contained urea was slower to ignite than the one without urea. Carbon dioxide was a particularly prominent ingredient of the preignition gas from the fertilizer containing urea.

The high dolomite content of the 15–0–14 is presumably the cause of the difficulty in igniting this fertilizer, and of the high percentages of carbon dioxide and ammonia in the preignition gas.

Table VII. Effect of Nitrate and Chloride on Ignition Characteristics

Sample[a] No.	Cl Added as KCl, %	NO_3 N Added, % As NH_4NO_3	NO_3 N Added, % As KNO_3	Temp., ° F., When Sample Removed from Bath: Bath	Temp., ° F., When Sample Removed from Bath: Sample	Max. Sample Temp., ° F., after Removal	Decompn. Self-Sustaining after Removal from Bath	Rate of Burning after Removal from Bath
1	10	0.68	0	464	446	514	Yes	Very slow
2	10	1.13	0	464	419	536	Yes	Slow
3	10	2.26	0	451	426	545	Yes	Very vigorous
4	10	3.39	0	446	401	635	Yes	Very vigorous
5	10	0	0.68	509	477	531	No	...
6	10	0	1.13	491	471	513	No	...
7	10	0	2.26	478	448	622	Yes	Slow
8	10	0	3.39	482	442	608	Yes	Slow
9	0	2.26	0	486	430	493	No	...
10	1	2.26	0	469	410	554	Yes	Very vigorous
11	3	2.26	0	428	392	545	Yes	Very vigorous
12	5	2.26	0	455	392	541	Yes	Moderately fast
13	10	2.26	0	464	444	554	Yes	Vigorous

[a] Ammoniated superphosphate mixed with indicated quantities of KCl, NH_4NO_3, and KNO_3.

Table VIII. Ignition of Fertilizers with a Concentrated Source of Heat[a]

Grade	10–10–10	10–10–10	8–16–16	12–12–12
Method of preparation	Batch ammoniation	Batch ammoniation	Batch ammoniation	Nitraphosphate process
Source of nitrogen	Nitrana 2	Urana 15	Nitrana 2	NH_3 and HNO_3
Nitrate N, %	2.85	1.7	2.3	5.3
Cl, %	7.8	7.8	12.3	9.4
Heat input				
15 watts	N.T.[b]	N.T.	N.T.	No ignition up to 240 hr.
20 watts	N.T.	N.T.	N.T.	Ignited after 36 hr.
25 watts	Ignited after 2.6 hr.	No ignition up to 94 hr.	No ignition up to 94 hr.	N.T.
35 watts	N.T.	No ignition up to 94 hr.	No ignition up to 94 hr.	N.T.

[a] Source of heat was 100-watt bulb with voltage adjusted to produce desired watts.
[b] Not tried.

Mixtures of Ammoniated Superphosphate with Salts

Tests were made on mixtures of ammoniated superphosphate containing different percentages of potassium chloride and potassium or ammonium nitrate. One-hundred-gram samples in 125-ml. Florence flasks were heated in a salt bath, with results shown in Table VII.

The first eight samples all contained 10% chlorine added as potassium chloride, together with varying amounts and kinds of nitrate. Samples 1 to 4 contained from 0.68 to 3.39% nitrate nitrogen added as ammonium nitrate. Samples 5 to 8 contained the same respective percentages of nitrate nitrogen as 1 to 4, but supplied as potassium nitrate. These samples required higher temperatures for spontaneous ignition than the ones with ammonium nitrate.

The last five samples all contained 2.26% nitrate nitrogen added as ammonium nitrate, but had different percentages of chlorine, added as potassium chloride. In sample 9 there was no chlorine, and this sample showed no reaction. One per cent chlorine gave a vigorous reaction and a substantial temperature rise. In this group, the mixture with 3% chlorine was most active, those with 5 and 10% chlorine showing smaller temperature rises and less vigorous reaction. Equation 4 calls for 2 equivalents of chloride to 4 equivalents of nitrate, or 2.8 parts of chlorine by weight to 2.26 parts of nitrate nitrogen.

Ignition by a Submerged Heater

The possibility of igniting a large mass of fertilizer by a submerged heater must be considered, a possible heater being an electric light bulb inadvertently covered by the fertilizer, and not turned off.

A heater submerged in a very large mass of fertilizer will attain a certain maximum temperature, after which heat will flow away as fast as it is supplied. The maximum temperature attained depends directly upon the power input and inversely upon the diameter of the heat source, assumed to be a sphere. For example, a 25-watt input in a sphere of 20-cm. (about 8 inches) diameter could raise the temperature at its center by about 180° F. (100° C.), not enough to cause spontaneous ignition, except in very exceptional circumstances. The same 25-watt input in a sphere of 10-cm. diameter could raise the temperature by about 360° F. (200° C.), which would frequently be sufficient for ignition.

Table VIII shows the results of some experiments in which samples of fertilizer contained in a metal box, the same 1-foot cube described previously, were heated by means of an electric light bulb at the center of the box. This was a 100-watt bulb intended to operate at 115 volts; the applied voltage was controlled with a variable transformer to give the desired power input, 15 to 35 watts. The data show that two of the fertilizers in Table VIII (only the 12–12–12 nitraphosphate is one of the six described in Table I) were ignited with heat inputs of 20 to 25 watts, and that three failed to ignite with even 35 watts.

Spontaneous Heating

None of the six fertilizers of Table I ignited spontaneously at 266° F. (130° C.), but all showed appreciable rates of gas evolution at this temperature. In these tests the temperature was held at 266° F. by the controlled oil bath, but in a pile of fertilizer the temperature would rise if the rate of liberation of heat exceeded the rate of loss.

To consider a specific case, the rate of gas evolution by the 16–8–8 prereactor (Nitrana) was 8.6 μmoles per hour per 100 grams at 266° F. The corresponding rate of heat evolution depends upon the relative quantities of material entering into the different reactions, and these are not known

definitely. However, by making reasonable assumptions, one can calculate that the heat would be enough to raise the temperature on the order of 0.5° F. per day if there were no loss. If heat could not be dissipated so quickly from the interior of the pile, the temperature would rise and the reaction would be accelerated. Because of uncertainties of the calculation and the variations in heat transfer conditions possible, an exact point at which rising temperature of the pile would be encountered cannot be determined. For this fertilizer in a large pile, 266° F. (130° C.) would be in the uncertain range. It would consequently be safer to make a practice of cooling it below 212° F. (100° C.) before storing.

Effect of Organic Matter

A set of three fertilizers was made on a laboratory scale. These were basically 8–7–4's, with such additional plant food as the filler contained. The filler amounted to 580 pounds per ton of a fertilizer. In one it was inorganic, a mixture of sand and expanded vermiculite, in another it was cottonseed meal, and in the third it was activated sewage sludge. The fertilizers with the organic fillers consistently evolved more gas than the straight inorganic one. Nevertheless, the inorganic one gave prompt spontaneous ignition when heated to 468° F. (242° C.), while neither of those with organic filler ignited spontaneously even when heated to 545° F. (285° C.), the highest convenient temperature.

These tests were made on well ammoniated fertilizers. The work of Hardesty and Davis (*2*, *3*) has shown that underammoniated fertilizers containing organic material can heat spontaneously, even starting at room temperature, to such a degree that they ignite. Moreover, the test conditions were unsuitable for showing the spontaneous heating due to oxidation of organic matter. Thus, even though these fertilizers did not show a sudden, sharp ignition like the inorganic ones, they would be subject to spontaneous heating and possible destruction if placed in pile storage while hot.

Acknowledgment

The authors thank their colleagues who have contributed to both the theoretical and experimental parts of the work.

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PLANT ANALYSES

Rapid Determination of Nitrate and Nitrite in Plant Material

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Because of its rapidity and simplicity, this modification of the Nelson, Kurtz, and Bray method is well adapted for measuring day-to-day changes in nitrate or nitrite or for other applications in which the basic plant material remains essentially constant. Modifications suggested are use of copper and slightly more reagent powder, and allowance of more latitude in the time schedule of operations.

A MODIFICATION of the Nelson, Kurtz, and Bray (*2*) method for the determination of nitrate and nitrite determines nitrite by diazotization of sulfanilic acid and coupling with 1-naphthylamine to form a red dye. Nitrate must be reduced to nitrite for this analysis. The modifications suggested are the use of copper, the use of slightly more reagent powder, and the allowance of more latitude in the time schedule of operations than was proposed by the original authors.

Procedure

For determination of nitrate in the absence of nitrite, an aqueous extract of plant material is made, usually at the ratio of 1 gram of fresh or 0.1 gram of dried plant material to 20 ml. of water. To 1 ml. of this extract are added 9 ml. of 20% acetic acid solution containing 0.2 p.p.m. of copper as copper sulfate. By the use of a measuring scoop, 0.8 gram (between 0.6 and 1.0 gram) of the powder described by Nelson, Kurtz, and Bray (*2*) is added. This powder is an intimate mixture of 100 grams of barium sulfate, 75 grams of citric acid, 10 grams of manganous sulfate dihydrate, 4 grams of sulfanilic acid, 2 grams of powdered zinc, and 2 grams of 1-naphthylamine. The sample is then shaken for about 15 seconds, and is similarly shaken 3 minutes later. After 3 minutes more the sample is shaken for the third time and is centrifuged for 3 minutes at 1000 × G. The supernatant solution is poured through a small loose plug of borosilicate glass wool, the light absorbance is measured at a wave length of 520 mμ, and the amount of nitrate is calculated from a standard curve. The time between shakings is relatively unimportant; it can be halved or doubled with little change in results. Usually the powder is added to eight sample tubes in succession, the tubes are shaken successively by hand at 3-minute intervals, and all eight tubes are centrifuged simultaneously.

The same procedure may be used for the determination of nitrite in the absence of nitrate, but if nitrite is to be determined in the presence of nitrate, the zinc, manganese sulfate, and cupric sulfate should be omitted.

The determination of nitrate in the presence of nitrite requires two runs, one with the zinc, manganese sulfate, and cupric sulfate and the other without these substances. Different standard curves must be made for nitrate and nitrite, as not all of the nitrate originally present appears as nitrite after the reduction (Figure 1).

The water should be as free from copper as is possible, because the final

definitely. However, by making reasonable assumptions, one can calculate that the heat would be enough to raise the temperature on the order of 0.5° F. per day if there were no loss. If heat could not be dissipated so quickly from the interior of the pile, the temperature would rise and the reaction would be accelerated. Because of uncertainties of the calculation and the variations in heat transfer conditions possible, an exact point at which rising temperature of the pile would be encountered cannot be determined. For this fertilizer in a large pile, 266° F. (130° C.) would be in the uncertain range. It would consequently be safer to make a practice of cooling it below 212° F. (100° C.) before storing.

Effect of Organic Matter

A set of three fertilizers was made on a laboratory scale. These were basically 8–7–4's, with such additional plant food as the filler contained. The filler amounted to 580 pounds per ton of a fertilizer. In one it was inorganic, a mixture of sand and expanded vermiculite, in another it was cottonseed meal, and in the third it was activated sewage sludge. The fertilizers with the organic fillers consistently evolved more gas than the straight inorganic one. Nevertheless, the inorganic one gave prompt spontaneous ignition when heated to 468° F. (242° C.), while neither of those with organic filler ignited spontaneously even when heated to 545° F. (285° C.), the highest convenient temperature.

These tests were made on well ammoniated fertilizers. The work of Hardesty and Davis (*2*, *3*) has shown that underammoniated fertilizers containing organic material can heat spontaneously, even starting at room temperature, to such a degree that they ignite. Moreover, the test conditions were unsuitable for showing the spontaneous heating due to oxidation of organic matter. Thus, even though these fertilizers did not show a sudden, sharp ignition like the inorganic ones, they would be subject to spontaneous heating and possible destruction if placed in pile storage while hot.

Acknowledgment

The authors thank their colleagues who have contributed to both the theoretical and experimental parts of the work.

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PLANT ANALYSES

Rapid Determination of Nitrate and Nitrite in Plant Material

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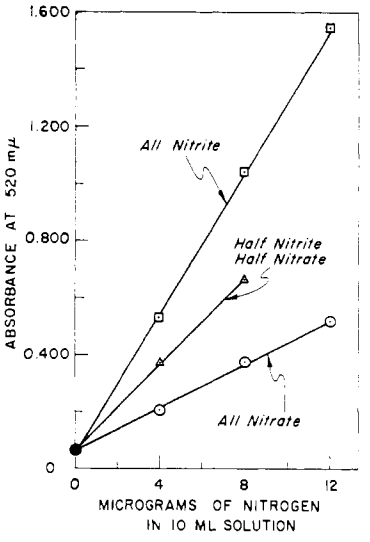

Figure 1. Standard curves for nitrate, nitrite, and a mixture of nitrate and nitrite

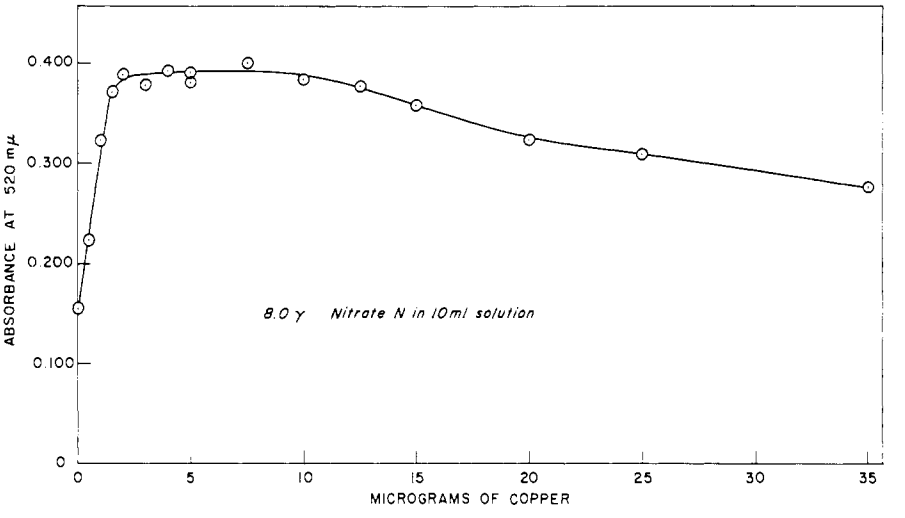

Figure 2. Dependence of nitrate determination on presence of copper

10 ml. of test solution must not contain more than 1.25 p.p.m. of copper. The water used in this work has been tested several times by the dithizone (*3*) method and has consistently been shown to contain less than 0.002 p.p.m. of heavy metals. Further, the electrical conductivity of the water was never higher than could be accounted for by dissolved carbon dioxide in equilibrium with the atmosphere. Water of this purity was obtained either by ion exchange with Rohm and Haas Amberlite MB-3 resin or by distillation in a borosilicate glass still. Water from these two sources gave identical results.

Standard potassium nitrate, potassium nitrite, and calcium nitrate samples, without plant material, were analyzed by this method, but with variation in the amount of copper used in the powder mixture.

Results and Discussion

The reduction of nitrate to nitrite in this method is dependent upon small amounts of copper salts. As is shown by Figure 2, maximum color is developed when the 10 ml. of test solution contain between 1.5 and 12.5 γ of copper. This is apparently a specific requirement for copper, in that cupric sulfate and cuprous chloride were equally effective at several concentrations, while individual applications of several other salts (cobalt chloride, potassium dichromate, sodium tungstate, ceric sulfate, tin chloride, and ammonium molybdate at concentrations of 0.1 μmole in the 10 ml. of solution, and 0.01, 0.1, and 1.0 μmole of ferrous sulfate) were completely ineffective in producing the required color.

This copper requirement was not found by the original investigators, presumably because their distilled water contained traces of copper from a defective tin-lined copper still.

More than the 12.5 γ of copper interferes with the determination of nitrite as well as of nitrate, but below this amount copper seems to have no other effect than that upon the reduction of nitrate.

Table I shows that nitrate and nitrite can be recovered after being added to plant material. Table II compares the results of this modified method to those obtained by the more accurate method of Johnson and Ulrich (*1*) for nitrate determination. The differences between the values obtained with the two methods are in the order of 10% for corn leaf samples containing more than 10 p.p.m. of nitrate nitrogen on a dry weight basis. Samples containing less nitrate than this cannot usually be analyzed with accuracy because the total quantity of plant material must be kept low to avoid interference with the analysis.

Table I. Recovery of Nitrate and Nitrite Added to Plant Leaf Material

Sample	Originally Present, γ	Added, γ	Total Found, γ	Recovery, % of Added
		Nitrate Nitrogen		
Corn 1	1.80	4.00	6.08	106
Corn 2	1.46	4.00	5.28	96
Magnolia	1.23	4.00	6.10	122
Soybean 1	6.02	4.00	9.80	94
		Nitrite Nitrogen		
Corn 1	0.05	4.00	4.00	99
Soybean 2	0.08	4.00	3.95	97
Soybean 3	0.10	2.00	2.25	108

Table II. Nitrate Determination in Corn Leaf Tissue by Two Methods

Sample	P.P.M. Nitrate Nitrogen Found (Dry Weight Basis): Modified Nelson, Kurtz, and Bray method	Johnson and Ulrich method
A	115	124
B	507	472
C	700	630

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Plant Damage and Eye Irritation from Ozone-Hydrocarbon Reactions

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Gas phase reaction products of several ozone-hydrocarbon mixtures, including mono-olefins, a diolefin, and aromatics, were assessed for their relative ability to damage pinto bean plants and to irritate eyes. Plant damage was obtained from all reactions whose products, after cleavage at the double bond, contained three or more carbon atoms; ozone reactions with propylene and 2-butene produced no phytotoxicant. Injury was markedly reduced by attaching a methyl group at the double bond of a straight-chain olefin; however, there was no difference in injury between cis- and trans- forms of a given olefin. None of the reactions irritated eyes above that amount reported for clean, carbon-filtered air.

OXIDANT plant damage and eye irritation are two characteristics of air pollution in the Los Angeles area. Haagen-Smit *et al.* (*4*) first reported that plant damage indistinguishable from that found in the field could be reproduced by reaction products of ozone and certain hydrocarbons. Lachrymatory effects were experienced with some of the mixtures, but specific examples were not cited. Reviews of the effects of these products on plants and the chemistry of the reactions are discussed elsewhere (*2, 5, 6*).

Recent work by Arnold (*1*) showed that the phytotoxicants from reactions of ozone with 2-pentene and with 3-heptene were short-lived, having half lives of 3 and 15 minutes, respectively. In contrast, eye irritants produced from irradiated auto exhausts at Stanford Research Institute (*3*) were stable, having a half life of several hours. It was suggested (*2*) that the phytotoxicant and eye irritant were not the same material.

In a study of the relative phytotoxicity of several ozone-hydrocarbon mixtures as a possible aid to identifying the phytotoxicant, it became of interest to determine if eye irritants were also formed. The present paper discusses these experiments.

Methods and Materials

Plant Fumigation Chamber and Eye Irritation Booths. A 16-cubic foot, glass and aluminum, fumigation chamber was constructed and operated as a stirred flow reactor as described previously (*2*). The chamber was set up in a glasshouse provided with activated carbon filters and air conditioning equipment. Natural sunlight was used for plant illumination and thus replaced the fluorescent tubes used in earlier experiments cited above. Maximum illumination was about 1700 foot candles on a bright day.

Except for slight modifications, the eye irritation booths and related equipment were the same as those developed for a similar purpose by the Stanford Research Institute (*3*). Three such booths were constructed adjacent to the plant exposure chamber. The exhaust air from the chamber was run in an all-glass system to a manifold and then through three air flow lines, one to each eye exposure booth. The end of each line was connected to a loose-fitting, plastic face mask. A valve system near the manifold permitted switching from air in the chamber to the filtered greenhouse air, defined as "clean air," so that persons sitting in the booths could be given either the experimental fumigants or clean air. When the subjects received clean air, air from the chamber was exhausted to the out-of-doors.

The air flow rate through the chamber was 36 liters per minute and was split equally between the three booths. This rate gave a residence time in the chamber of 13 minutes.

Fumigants. Ozone, ozone-hydrocarbon mixtures, and acrolein, a known eye irritant, were used in these studies.

Ozone was prepared at the desired concentration by passing cylinder oxygen through a high voltage discharge tube and into the stirred flow reactor. Steady-state concentrations were measured with a Kruger photometer.

Sixteen hydrocarbons were used including mono-olefins, a diolefin, and three aromatics. These materials were selected to provide a range in numbers of carbon atoms and of configuration.

Liquid hydrocarbons were dispensed from a gear-driven hypodermic syringe (*4*) and carried as vapors into the stirred flow reactor in a stream of oxygen. Gaseous materials were metered into a metered oxygen stream, and the resulting dilution was metered into the reactor. Hydrocarbon concentrations were calculated, as no instrument was available for measuring them.

Two concentrations of ozone and/or ozone-hydrocarbon mixtures were used. These were approximately 0.6 p.p.m. ozone to 10 p.p.m. hydrocarbon (low), and 2.5 p.p.m. ozone to 50 p.p.m. hydrocarbon (high).

Acrolein was diluted in water and the mixture dispensed from a syringe into a stream of oxygen as noted above. Concentrations were determined by absorbing the vapors in a buffered semicarbazide hydrochloride solution and reading the absorbance on a Beckman DU spectrometer.

Eye Irritation Panel. Thirty-six students, 26 male and 10 female, were selected from the student body of the Riverside campus. The only screening attempted was to use those students whose medical record showed freedom from chronic upper respiratory or eye troubles.

During exposures, the subjects wore activated carbon respirators so that they breathed clean air and only the eyes were exposed to the test mixture. Subjects were not aware of what they were receiving and were not able to see each other or view the equipment. Each student recorded the degree of irritation as none, medium, or severe on data sheets every 30 seconds during a 5-minute exposure. For statistical calculations, the values of 0, 1, and 2 were assigned, respectively, to the responses of none, medium, and severe. The maximum value recorded by a subject during a test was used as the response of that subject to a given material.

Plant Material. Pinto bean plants, 14 days old, were used as the test material for damage assessment. Four

Table I. Plant Damage and Eye Irritation from Carbon-Filtered Air, Acrolein, Ozone, and Ozone-Hydrocarbon Reactions

(13-minute residence in a stirred flow system)

Fumigant and Concn., P.P.M.[a]		Injury Index[b] 35 min.	Injury Index[b] 70 min.	Eye Irritation, Max. = 2.000
Carbon-filtered air		0.0	0.0	0.361
		0.0	0.0	0.265
		0.0	0.0	0.088
Acrolein				
0.06 (calcd.)		...	...	0.471
1.3–1.6		...	1.0(0.8) [0.5–1.2]	1.182
2.0–2.3		...	1.1(1.0) [0.7–1.4]	1.476
Ozone	Low	1.4	3.1	0.270
	High	5.6	8.3	0.090
Propylene	Low	0.0	0.0	0.250
	High	0.0	0.0	0.235
1-Butene	Low	0.0	0.0	0.114
	High	4.1(3.9) [3.2–4.6]	5.3(5.3) [4.8–5.8]	0.152
3-Methyl-1-butene	Low	0.03(0.03) [0.02–0.04]	0.2(0.1) [0.06–0.2]	0.188
	High	7.1(7.3) [6.8–7.8]	8.6(8.7) [8.3–9.1]	0.235
trans-2-Butene	Low	0.0	0.0	0.303
	High	0.0	0.0	0.286
1-Pentene	Low	0.6(0.4) [0.2–0.5]	1.3(1.1) [0.8–1.4]	0.156
	High	9.2(9.5) [9.3–9.8]	9.1(9.6) [9.1–9.8]	0.382
2-Pentene	Low	0.0	0.0	0.182
	High	0.9(0.5) [0.4–0.6]	2.5(2.2) [1.7–2.7]	0.088
cis-3-Methyl-2-pentene	Low	0.0	0.0	0.110
	High	0.0	0.4(0.2) [0.1–0.4]	0.090
1-Hexene	Low	0.8(0.5) [0.3–0.7]	2.6(2.3) [1.7–2.9]	0.167
	High	6.7(7.2) [6.3–8.1]	9.8(9.9) [9.8–10.0]	0.281
		...	7.7(8.1) [7.5–8.6][c]	None[d]
2-Methyl-1-hexene	Low	0.0	0.3(0.3) [0.1–0.5]	0.180
	High	0.1(0.05) [0.02–0.1]	1.1(0.9) [0.6–1.2]	0.110
		...	1.1(0.8) [0.5–1.1][c]	None[d]
cis-3-Hexene	Low	0.2(0.1) [0.07–0.2]	1.3(1.1) [0.7–1.5]	0.286
	High	2.8(2.6) [2.1–3.0]	5.5(5.8) [4.7–7.0]	0.333
		...	9.7(9.8) [9.7–9.9][e]	None[d]
trans-Hexene	Low	0.03(0.03) [0.02–0.05]	0.3(0.2) [0.1–0.3]	0.090
	High	1.5(1.4) [1.2–1.6]	3.4(3.4) [3.0–3.8]	0.210
		...	9.1(9.5) [9.1–9.8][e]	None[d]
3-Heptene	Low	0.4(0.2) [0.1–0.3]	1.4(1.2) [1.0–1.5]	0.161
	High	9.8(9.9) [9.9–10.0]	9.9(9.9) [9.9–10.0]	0.212
1,3-Butadiene	Low	0.0	0.0	0.182
	High	1.0(0.8) [0.6–1.0]	1.5(1.3) [0.9–1.7]	0.353
Benzene	Low	0.5(0.3) [0.2–0.4]	1.2(1.0) [0.7–1.3]	0.167
	High	2.2(2.1) [1.8–2.3]	1.6(1.5) [2.0–2.5]	0.265
Toluene	Low	0.0	0.0	0.147
	High	0.6(0.4) [0.2–0.6]	1.3(1.1) [0.7–1.5]	0.229
Cumene	Low	0.2(0.07) [0.04–0.1]	1.0(0.7) [0.5–0.9]	0.121
	High	2.3(2.2) [2.0–2.4]	2.0(1.9) [1.7–2.2]	0.235

[a] Only the hydrocarbons are listed for ozone-hydrocarbon reactions. Low, 0.6 p.p.m. ozone to 10 p.p.m. hydrocarbon; high, 2.5 p.p.m. ozone to 50 p.p.m. hydrocarbon.
[b] Index in ( ) corresponds to average angle; index range in [ ] corresponds to 99% confidence limits for average angle.
[c] Average of 4 experiments.
[d] Regular panel not available; value is response of 4 to 6 staff members.
[e] Average of 2 experiments.

sets of plants were placed in the chamber during four successive 35-minute periods and two sets were exposed during two cumulative 70-minute periods. Each set consisted of five pots, each containing three plants with two primary leaves per plant, or a total of 30 leaves per set. Injury to the primary leaves of exposed plants was estimated the second day after exposure as per cent of leaf surface damaged and recorded on an injury scale of 0 to 10, wherein 10 indicated 100% injury. For calculation of the 99% confidence limits, the scale of observations (per cent injury) was transformed to angles (angle = arcsin $\sqrt{\text{percentage}}$) to stabilize the variance (*7*).

Test Procedure. Plants and eyes were exposed simultaneously to a given fumigant. The selection of fumigant and concentration was randomized and only one combination was run on any one day. Approximately 31 subjects of the total panel were exposed each day. After a given fumigant had reached steady-state concentration, plants were placed in the chamber and the exhausts passed over the subjects' eyes. In ozone-hydrocarbon mixtures, the ozone was first brought to steady state, the hydrocarbon added, and the experiment begun when the reaction had reached steady state as indicated by the reduced ozone level on the photometer.

Results

The results of the experiments are summarized in Table I, showing the fumigants, concentrations, plant injury indexes corresponding to the average angles, the 99% confidence limits, and eye irritation values. The injury index indicates oxidant type damage except for that produced by ozone alone, which was typical of ozone. Damage from oxidants and ozone occurs on the lower and upper leaf surfaces, respectively, and is easily distinguished on the pinto bean (*5*).

Plant Damage. Plant damage was obtained at one or both concentrations with all of the hydrocarbons except propylene and 2-butene. This is the first report of damage for the six following materials: 3-methyl-1-butene, *cis*-3-methyl-2-pentene, *cis*- and *trans*-3-hexene, toluene, and cumene. Propylene and 2-butene give reaction products of one and two carbons; plant damage has never yet been obtained in ozone reactions with such materials. Haagen-Smit *et al.* (*4*) proposed that the most reactive hydrocarbons, with respect to formation of phytotoxicant, were those giving reaction products with three to five carbon atoms, and even those with three were sometimes inactive. It has since been reported (*2*) that compounds such as 2-pentene, giving rise to three-carbon reaction products, will produce phytotoxicants if the concentration and residence time are optimal. This is further borne out by the present results with 3-methyl-1-butene and *cis*- and *trans*-3-hexene, all of which give three-carbon reaction products.

In general, there is agreement with results of previous work in that the low concentration of 1-pentene, 1-hexene, and 3-heptene approached threshold values for plant damage with approximately $^1/_2$-hour exposures, and 1-hexene gave the most damage. At the high concentration, either at 35- or 70-minute exposures, damage from 1-pentene and 3-heptene reactions exceeded that from 3-hexene; damage from 3-methyl-1-butene and the two forms of 3-hexene also was equal to or exceeded that from 1-hexene.

Special attention was given to comparing the phytotoxicity of a straight-chain olefin with one wherein a methyl group was attached at the double bond, and cis and trans forms of a given material. For this purpose comparisons were made between 1-hexene and 2-methyl-1-hexene and between *cis*- and *trans*-3-hexene. The addition of a methyl group at the double bond markedly reduced plant damage and it is suggested that the methyl group interfered in some way with phytotoxicant formation. Changing from cis to trans forms did not alter level of phytotoxicity.

Oxidant damage was produced with about 2 p.p.m. of acrolein alone. While damage was not severe, it was definite and indistinguishable from the underside bronzing typical of oxidant damage. Haagen-Smit *et al.* (*4*) had shown that acrolein produced atypical damage on some of the test plants used.

Eye Irritation. No eye irritation was obtained with any ozone-hydrocarbon mixture that was appreciably higher than that reported for clean air. Neither was irritation reported for ozone alone, even at high concentrations. For comparison of acrolein (a known lachrymator) with carbon-filtered air, the frequencies for none, medium, and severe irritation resembled a Poisson distribution; one of the appropriate transformations is $y = \sqrt{x+1/2}$ (*7*).

Acknowledgment

Thanks are extended to Kenneth Greenlee, The Ohio State University, for preparing and supplying certain hydrocarbons, and to C. H. Hine and Frank Ivanhoe, University of California Medical School, San Francisco, for supplying the method of acrolein analysis.

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MINERAL SUPPLEMENTS

Copper, Nickel, and Cobalt Content of Oyster Shells

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The copper, nickel, and cobalt content in oyster shells has been found to be 33, 30, and 1 p.p.m., respectively. These quantities of copper and cobalt are too low to be significant in correcting soil and plant deficiencies by the application of oyster shells as liming material, but the cobalt present in oyster shells might be beneficial in poultry nutrition. Unlike copper, nickel is present in higher quantity in the shell of oysters than in the edible portion. The concentration of copper and cobalt from sea water to oyster shells is about the same, whereas nickel appears to be accumulated to a greater degree.

Oyster shells play a minor role in agriculture as a mineral supplement in poultry nutrition and occasionally as a soil additive in the form of lime or limestone. There does not appear to be a recorded observation on the cobalt or nickel content of oyster shells, and only a single reference to the quantity of copper in this material has been found.

A composite 10-pound sample of shells of the common oyster, *Ostrea virginica*, gathered on several occasions from different locations off the coast of Prince Edward Island, Canada, was thoroughly freed from organic matter by scrubbing with a stiff-bristle brush in fresh water. The shells were rinsed ten times in distilled water, dried overnight at 105° C., crushed, and pulverized to approximately 65 mesh. From this bulk sample a final 250-gram analytical sample was obtained by riffling, and this was pulverized to −100 mesh. The washing and drying procedures used on these shells left such small quantities of organic matter and moisture that their presence had no effect on the concentration of metals in the parts per million range.

Standard colorimetric procedures were employed to determine nickel as nickel dimethylglyoxime in alkaline solution, and cobalt by the Nitroso-R salt procedure. After the addition of dimethylglyoxime and removal of nickel and cobalt by extraction with carbon tetrachloride, copper was determined with sodium diethyldithiocarbamate (*5*). The following results, in parts per million, were obtained: copper, 33; nickel, 30; and cobalt, 1.

The quantities of these metals found in sea water by different investigators show considerable variation. This is not surprising when one considers the influence of land drainage, shore rock composition, currents, and other factors affecting the composition of sea water, as well as the difficulties in sampling and analysis for trace elements. The range of values published in recent years for copper, nickel, and cobalt in sea water is illustrated in Table I.

The figures reported by Young, Smith, and Langille (*11*) for copper and cobalt are of particular significance, as their samples were likewise taken from the sea around the Atlantic provinces of Canada. The average content of copper found by Young, Smith, and Langille agrees well with the mean compiled by Vinogradov (*10*), and their average cobalt value of 0.5 γ per liter shows good agreement with the averages listed by

Special attention was given to comparing the phytotoxicity of a straight-chain olefin with one wherein a methyl group was attached at the double bond, and cis and trans forms of a given material. For this purpose comparisons were made between 1-hexene and 2-methyl-1-hexene and between *cis*- and *trans*-3-hexene. The addition of a methyl group at the double bond markedly reduced plant damage and it is suggested that the methyl group interfered in some way with phytotoxicant formation. Changing from cis to trans forms did not alter level of phytotoxicity.

Oxidant damage was produced with about 2 p.p.m. of acrolein alone. While damage was not severe, it was definite and indistinguishable from the underside bronzing typical of oxidant damage. Haagen-Smit *et al.* (*4*) had shown that acrolein produced atypical damage on some of the test plants used.

Eye Irritation. No eye irritation was obtained with any ozone-hydrocarbon mixture that was appreciably higher than that reported for clean air. Neither was irritation reported for ozone alone, even at high concentrations. For comparison of acrolein (a known lachrymator) with carbon-filtered air, the frequencies for none, medium, and severe irritation resembled a Poisson distribution; one of the appropriate transformations is $y = \sqrt{x+1/2}$ (*7*).

Acknowledgment

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MINERAL SUPPLEMENTS

Copper, Nickel, and Cobalt Content of Oyster Shells

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The copper, nickel, and cobalt content in oyster shells has been found to be 33, 30, and 1 p.p.m., respectively. These quantities of copper and cobalt are too low to be significant in correcting soil and plant deficiencies by the application of oyster shells as liming material, but the cobalt present in oyster shells might be beneficial in poultry nutrition. Unlike copper, nickel is present in higher quantity in the shell of oysters than in the edible portion. The concentration of copper and cobalt from sea water to oyster shells is about the same, whereas nickel appears to be accumulated to a greater degree.

Oyster shells play a minor role in agriculture as a mineral supplement in poultry nutrition and occasionally as a soil additive in the form of lime or limestone. There does not appear to be a recorded observation on the cobalt or nickel content of oyster shells, and only a single reference to the quantity of copper in this material has been found.

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Standard colorimetric procedures were employed to determine nickel as nickel dimethylglyoxime in alkaline solution, and cobalt by the Nitroso-R salt procedure. After the addition of dimethylglyoxime and removal of nickel and cobalt by extraction with carbon tetrachloride, copper was determined with sodium diethyldithiocarbamate (*5*). The following results, in parts per million, were obtained: copper, 33; nickel, 30; and cobalt, 1.

The quantities of these metals found in sea water by different investigators show considerable variation. This is not surprising when one considers the influence of land drainage, shore rock composition, currents, and other factors affecting the composition of sea water, as well as the difficulties in sampling and analysis for trace elements. The range of values published in recent years for copper, nickel, and cobalt in sea water is illustrated in Table I.

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(7)		2	
(8)			0.23–0.32
(10)	20	3	1
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other investigators. If the respective values of 18, 2, and 0.5 γ per liter are accepted for copper, nickel, and cobalt in sea water, results of the present work indicate that copper and cobalt are concentrated in the oyster shell to nearly the same extent, 1.8×10^3 and 2×10^3, respectively. The concentration of nickel, however, from sea water to shell is appreciably higher at 1.5×10^4.

Copper in the edible portion of oysters has been reported by various workers to vary over the wide range of about 5 to 2000 p.p.m. in the living matter. A fair average of the compiled data of Vinogradov for *Crassostrea virginica* would be approximately 1100 p.p.m. of copper. As the edible portion of oysters contains about 87% water, it is evident that on the dry basis this part, even for the lower ranges of copper recorded in the literature, contains more of this element than found in the shell in the present study. The single reference to copper in oyster shells which was noted (*9*) gave a value of 25 p.p.m., which is close to the findings reported here.

Figures quoted by Vinogradov on nickel in gills, mantle, and hepatopancreas of *Crassostrea angulata* are 1.3, 0.9, and 1.0 p.p.m., respectively, and a value for nickel in the edible portion of *Crassostrea sp.* is given as 1.7 p.p.m. No reported reference to nickel in the shell was found. The author's value of 30 p.p.m. indicates that, unlike copper, there is considerably more nickel in the shell of oysters than in the edible portion.

Cobalt does not appear to have been determined in either edible portion or shell of oysters, though the element has been recorded in living matter and various organs of a few genera of Mollusca (*10*). The limited data indicate that, in general, cobalt is present in smaller quantity than nickel and that the nickel-to-cobalt ratio is about the same as that found in sea water.

Although the cobalt content of oyster shells is higher than that of most plant products, it is far too low to serve as a source of this element in cobalt-deficient areas by the application of lime derived from oyster shells. In regions of the world where maintenance of health in ruminants requires the addition of a cobalt supplement to fertilizer or lime, 2 pounds of the commercial sulfate per acre will last for 3 to 5 years. In other words, about 0.1 pound of cobalt per year will be needed, whereas the application of even 5 tons per acre of oyster shells would furnish only 0.01 pound of cobalt.

The copper content of oyster shells is probably too low for it to exert any significant effect in correcting a deficiency of this element in plants and soils. In various soils throughout the world where copper deficiencies for livestock, citrus fruits, or other crops occur, an application of commercial sulfate varying from 5 to 50 pounds per acre has been employed, and the effect usually lasts at least 3 years. A minimum of about 1 pound of copper per acre per year is thus indicated, whereas even 5 tons per acre of oyster shells would furnish only about 0.3 pound of copper.

There have been several reports on the beneficial effect from cobalt additions to poultry rations (*1*, *3*). From 1 to 12 p.p.m. of cobalt in the feed have resulted in increased growth. It is possible, therefore, that the minute quantities of cobalt in oyster shells may have significance in poultry nutrition.

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DIETARY PROTEIN VALUES

Complete *vs.* Total Protein in the Evaluation of Diets

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RICHARD J. BLOCK

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THE PROTEIN CONTENT OF FOODS is customarily determined by multiplying the nitrogen content of the food by one of several factors which range in magnitude from 5.7 to 6.66. The misleading impression that such factors may impart concerning the real protein content of mixed foods has been discussed (*5*). However, even if the protein content is determined by more adequate means, the result may or may not describe the value of this protein for nutritive purposes. The concept of "complete protein" was introduced (*7*) to define more clearly the quantity of protein which would be available to the organism for repair and synthesis of tissue. Complete protein is, by this definition, that fraction of the total dietary protein which is completely utilized for anabolic purposes. As the pattern of essential amino acids required by an animal appears to depend on its physiological state (growth, tissue repair, pregnancy, lactation, adult maintenance), the amino acid composition of the complete protein will vary with the animal's requirements. In this study, complete protein is represented by the pattern of essential amino acids needed

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Determination of the total protein content of food is not an adequate index of its nutritive value. Experiments in which rats were fed diets containing approximately equal amounts of total (crude) protein but differing from each other in their content of several essential amino acids and in their content of complete protein, show that the nutritive value of a food as a source of protein is closely correlated with its content of complete protein.

for growth of the young rat. The complete protein content of a food or diet is calculated from the equation:

$$P_c = Q \times \frac{A}{Ar}$$

where

P_c = percentage of complete protein in food
Q = percentage of total protein N × 6.25
A = quantity of limiting amino acid, grams per 16.0 grams N
Ar = requirement for limiting amino acid, grams per 16.0 grams N

The complete protein content cannot, by definition, exceed the total protein of the diet.

The experiments described here show that when various mixtures of white flour, lactalbumin, and lysine are fed to rats at the same level of total protein (N × 6.25), there is no relationship between the growth rate and the total protein (N × 6.25) content of the diets. On the other hand, when the complete protein content of the diets is used as the criterion, then, in accord with previous experiments (*7*), the growth response, the food consumption, etc., are significantly related to the amount of complete protein in the diets.

Table I. Growth Response of Rats on Diets of Equal Total Protein but Different Complete Protein Content

Supplement per 100 G. Wheat Flour		Protein		Weight Gain		
Lactalbumin, g.	Lysine. HCl, mg.	Total, %	Complete,[a] %	4-week, g.	Av. daily, %	Limiting Amino Acid Assumed
33	0	15.2	15.2	121	4.09	Cys-Met
25	0	15.3	15.3	130	4.21	Cys-Met
19	0	15.2	14.9	134	4.25	Cys-Met
14	0	15.1	14.0	129	4.18	Cys-Met
11	0	15.2	13.5	121	4.09	Cys-Met
8	0	15.2	12.6	110	3.92	Lysine
0	580[b]	15.1	11.6	82	3.39	Cys-Met
0	450[b]	15.0	11.6	96	3.67	Cys-Met and Lysine
0	710[b]	15.1	11.5	91	3.59	Cys-Met
0	760[b]	15.0	11.3	88	3.65	Cys-Met
0	830[b]	15.0	11.3	91	3.59	Cys-Met
5	0	14.9	10.4	85	3.45	Lysine
0	330[b]	15.1	10.3	93	3.57	Lysine
0	450	14.9	9.8	76	3.25	Cys-Met
0	330	14.9	9.8	76	3.25	Cys-Met
3	0	15.1	9.1	65	2.97	Lysine
0	220[b]	15.1	8.9	72	3.17	Lysine
0	220	15.1	8.9	67	3.07	Lysine
1.3	0	15.1	7.6	38	2.11	Lysine
0	100	15.0	7.5	40	2.18	Lysine

[a] Complete protein calculated on basis of limiting amino acid shown in column 7.
[b] Also contained 100 mg. of DL-methionine, 50 mg. of DL-threonine (allo-free), and 40 mg. of DL-tryptophan.

Experimental

Diets. Mixtures of high protein wheat flour (N = 2.67%) and lactalbumin (N = 12.8%) or of wheat flour and lysine.HCl (95% L-lysine.HCl) were added to starch, corn oil, minerals, and vitamins, so that all the final diets contained 15% total protein (N × 6.25). Details of the preparation of the diets have been described (*7*).

Amino Acids. The amino acid compositions of the diets were calculated from the composition of wheat flour (*5, 8*) and lactalbumin (*6*) and confirmed, in part, by analyzing the diets for lysine by paper chromatography (*6*).

Feeding. One hundred and twenty weanling male rats, weighing 45 to 50 grams each, were randomly distributed among 20 groups of six rats each. The animals were housed in individual cages and each animal received, *ad libitum*, water and one of the experimental diets. Individual records were kept of the weekly weight gains and the food consumption for the 28 days of the experiment.

Results and Discussion

Table I shows the total and complete protein contents of the diets and the weight gains. The dietary level of complete protein was calculated using the equation given above. In this calculation it was assumed that complete protein contains 5.3 grams of lysine, 4.6 grams of valine, 5.0 grams of sulfur amino acids (cystine plus methionine), 3.0 grams of threonine, and 1.1 grams of tryptophan per 16 grams of nitrogen (*1, 2*), although the following pattern was suggested by one of us (*4*) in 1945: arginine 4.7, histidine 2.0, lysine 5.2, tyrosine plus phenylalanine 8.6, tryptophan 1.1, cystine plus methionine 4.1, and threonine 3.6. The evidence that complete protein contains approximately 5.3 grams of lysine per 16 grams of nitrogen is considerable (*7*); however, the values for the remaining essential amino acids are less well documented.

In agreement with Bender's findings (*3*), the maximum growth rate attained on wheat flour supplemented with lysine does not exceed that corresponding to 65 to 70% complete protein. If the assumed estimate of amino acid requirements is correct, the second limiting essential amino acid in flour should be cystine plus methionine. However, experiments reported in the literature (*3, 11*) suggest that threonine is the second limiting amino acid in wheat proteins. This discrepancy suggests that the estimated requirements for the sulfur amino acids are too high and those for threonine too low. There is considerable evidence that the threonine requirement is in the order of 3.0 to 3.8 grams per 16.0 grams of nitrogen (*4, 5*) when amino acid mixtures simulating good animal proteins are used in the test ration. If the requirements for threonine are higher when wheat proteins are the source of the amino acids, the possibility of an amino acid imbalance causing this increased need should be considered. However, the levels of the sulfur amino acids, and of threonine, valine, and tryptophan in wheat proteins, are such that an error of 10 to 20% in the estimated requirements for any of these would change the identity of the second and third limiting essential amino acid without appreciably affecting the calculated quantity of complete protein.

In spite of these possible sources of error, statistical analysis of the results reveals that there is a significant relationship (correlation coefficient, r = 0.93) between the calculated amount of complete protein in the diet and the growth response (Figure 1) and that

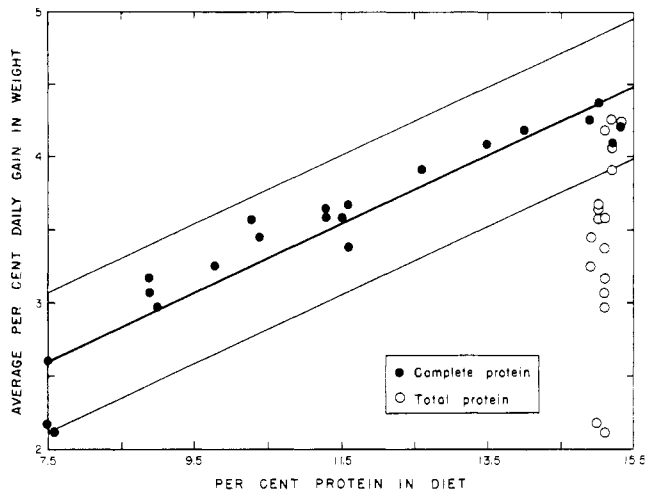

Figure 1. Correlation of average daily weight gain with complete and total protein

Fine lines represent range within which 95% of values may be expected to fall

there is a lack of correlation between the total protein in the diet and the growth response.

The results described in this paper demonstrate again that the nutritive value of foods is not adequately expressed by their total protein content (N × a factor) and that a better, although certainly not perfect, method would be the evaluation of foods on the basis of their complete protein content. This method of protein evaluation is not something for the distant future, but can be used with reasonable confidence at present. The amino acid composition of the important food proteins (*6*, *8*, *10*) and the amino acid pattern of complete protein (*2*, *4*) are already known with sufficient accuracy to be used in many practical applications. One further point is that the amino acids in the food which are being evaluated must be available to the organism. If one or more essential amino acids have been rendered partly unavailable, because of underheating in the case of many legumes or by overheating (toasting) in the case of certain cereals, the amount of this loss must be taken into account and the quantity of complete protein must be corrected accordingly.

The concept of complete protein is also useful in another respect. For example, certain methods of processing milk result in the biological loss of approximately 10% of its lysine (*9*). This would be of importance when the milk proteins are employed to supply lysine to lysine-deficient diets. However, in diets in which lysine is present in relative abundance but total protein is limiting, the inactivation of 10% or so of the lysine is immaterial. Milk proteins are approximately 80% complete, as they contain 4 grams of cystine plus methionine per 16 grams of nitrogen and the assumed requirement is 5 grams. As milk contains 8.0 grams of lysine per 16 grams of nitrogen (requirement is 5.3 grams), almost one half of the lysine in milk could be lost during processing without diminishing the amount of complete protein. Such an extensive loss of lysine is never caused by modern methods of processing milk.

Acknowledgment

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REVIEW OF FILLED MILK

Nutritional Evaluation of the Replacement of the Fat in Whole Cow's Milk by Coconut Oil

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THERE IS GROWING CONCERN among nutritionists and others regarding the far-reaching implications of the use of additives and substitutes in industrially prepared foods. In various milk-deficit areas of the world coconut oil mixed with nonfat milk solids is offered as a replacement for whole milk, for all purposes, including the feeding of infants.

It is desirable to know to what extent nutritional intakes are jeopardized by this substitution, especially in the feeding of children who in some regions, may be already on suboptimal diets. The purpose of this study is to review the findings and facts that bear directly or indirectly on the comparative nutritional qualities of milk fat and coconut oil, and the implications of the substitution of the one for the other in whole cow's milk.

Fats other than coconut oil do not appear to be extensively used as a substitute for milk fat in the production of filled milks, except in the United States. Coconut oil is the cheapest of the usable oils in most tropical countries. WHO reports that some olive oil is used in Spain and peanut oil in El Salvador. Hydrogenated cottonseed oil appears to be the substitute fat generally employed in the United States.

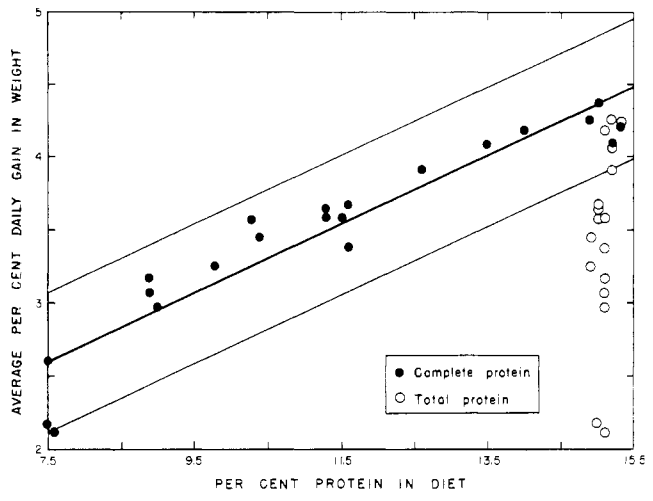

Figure 1. Correlation of average daily weight gain with complete and total protein

Fine lines represent range within which 95% of values may be expected to fall

there is a lack of correlation between the total protein in the diet and the growth response.

The results described in this paper demonstrate again that the nutritive value of foods is not adequately expressed by their total protein content (N × a factor) and that a better, although certainly not perfect, method would be the evaluation of foods on the basis of their complete protein content. This method of protein evaluation is not something for the distant future, but can be used with reasonable confidence at present. The amino acid composition of the important food proteins (*6*, *8*, *10*) and the amino acid pattern of complete protein (*2*, *4*) are already known with sufficient accuracy to be used in many practical applications. One further point is that the amino acids in the food which are being evaluated must be available to the organism. If one or more essential amino acids have been rendered partly unavailable, because of underheating in the case of many legumes or by overheating (toasting) in the case of certain cereals, the amount of this loss must be taken into account and the quantity of complete protein must be corrected accordingly.

The concept of complete protein is also useful in another respect. For example, certain methods of processing milk result in the biological loss of approximately 10% of its lysine (*9*). This would be of importance when the milk proteins are employed to supply lysine to lysine-deficient diets. However, in diets in which lysine is present in relative abundance but total protein is limiting, the inactivation of 10% or so of the lysine is immaterial. Milk proteins are approximately 80% complete, as they contain 4 grams of cystine plus methionine per 16 grams of nitrogen and the assumed requirement is 5 grams. As milk contains 8.0 grams of lysine per 16 grams of nitrogen (requirement is 5.3 grams), almost one half of the lysine in milk could be lost during processing without diminishing the amount of complete protein. Such an extensive loss of lysine is never caused by modern methods of processing milk.

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The fat of cow's milk in the light of modern nutritional knowledge is significantly preferable to coconut oil. The latter is low in unsaturated fatty acids, including linoleic acid, high in saturated fatty acids, and especially high in lauric acid, compounds of which have a toxic effect when fed under certain conditions. Milk fat carries with it important milk nutrients, not present in skim milk or coconut oil. Cow's milk fat more closely approaches human milk fat in composition. Feeding experiments with animals and man have demonstrated two special conditions under which milk fat rates higher nutritionally than coconut oil—during the growth period and when the diet otherwise is marginal. Full-cream cow's milk is significantly preferable to a substitute, the fat content of which is coconut oil, for consumption by the young in areas where dietaries are not all they should be.

Chemical Composition of Fats

Chemically, milk fat bears no resemblance to coconut oil, which in its turn does not resemble any other fats or vegetable oils. Table I illustrates differences that are important from the nutritional point of view.

Coconut oil is lowest with respect to the essential fatty acid, linoleic acid. It is low in unsaturated fatty acid content and very high in saturated. It is astonishingly high in lauric acid; no other commonly consumed fat compares in this respect.

Coconut oil differs greatly from the class of fats often referred to as vegetable oils. Corn oil is listed in the table for comparison. When the term "vegetable oils" is used in contradistinction to "animal fats," coconut oil should be marked as an exception to either class. An excellent example illustrating the different dietary behavior of coconut oil and corn oil was described by Ahrens *et al.* (*1*). Blood cholesterol in a 27-year-old male increased sharply when 40% coconut oil was included in the diet. When coconut oil was replaced with corn oil, the cholesterol level dropped just as rapidly.

Cow's milk fat contains butyric acid, the glyceride of which is readily absorbed and metabolized, as has been demonstrated in the feeding of premature (*35*). Coconut oil contains none.

Oleic acid, though not essential, makes a material contribution to a good diet. Its nutritional role has not been fully defined. Cow's milk fat contains a normal amount of this constituent, whereas coconut oil is one of the edible fats lowest in this respect.

Fatty Acids

Linoleic acid has been shown to be essential for maintaining good nutritional status. The needed supply can come only from food. Among other essential fatty acid–deficiency symptoms are skin disorders, especially in the growing young. The level of cholesterol in the blood is observed to rise when there is a deficiency of essential fatty acid. The majority of researchers in this field contend that among the causative factors in cardiovascular disorder are conditions which permit high levels of cholesterol in the blood.

Fats extremely high in saturated fatty acids, such as coconut oil, have been found to be a factor in increasing the cholesterol content of the blood, in part by reducing the effectiveness of linoleic acid (*11*).

The development of fatty livers has been used by investigators as an indication of an inadequate diet. It has been concluded by Stetten and Salcedo in feeding choline-deficient diets that intake of saturated fat proportionately increases fatty infiltration of the liver (*36*).

A well balanced fat should be neither high in saturated fats nor low in total unsaturated fatty acids (*18*). The ratio of saturated to unsaturated fatty acids in coconut oil as compared with milk fat is extremely wide (Table I).

Cow's milk fat does not average as high as human milk fat in essential fatty acid content, yet from clinical observations it must be concluded to be a satisfactory carrier of these nutrients in the infant's diet. Any fat that contains a lesser amount or causes an increased need for essential fatty acid is inadvisable as a substitute for milk fat. With respect to human milk fat, however, it has been demonstrated that the linoleic acid content may vary radically depending on the dietary intake of the mother, in some cases down to less than half of the cow's milk fat average (*19, 20*).

Table I. Fatty Acids in Certain Food Fats (*40*)

Fatty Acids	Percentage of Total Fatty Acids			
	Cow's milk fat[a]	Human milk fat	Coconut oil[b]	Corn oil[c]
Linoleic (essential)	3.0	7.8	0.8	54.1
Oleic	35.0	36.5	4.0	30.0
Total unsaturated	41.2	51.8	4.8	87.6
Total saturated	58.8	48.2	95.3	12.4
Butyric, C_4	3.2	0.4	...	...
Caproic, C_6	1.8	0.1	0.4	...
Caprylic, C_8	0.8	0.3	6.5	...
Capric, C_{10}	1.4	2.2	9.0	...
Lauric, C_{12}	3.8	5.5	55.1	...
Myristic, C_{14}	8.3	8.5	16.9	0.1

[a] U. S. ("selected value"). [b] Philippine origin. [c] Commercial, refined.

Short-Chain Fatty Acid Content

Studies show that fats composed of fatty acids in the short-chain class are readily digested, absorbed, and transported. The passage is direct from the intestine through the portal circulation rather than the lacteal (*7, 27*).

While both milk fat and coconut oil are higher in short-chain fatty acids than most edible fats, they differ when it comes to the individual acids. Milk fat is higher in the C_4 and C_6 fatty acids, and the reverse is true in the C_8 and C_{10} range.

Lauric Acid. The depot fat of man on a normal diet contains about 0.1% of lauric acid (*17*). The content of this fatty acid, expressed as percentage of total fatty acids in cow's milk fat, averages 3.8%, rising to an average of 5.5% in human milk fat. The extremely high figure of 55.1% for coconut oil is approached in no other edible fat.

A number of research studies have indicated that lauric acid and its compounds have a deleterious effect when fed in large amounts, particularly where the diet is suboptimal.

Cox (*8*), in feeding young rats with individual saturated fatty acid esters, observed a peculiarly high mortality rate among those receiving ethyl laurate. Similar results in feeding rats and other animals with various lauric acid esters, including the glyceryl ester, were later reported by Strach, Loeschke, and Blum (*37*), Stetten and Salcedo (*36*), Kesten, Salcedo, and Stetten (*24*),

Keane, Cohn, and Johnson (*22*), Schön *et al.* (*34*), and Dryden, Gleis, and Hartman (*12*).

In all cases the toxic action of the lauric acid compounds was particularly marked where the diets were suboptimal nutritionally and where the test animals were in the early growing period.

Feeding Experiments with Commercial Filled Milk

Only a few studies have been reported wherein coconut oil–containing "filled milks" purchased on the market have been compared with competitive whole milk products.

Freeman and Ivy (*13*) compared a commercial filled milk with evaporated milk in feeding rats. Differences between the feeding regimens began to appear at 49 days; the filled milk group showed diminished growth and a greater incidence of loose stools.

Boutwell *et al.* (*6*) in their studies on the comparative nutritional value of milk fat and other fats and oils, including coconut oil, were led to the conclusion that filled milks are inferior to whole milk in growth-promoting properties when fed to young rats.

Coconut oil–filled concentrated milks, as sold commercially, differ from the milks they are designed to imitate also in composition. Imitation evaporated milk is generally standardized at 6% coconut fat as against 7.9 to 8.0% milk fat in evaporated milk. The nonfat portion of the filled milk, however, is often higher than that of evaporated milk.

Coconut Oil in Laboratory Diets

There are a number of reports of synthetic rations in which milk fat was incorporated on the one hand and various other fats on the other, including coconut oil. In a recent publication from the Yale Nutrition Laboratory, Barboriak *et al.* (*3*) observed 600 rats for periods up to 2 years on high-fat diets which included cow's milk fat and coconut oil. After several months of high-fat feeding, 61.6 or 81% of the calories, more fat storage was observed in the milk fat–fed rats than in those on diets containing coconut oil. It was concluded that milk fat is better digested and metabolized. The investigators found that coconut oil had the lowest growth-promoting value of the tested fats.

Numerous other reports in the literature demonstrate under various feeding conditions that coconut oil does not support the growth and well-being of young animals as well as other fats (*4, 14, 16, 33, 39*).

An especially important study from the practical point of view was recently published by Kehar and associates from India (*23*). Here, ghee (highly heated milk fat in practically anhydrous form) was made a part of a diet given to rats over a three-generation period. Coconut oil was one of the other fats fed for comparison. One of several rations used in the experiment was designed to represent the kind of suboptimal diet often consumed in India. Under such feeding conditions the ghee-fed animals grew better than those on diets containing coconut oil or the other fats. No significant differences were observed among the fats, however, when the animals were on a liberal diet.

In an earlier study in India, De and Karkun (*9*) fed to three human subjects rice-fish diets with various fats. Negative calcium balances were exhibited when coconut oil was the fat used, while milk fat maintained the subjects in positive calcium balance. The investigators also compared milk fat and coconut oil in feeding similar diets to rats. Growth rates were observed to be greater on the rations containing milk fat.

In Japan, Tange (*38*) found that the growth of rats fed butter was superior to the growth of those receiving coconut oil. However, when 2% yeast was added to the ration the differences were insignificant.

The coefficients of digestibility of cow's milk fat and coconut oil are similar —both over 97%. Absorption rates differ markedly, particularly in the earlier stages of digestion. Deuel (*10*) reports that in rat feeding fat absorbed in 4 hours was 71% for milk fat and 47.4% for coconut oil. Fat absorbed in 1 hour per 100 sq. cm. of surface was calculated to be 62.3 mg. for milk fat and 41.2 mg. for coconut oil.

Dietary Interrelationships

Dietary interrelationships constitute an area in nutrition that has had much attention. The particular superiority of cow's milk fat over other fats in promoting growth when the carbohydrate of the ration is lactose has been demonstrated by Boutwell and associates (*6*).

Nutrient relationships need more investigation; it may be found that the utilization of other important dietary elements is partially aided by the normal fat of the milk, a situation that does not obtain when other food fats are substituted (*2, 41*).

An extraordinary interrelationship in feeding 10% hydrogenated coconut oil along with 20% palmitic acid and 1% cholesterol to weanling mice was observed by Bosshardt, Kryvokulsky, and Howe (*5*). Complete cessation of growth occurred when the combination was fed; but the exclusion of any one of the three alleviated the adverse growth effect. Trace amounts of oleic and linoleic acids in the mixture as contributed by the coconut oil were believed to be a causative factor.

Milk Fat Nutrients Not Present in Coconut Oil

Milk may well be expected to contain factors that contribute to its nutritional value, other than protein, carbohydrate, minerals, vitamins, and fat, and are not to be found in the plant fats in identical form. Cow's milk fat is especially rich in them. Many have been isolated and studied, but much of the fraction remains unidentified (*21*).

Lecithin is present in cow's milk in substantial quantities—average 0.057 gram per 100 ml.; in human milk 0.078 gram per 100 ml. (*26*). On separation of cream from the nonfat portion of the milk, the lecithin accompanies the fat predominantly. Butter averages about 0.2% lecithin.

Dietary lecithin aids fat digestion and absorption. The oral administration of lecithin has been shown to reduce the level of blood cholesterol in hypercholesterolemic patients (*28*).

Some plants contain lecithin-like compounds, as in the case of soybeans (*17*). The presence of such substances in coconut oil has not been reported.

Other Phospholipides are contained in milk. Like lecithin they are mostly fat-soluble and pass into the cream on separation.

McGillivray finds evidence for the conclusion that the phospholipide content of dairy products is responsible for their greater effect on blood coagulation relative to vegetable fats (*25*). The feeding of cream stimulates the release of heparin into the blood stream, with resulting lipemia clearing (*30*). The evidence would seem to indicate, however, that the relationship among fat feeding, coagulation, and atherosclerosis should be investigated more extensively (*32*).

Plant Sterols, such as the sitosterols, contained in many vegetable oils, when present in the diet, have been credited by some investigators with having a favorable influence in holding blood cholesterol to lower levels, either by retarding absorption of dietary cholesterol or by exerting their influence metabolically. The general conclusion, however, is that these substances may be expected to have no more than minimal effect (*7, 31*). Whatever benefit there may be, it has not been demonstrated that coconut oil is a carrier of the sitosterols.

Cholesterol is present in cow's milk, though to a lesser extent than in human milk—0.014 and 0.020 gram per 100 ml., respectively (*26*).

Because all animal fats contain some cholesterol whereas vegetable fats do not, it has often been postulated that animal fats, including milk fat, should be shunned in favor of vegetable oils. The National Research Council Committee on Fats in Human Nutrition,

however, concludes that in man dietary cholesterol has but minor influence on plasma cholesterol levels (7).

Trace Substances in milk fat, hitherto not recognized, are being identified by the newly perfected methods now available to the laboratories. For example, Hansen, Shorland, and Cooke have identified fatty acids in the range of C_{20} to C_{26} which are believed to result from the direct assimilation of dietary lipides by the cow (*15*).

Conclusions

Despite innumerable studies comparing the nutritional value of fats, there are many unanswered questions. For instance, with certain dietary regimens while milk fat induces a more rapid growth than most fats in young animals, longevity sometimes is significantly reduced (*29*). In practical infant nutrition, however, a parallel manifestation would seem unlikely. Current pediatric literature emphasizes the importance of providing the infant and child with a favorable nutritional environment as an essential condition for optimal health in later decades.

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BEEF AROMA

Some Volatile Constituents of Cooked Beef

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This study was undertaken to identify as many as possible of the volatile compounds responsible for the characteristic odor of cooked beef. While it is a common observation that this aroma develops only after heating, the literature affords relatively little information as to the nature of the compounds responsible.

Experimental Procedures and Results

Beef Broth. Fresh lean beef (round steak, U. S. Choice or U. S. Good) was trimmed free of fat, passed through a meat grinder, and refluxed with an equal weight of water for 3 hours. Two drops of Dow-Corning antifoam A were added before refluxing to prevent excessive foaming. After cooling and filtering, the broth was distilled to one third to one half of the original volume at atmospheric pressure either at its natural pH of 5 to 6 or after pH adjustment. Most of the characteristic odor was present in the distillate. Unless otherwise specified, only the distillate was used for chemical studies.

Basic Fraction. When the broth was to be examined for volatile basic compounds the distillate was collected in 2*N* acid, either sulfuric or hydrochloric, or the distillate was allowed to pass directly into a 2.6 × 28 cm. Dowex 50 (H^+) column and the column was subsequently eluted with 1*N* hydrochloric acid. The acidic solution obtained by either of these

however, concludes that in man dietary cholesterol has but minor influence on plasma cholesterol levels (7).

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BEEF AROMA

Some Volatile Constituents of Cooked Beef

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This study was undertaken to identify as many as possible of the volatile compounds responsible for the characteristic odor of cooked beef. While it is a common observation that this aroma develops only after heating, the literature affords relatively little information as to the nature of the compounds responsible.

Experimental Procedures and Results

Beef Broth. Fresh lean beef (round steak, U. S. Choice or U. S. Good) was trimmed free of fat, passed through a meat grinder, and refluxed with an equal weight of water for 3 hours. Two drops of Dow-Corning antifoam A were added before refluxing to prevent excessive foaming. After cooling and filtering, the broth was distilled to one third to one half of the original volume at atmospheric pressure either at its natural pH of 5 to 6 or after pH adjustment. Most of the characteristic odor was present in the distillate. Unless otherwise specified, only the distillate was used for chemical studies.

Basic Fraction. When the broth was to be examined for volatile basic compounds the distillate was collected in 2*N* acid, either sulfuric or hydrochloric, or the distillate was allowed to pass directly into a 2.6 × 28 cm. Dowex 50 (H^+) column and the column was subsequently eluted with 1*N* hydrochloric acid. The acidic solution obtained by either of these

The volatile fraction from lean beef cooked in boiling water was shown to contain hydrogen sulfide, ammonia, acetaldehyde, acetone, and diacetyl. In addition the presence of formic, acetic, propionic, butyric, and isobutyric acids, and of dimethyl sulfide was tentatively established. Volatile alcohols and esters were absent. The amount of hydrogen sulfide obtained after 3 hours of boiling was 6 to 8 mg. per kg. of beef, but much larger amounts were evolved after boiling for 7 days.

Table I. Volatile Carbonyl Compounds from Beef Broth

Compound, DNP Derivative	Melting Points, ° C. Isolated	Authentic	Mixed	Additional Evidence
Acetone	121–3	124–5	123–4	Paper chromatography, infrared spectrum, x-ray diffraction
Acetaldehyde	155–7	156–7	155–7	Same as for acetone
Diacetyl	d. >300	d. >300	d. >300	Infrared spectrum, ultimate analysis

methods was evaporated to dryness, the residue made alkaline with sodium hydroxide, and the mixture then redistilled into redistilled aqueous hydrochloric acid. After vacuum evaporation of the resulting solution, a white salt was obtained in a yield of approximately 0.10 gram per kg. of fresh lean beef.

This product gave a negative test for carbon by the method of Pepkowitz (*8*). Upon addition of sodium hydroxide, it liberated a volatile base which turned red litmus paper blue and gave the odor of ammonia. It was, therefore, concluded that this salt was ammonium chloride. This conclusion was confirmed by decomposing a portion of the salt with sodium hydroxide and aerating the volatile base into a solution of phenylisothiocyanate. A solid derivative formed almost immediately which, after washing with Skellysolve B and 50% ethyl alcohol and recrystallizing from 95% ethyl alcohol, melted at 157°–158° C. This product showed no melting point depression when mixed with known phenylthiourea.

Carbonyl Compounds. Fresh broth prepared as described above was distilled into a flask which was immersed in an ice bath and contained a 0.2% solution of 2,4-dinitrophenylhydrazine (DNP) in 2*N* hydrochloric acid. The precipitate of crude dinitrophenylhydrazones was filtered off, dried, and weighed. The yield was approximately 0.09 gram per kg. of fresh beef.

The crude mixture of DNP derivatives so obtained was stirred up repeatedly with 30- to 40-ml. portions of boiling ethyl alcohol and the insoluble portions were filtered off. About 0.04 gram of this insoluble fraction, presumably consisting of polycarbonyl derivatives, was obtained per kg. of beef. The ethyl alcohol solution containing the monocarbonyl derivatives was evaporated to dryness, the residue dissolved in warm 1 to 1 benzene to heptane, and the solution poured onto the top of a 2.2 × 10 cm. column of powdered anhydrous magnesium sulfate. The column was developed with the same solvent mixture which was passed through under pressure, until the carbonyl derivatives were eluted and the effluent became almost colorless. Under these conditions the unreacted dinitrophenylhydrazine reagent remained at the top of the column.

The effluent was evaporated to a small volume and a convenient amount chromatographed on Whatman No. 1 paper according to the method of Huelin (*5*). The developed chromatogram showed two yellow spots with R_f values of 0.26 and 0.40 which corresponded to those of the DNP derivatives of acetone and acetaldehyde, respectively. Judging from the intensities of the spots on the chromatograms, the acetaldehyde derivative was the major component.

To obtain sufficient material for identification, the concentrated effluent from the magnesium sulfate column was applied to a number of papers in a narrow, intensely colored band. After development of the chromatograms the bands corresponding to the above two spots were separately eluted with 95% ethyl alcohol and the eluates pooled and evaporated to dryness. The derivatives were recrystallized from 95% ethyl alcohol to a constant melting point. As shown in Table I these two products proved to be identical with the derivatives of acetone and acetaldehyde, respectively. In addition to melting point and paper chromatographic data, infrared tracings and x-ray diffraction patterns were obtained for each of the dinitrophenylhydrazone derivatives. In all cases the results with the isolated and known compounds were identical.

The ethyl alcohol–insoluble polycarbonyl fraction was recrystallized from nitrobenzene without previous passage through the magnesium sulfate column. Comparison of the melting point and infrared spectrum of these crystals with those of an authentic sample of diacetyl - *bis*- 2,4 - dinitrophenylhydrazone showed that they were identical (Table I).

Analysis. Calculated for $C_{16}H_{14}O_8N_8$: C, 43.05; H, 3.16; N, 25.11. Found: C, 43.20; H, 3.33; N, 25.04.

Fatty Acids. Fresh broth was acidified with sulfuric acid to pH 1 and distilled at atmospheric pressure to two thirds of the original volume. The distillate was collected in a receiver cooled in an ice bath and was titrated to a phenolphthalein end point with sodium hydroxide. Approximately 1 meq. of acids was obtained per kg. of fresh meat.

The neutralized distillate was evaporated to dryness in vacuum and the salts decomposed with 6*N* sulfuric acid. The free fatty acids were taken up in ether and a solution of diazomethane in ether was added in small portions until a faint yellow color persisted. Most of the solvent was evaporated from the resulting solution of methyl esters by placing the mixture in an open Erlenmeyer flask and warming gently. No effort was made to remove the last traces of ether for fear that volatile esters might also be evaporated. The remaining solution was then subjected to gas chromatography on a Model 154 Perkin-Elmer Vapor Fractometer. The column used was 5 mm. in internal diameter and 2 meters long, and was filled with one part of diisodecylphthalate on four parts of 60- to 80-mesh Celite 505 (Johns-Manville Co., filter aid) support. The conditions and results are shown in Figure 1.

In addition to peaks caused by air and ether, five more peaks corresponding to the methyl esters of formic, acetic, propionic, isobutyric, and butyric acids were observed. The retention times of the esters checked very closely with those of the known samples. However, no additional evidence was obtained to verify the tentative identification of the esters as listed above.

Sulfur Compounds. In order to look for volatile sulfur compounds the vapor arising from the simmering meat was carried by a stream of high purity nitrogen gas through a reflux condenser, then through a wash bottle containing 20 ml. of 2*N* sulfuric acid for removal of ammonia, and then into various trapping solutions. When either aqueous lead acetate or mercuric cyanide solutions were used as trapping agents, black precipitates formed. The black precipitate in the lead acetate trap was

filtered off, washed, and dried, and subsequently found to give a negative test for carbon by the method of Pepkowitz (*8*). The black precipitate obtained in the mercuric cyanide trap gave no odor when warmed with dilute hydrochloric or sulfuric acids. These results indicate the presence of hydrogen sulfide and the absence of volatile mercaptans, as mercaptans are released when their mercuric salts are warmed with dilute mineral acid (*7*). It is possible that mercaptans might have been oxidized to the corresponding disulfides during passage of the vapors through the 2.N sulfuric acid trap and the other compartments of the absorption train (*3*). However, no disulfides were detected either, hence no evidence was obtained in the present work that the vapors contained mercaptans.

A quantitative determination of the hydrogen sulfide released from the cooked beef was carried out by the methylene blue method as described by Sands and coworkers (*9*) and by Marbach and Doty (*7*). The vapor above the simmering beef was aerated with high purity nitrogen into 2% zinc acetate as the trapping solution. This was replaced by fresh solution as soon as any visible turbidity developed. After 3 hours the turbid mixtures were pooled and diluted to 500 ml. with 2% zinc acetate, and the color was developed as previously described (*9*). Absorbancy readings were obtained on a Bausch and Lomb Spectronic No. 20 colorimeter at 665 mμ. The readings were converted into equivalent hydrogen sulfide values by interpolation on a standard curve prepared with known quantities of sodium sulfide (*7*). In two determinations the amount of hydrogen sulfide found varied from 6 to 8 mg. per kg. of fresh beef.

When the vapors from the cooking meat were passed first through a sulfuric acid trap and then into a mercuric chloride trap a white precipitate was obtained in the latter. This product showed no crystalline form when examined under the microscope and began to decompose at 250° C. On treatment with 6.N hydrochloric acid no odor was detected. This result indicated that disulfides were absent. However, the precipitate gave rise to an odor very similar to that of dimethyl sulfide when treated with 10% sodium hydroxide. This behavior is characteristic of volatile thioethers (*1*).

The quantities of volatile thioether obtained were so small that positive identification by classical methods was practically impossible. The vapor obtained from 1.35 kg. of beef after decomposition of the mercuric chloride salt with sodium hydroxide was passed through the Perkin-Elmer gas chromatography apparatus, but failed to result in a detectable peak. Subsequently, the gas chromatographic analysis of a similar thioether preparation was repeated on a Barber-Colman gas-liquid chromatograph Model 10 with the very sensitive strontium-90 detector (*6*). The column employed was 8 feet long and was filled with five parts of silicone oil on 100 parts of Chromosorb W support (Murray and Baker Co., Dagenham, England). In addition to peaks due to air and water, a small peak corresponding in retention time to authentic dimethyl sulfide was obtained.

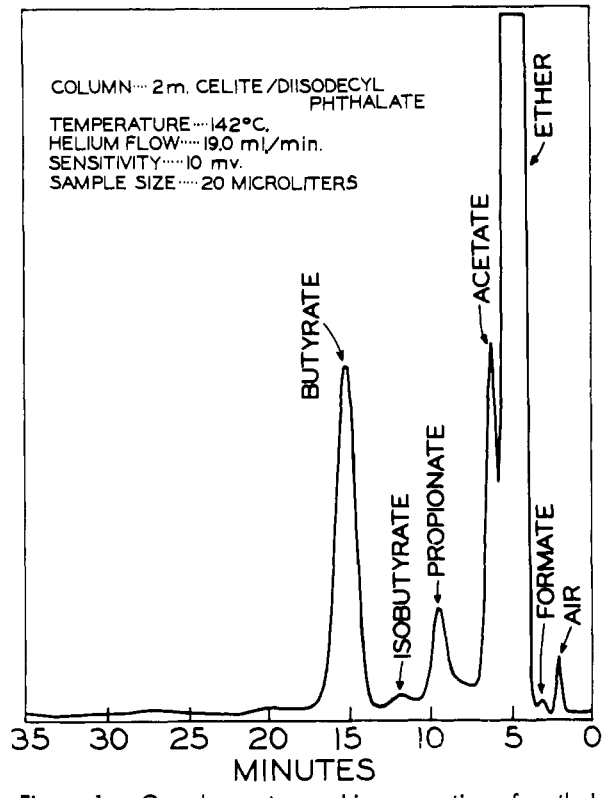

Figure 1. Gas chromatographic separation of methyl esters of volatile fatty acids of cooked beef

Discussion

The results obtained in the present study establish the presence of a number of compounds in the volatile fraction of cooked beef in addition to hydrogen sulfide, previously reported by Crocker (*2*). The compounds which have been firmly identified include ammonia, acetaldehyde, acetone, and diacetyl. In addition, reasonably good evidence for the presence of formic, acetic, propionic, isobutyric, and butyric acids and of dimethyl sulfide was obtained. An attempt to find volatile alcohols in the distillate from the broth by preparation of 3,5-dinitrobenzoyl esters was unsuccessful. Similarly, a qualitative test for esters by means of alkaline hydroxylamine followed by ferric chloride was negative. It seems probable, therefore, that volatile alcohols and esters were not present in appreciable amounts in the beef broth.

While the present report was in preparation, a paper by Hornstein *et al.* (*4*) appeared which described the identification of small amounts of carbonyl compounds, ammonia, and hydrogen sulfide in the volatile fraction of heated beef extract. Their results agree well with those of the present study.

Hydrogen sulfide, ammonia, diacetyl, and acetaldehyde appear to be some of the major components responsible for the characteristic aroma of cooked beef. All of these substances seem to be liberated during cooking from essentially odorless precursors. Whether the ammonia and hydrogen sulfide are derived from the amino and sulfhydryl groups of the meat proteins has not, to the authors' knowledge, been established. It was noted in the present study that greatly increased amounts of hydrogen sulfide were evolved from the meat if the heating period were prolonged. For example, the white precipitate in the mercuric chloride trap (presumably mostly $HgCl_2.2HgS$) obtained after boiling and aerating continuously for 3 hours and, in another experiment, for 7 days weighed 0.04 and 1.34 gram per kg. of beef, respectively.

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MEAT FLAVOR CHEMISTRY

Flavor Studies on Beef and Pork

IRWIN HORNSTEIN and PATRICK F. CROWE

Meat Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

The odor responses, and the chemical compounds isolated, from the volatile pyrolysis products of lyophilized cold water extracts of lean beef and lean pork were found to be similar. The flavor precursors in lean meat are low molecular weight compounds present in the dialyzable portion of the cold water extracts of the raw lean meats. Beef and pork fat when heated produced dissimilar aromas. Free fatty acids and carbonyls were determined in these fats before and after heating. The results suggest that, on heating, the lean portions of pork and beef contribute an identical meaty flavor to these meats, while the characteristic flavor differences in pork and beef reside in the fat.

PRIMARY EMPHASIS has been placed on the odor constituents of meats. The authors previously studied lean beef and found the flavor precursors present in the raw meat to be cold water–extractable. This extract was lyophilized, and the resulting powder heated under vacuum and fractionated into two major portions. The more volatile fraction has been studied (*7*). Investigation of the less volatile fraction (fraction I) is reported in this paper. Lean pork has also been subjected to the techniques previously applied to lean beef and its volatile constituents have been examined. In addition, beef and pork fat have been analyzed for free fatty acids and monocarbonyl compounds, and possible flavor precursor systems have been studied.

Experimental

Lean Beef, Fraction I. The powder obtained by the lyophilization of a cold water extract of raw, lean beef contains flavor precursors of cooked beef. The total volatiles produced by pyrolysis of this powder at 100° C. are trapped at liquid nitrogen temperatures and fractionated at room temperature under vacuum into two major fractions. The less volatile of these two fractions, fraction I, is a viscous residue of meaty aroma (*7*).

INITIAL OBSERVATIONS. Approximately 100 mg. of fraction I were obtained for every 30-gram batch of dried powder pyrolyzed. The pH of a water solution of 10 mg. of I per ml. varied from 3.5 to 4.0. The infrared spectrum of a film of I on rock salt plates was obtained on a Perkin-Elmer Model 137 Infracord spectrophotometer. Major peaks were recorded at 3.15, 5.81, 6.32, 7.12, 8.9, 9.6, and 11.7 microns (Figure 1). The ultraviolet spectrum of a water solution of 2 mg. of I per ml. was obtained on a Beckman DU spectrophotometer; a maximum was observed at 290 to 295 mμ. Elemental analysis of I was: C, 34.21%; H, 7.69%; N, 5.46%; S and P, absent; and O (by difference), 52.64%. The neutral equivalent of I was 216.

PAPER CHROMATOGRAPHY OF I. Fraction I was best separated on paper by developing the chromatogram with butyl alcohol saturated with water. The ascending chromatographic technique and apparatus described by Mitchell (*12*) were used to separate I on a milligram scale. Two Whatman No. 1 sheets, 8 × 8 inches, were streaked across the paper 1 inch from the bottom with 2 ml. of methanol containing 37.5 mg. of I, and the chromatogram was developed until the solvent front was 1 inch from the top. The papers were hung in a well ventilated hood to dry, and then a 0.5-inch strip was cut from the edge of the sheet and sprayed with 1% permanganate. Three bands appeared at R_f 0.82, 0.50, and 0.25. The remainder of each of these fractions was eluted with methanol, concentrated on a rotary evaporator, rechromatographed, and again eluted with methanol. An appropriate amount of each solution was placed on a rock salt plate and the solvent volatilized by heat. The infrared spectra for the fractions at R_f 0.82 and 0.25 were obtained. An insufficient amount of material of the components at R_f 0.50 was recovered to obtain an infrared curve. Ultraviolet spectra of water solutions containing 2 mg. per ml. of the fractions recovered at R_f 0.82 and 0.25, respectively, gave no characteristic peaks; when 3 ml. of water were added to the residue from the fraction at R_f 0.50, a slight increase in absorption at 290 to 295 mμ was observed.

TITRATION CURVES. Seven milliliters of the solution to be titrated, containing 77.0 mg. of fraction I, were placed in a small constant temperature cell equipped with electrodes attached to a line-operated pH meter. The solution was stirred magnetically and blanketed by nitrogen. A calibrated micropipet, made from a glass capillary and a vernier micrometer, was used to add small increments of alkali or acid. After each addition, the pH of the solution was recorded.

LABILE NITROGEN. Labile nitrogen in I was determined by comparing Kjeldahl nitrogen results with those obtained by the following modified Kjeldahl procedure. Twenty-five milligrams of I in 3 ml. of water were placed in a test tube ending in a standard-taper 24/40 joint connected to an adapter, through which 5 ml. of 30% sodium hydroxide were added and through which nitrogen gas was admitted. The nitrogen passed slowly over the magnet-

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ically stirred solution and into the trapping solution (*11*). The ammonia collected after 24 hours was determined by titration with 0.01*N* hydrochloric acid.

Optical Activity. The optical rotation of I was measured by means of a Keston spectropolarimeter attachment (Keston polarimeter Model D, Standard Polarimeter Co., New York, N. Y.) used with a Beckman Model DU spectrophotometer.

Determination of Lactic Acid. Lactic acid in I was determined by the method of Hullin and Noble (*8*).

Lean Pork. The techniques developed for lean beef (*7*) were applied to lean pork. Fat was removed as completely as possible from a cut obtained from a carcass kept at 0° to 4° C. for one week. The lean pork was ground, extracted with water, centrifuged, and filtered, and the water extract lyophilized to yield a powder containing 3% by weight of the starting material. Thirty grams of the freeze-dried pork extract were carried through the vacuum pyrolysis procedure devised for beef (*7*). The total volatiles trapped at −195° C. were fractionated under vacuum at room temperature to yield, as in the case of beef, a volatile fraction of an ammoniacal sulfurous odor and a nonvolatile fraction, pork I, of a fruity odor that turned to a meaty odor on exposure to air. The infrared spectrum of a film of pork I is shown in Figure 1. The ultraviolet spectrum of a water solution containing 2 mg. of pork I per ml. had an absorbance peak at 290 to 295 mμ. Pork I was chromatographed using techniques applied to I obtained from beef. Labile nitrogen and lactic acid were also determined.

The most volatile fraction obtained from pork was analyzed for carbonyls and acidic and basic components, using the procedures described in (*7*).

Beef and Pork Fat. Determination of Free Fatty Acids in Beef and Pork Fat. Depot fat was stored in a deep-freeze at −40° C. and samples were taken as needed. The free fatty acids and their concentration in the fat were initially determined. Twenty grams of fat were heated on a steam bath under nitrogen until molten, then filtered through several folds of cheesecloth. Ten ±0.2 grams of this filtrate were weighed into a 250-ml. Erlenmeyer flask and 30 ml. of petroleum ether added. The free fatty acids were separated from the fat by adsorption on an anion exchange resin, converted to their methyl esters, and identified and determined by gas chromatography, using the procedure and operating parameters described by Hornstein *et. al.* (*5*). The results are given in Table I.

The change, in kind and amount of free fatty acids present in fat, induced by heating under vacuum at 100° C., was studied. Twenty grams of fat were carried through the vacuum pyrolysis procedure (*7*). The total volatiles, however, were not further fractionated; instead, 10 ml. of water were added and the mixture was titrated directly with 0.01*N* sodium hydroxide. No titratable acidity was found. The residue in the pyrolysis chamber was analyzed for free fatty acids (*5*) and change in the kind of free fatty acids or in their concentration was observed.

The effect of heating fat at 100° C. in air was also studied. A wide-mouthed test tube, 5.5 cm. in diameter and 14.5 cm. deep, equipped with a standard taper 55/50 joint, was used as the heating chamber for 20 grams of fat. The use of a wide tube gave a high surface-to-volume ratio. Air at a flow rate of 2 liters per hour was first passed through a purification tower containing sodium carbonate admixed with silica gel, through a silica gel drying tower, and then across the surface of the heated fat and into a trap cooled in dry ice–isopropyl alcohol. No titratable acidity was found in the volatile fraction. The fat residues were analyzed for free fatty acids (Table I).

Determination of Carbonyl Compounds in Beef and Pork Fat. Samples of beef and pork fat were taken for "carbonyl" analyses from the same batches of fat used in the fatty acid studies and subjected to the same heat treatments. Volatile carbonyl compounds in the total condensate, from the samples heated under vacuum, were converted to their 2,4-dinitrophenylhydrazones (DNPH's) by the addition of 20 ml. of a 2,4-dinitrophenylhydrazine saturated solution of 2*N* hydrochloric acid. Steam-volatile carbonyl compounds present in the fat residues were steam distilled at a rate of 6 ml. per minute into an ice-cooled flask, containing 50 ml. of a similar mixture.

The heat treatment in air was like the procedure described for acids; the trapping solutions for the volatile carbonyl compounds were, however, different. Air, at a rate of 2 liters per hour, was first pulled through a purification trap containing 20 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid; secondly, through a silica gel drying tower; and then through the heat changer containing the fat. The final trapping solution was maintained at 0° to 4° C. and contained 20 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid. The steam-volatile carbonyl compounds in the residue were converted to DNPH's by steam distilling into a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid. The paper chro-

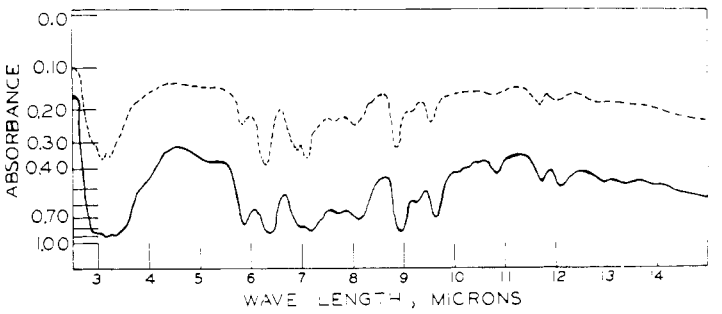

Figure 1. Infrared spectrum of fraction I obtained from lean beef

– – – Beef
—— Pork

Table I. Free Fatty Acids Found in Beef and Pork Fat

(Before and after heating in air for 4 hours at 100° C.)

	Beef, Mg./Gram		Pork, Mg./Gram	
Acid	Before	After	Before	After
Lauric	0.04	0.16	0.08	0.56
Myristic	0.49	2.04	0.54	1.39
Tetradecenoic[a]	0.36	2.24	..	..
Pentadecanoic[a]	0.06	0.15	..	..
Palmitic	2.24	4.91	2.89	3.62
Hexadecenoic[a]	1.31	4.98	1.64	3.45
Heptadecenoic[a]	0.19	0.44	..	..
Stearic	0.96	1.37	0.77	3.21
Oleic	9.24	19.74	17.01	28.52
Linoleic	0.58	1.34	5.45	13.27
Linolenic	..	..	1.04	1.45
Total	15.47	37.37	29.42	55.47

[a] Structure assigned on basis of retention volumes; factors for converting areas to concentrations obtained by interpolation from known factors for neighboring acids.

matographic methods used in separating, and quantitatively estimating the monocarbonyl DNPH's were described by Gaddis and Ellis (*3, 4*).

Flavor Precursors. Possible Precursor Systems. The technique developed for vacuum pyrolysis of the lyophilized powder containing lean beef flavor precursors was applied to the following systems: gelatin; gelatin plus glucose; egg albumin; egg albumin plus glucose; and egg albumin, glucose, and lactic acid.

The odors of the total condensate were noted for each system. No analytical data were obtained.

Dialysis Experiments. One hundred grams of lean ground beef were blended with 350 ml. of water. The resulting slurry was centrifuged and the supernatant liquid decanted through Whatman No. 1 paper to remove fat particles. The filtrate was concentrated by freeze-drying to approximately 75 ml. The liquid was then dialyzed against an equal volume of water through a seamless cellulose membrane with gentle shaking, overnight, in a cold room. This dialyzate was lyophilized to yield a white, fluffy powder. The contents of the dialysis tube were further dialyzed, overnight, against cold, running water, and the solution remaining in the tube was examined separately.

In another experiment, 75 ml. of concentrated beef extract were placed in a 100-ml. glass-stoppered graduate and dialyzed against 40 grams of Dowex 50, prepared by the method of Hunter, Houston, and Kester (*9*), and placed in the dialysis tube. The amino acids that dialyzed into the tube were absorbed on the ion exchange resin over a period of 60 hours at 4° C. The resin was then washed with distilled water and poured into a "chromatographic" column, and the absorbed amino acids were eluted with 350 ml. of 5*N* ammonium hydroxide. The first 75 ml. of eluate were discarded and the remainder was concentrated under vacuum to about 100 ml.

Table II. Carbonyl Compounds Isolated from Beef and Pork Fat Heated in Air at 100° C.

Fraction	Class	Concn. of Classes, μmoles Carbonyl/10 Grams Fat	Compounds Isolated from Each Class
	Beef Fat		
Volatile	Alkanal	0.039	Acetone[a,b] Propanal
	2-Enal	0.072	Acetaldehyde[c]
Steam-distilled fraction	Alkanal	0.070	Hexanal[b] Nonanal
	2-Enals	0.028	Hepta-2-en-1-al[d] Octa-2-en-1-al[d] Nona-2-en-1-al Deca-2-en-1-al[b] Undeca-2-en-1-al
	2,4-Dienals	0.021	Deca-2,4-dienal
	Pork Fat		
Volatiles	Alkanals	0.27	Acetone[a] Propanal Hexanal[b] Octanal Nonanal
	2-Enals	0.06	Acetaldehyde[c] Hepta-2-en-1-al[d] Octa-2-en-1-al[d] Nona-2-en-1-al[d] Deca-2-en-1-al[d] Undeca-2-en-1-al[d]
	2,4-Dienals	..	Deca-2,4-dienal[d]
Steam-distilled fractions	Alkanals	0.24	Hexanal[b] Octanal Nonanal Undecanal
	2-Enals	0.12	Hepta-2-en-1-al Octa-2-en-1-al Nona-2-en-1-al Deca-2-en-1-al Undeca-2-en-1-al
	2,4-Dienals	0.083	Hepta-2,4-dienal Nona-2,4-dienal Deca-2,4-dienal[b]

[a] Acetone isolated with alkanals.
[b] Major component in class.
[c] Acetaldehyde isolated with 2-enals.
[d] Trace amounts (estimated at less than 0.005 μmole).

Results and Discussion

The desirable aroma of fraction I obtained from beef disappeared when efforts at fractionation (regardless of technique) were made. Paper chromatography was selected as the least destructive separation procedure and was used to study I.

The major differences in the infrared spectra of the fractions separated from I by paper chromatography were observed at 6, 7, and 12 microns. An examination of the spectra-structure correlation in the infrared region (*1*) strongly suggested that I was, to a large extent, a mixture of an organic acid and its ammonium salt. This conclusion was further confirmed when titration curves for I exhibited buffering action in the pH regions 3.0 to 5.0 and 8.0 to 10.1, and analyses showed that all the nitrogen was labile. Kjeldahl nitrogen values were 6.06%. Lactic acid was a likely possibility for the unknown acid, since it is the end product of glycolysis. Qualitative tests (*2*) confirmed the presence of lactic acid, and the quantitative results indicated that approximately 87% of I was lactic acid. If one assumes that the labile nitrogen is present as ammonium lactate, then over 93% of I is accounted for. As I was found to be optically active, it was concluded that optically active lactic acid, presumably an end product of glycolysis, accounted for approximately 90% of fraction I and that approximately 5% of ammonia, formed during the pyrolysis of beef powder, was fortuitously trapped in the acid. In addition, at least one unknown material, with a strong ultraviolet absorbance at 290 to 295 mμ and possibly responsible for the "meaty" aroma, was also present in fraction I.

The odor characteristics of the corresponding fraction I from lean pork were identical to that from beef I. The infrared spectrum of fraction I, obtained from lean pork, was qualitatively identical to that of fraction I from lean beef. The ultraviolet spectrum for both beef I and pork I exhibited an identical absorbance peak at 290 to 295 mμ. The paper chromatographic separation of pork I yielded fractions identical in R_f values and of approximately the same concentration as the corresponding fractions from beef I. Nitrogen was again shown to be present in the form of labile ammonia, and approximately 85% of pork I was found to be lactic acid. The most volatile fraction obtained from lean pork was identical in odor to the corresponding fraction from lean beef. Carbonyl compounds found in this fraction were acetone, acetaldehyde, and formaldehyde. The only basic compound isolated was ammonia. Hydrogen sulfide and carbon dioxide were

isolated from the acidic volatile fraction. These results were similar to those previously obtained from the corresponding fraction isolated from lean beef. When separate 3% water solutions of lyophilized powders obtained from lean beef and lean pork were heated simultaneously, the aromas of both samples were judged to be of the same quality, differing only in intensity. Ten people, selected from the Meat Laboratory personnel, stated that the aroma could best be characterized as "meaty," five of the observers described the odor in terms of "beef," and only one judge detected anything reminiscent of a "pork" aroma.

The similarity of all results obtained from lean beef and lean pork, which is contrary to their different flavors, focused attention on the fat portions of these meats. Free fatty acids present in fat might be expected to contribute to taste, and, if volatile, also to odor. It was of interest, therefore, to study the changes in the nature and concentration of free fatty acids produced by heat. Any study pertaining to flavor in meat must also include a study of the carbonyl compounds derived from fat, since carbonyls are presumably responsible for both desirable and undesirable flavors in foodstuffs. For example, Patton *et al.* (*13*) states that 2,4-dienals are responsible for the "deep-fat-fried" odor common to many foods.

Gas chromatography was used to separate and determine the isolated methyl esters of the free fatty acids present in beef and pork fat. Free fatty acids were determined prior to heating, after heating at 100° C. in vacuum, and after heating at 100° C. in air. No change in the nature and little, if any, change in the concentration of the free fatty acids were noted as a result of heating in vacuum. However, heating in air resulted in marked changes in the concentrations of the various free fatty acids (Table I). The increase in free fatty acid concentration on prolonged heating in air can be attributed to the hydrolysis of the glycerides by the water present in the fat. In the vacuum distillation procedure, water is removed prior to heating and therefore hydrolysis is minimized.

The gas chromatographic technique used to isolate and determine the free fatty acids was standardized against a number of known pure fatty acids (*5*). The gas chromatograms for free fatty acids in beef and pork (Figure 2) have a number of unknown peaks; an attempt was made to identify these based on the gas chromatographic data. For saturated straight-chain and iso-fatty acid esters, a plot of the log of the retention volume against the number of carbon atoms yields different straight lines for each series (*10*). This also holds true for homologous series of paraffins, alcohols, methyl ketones, and esters (*14*).

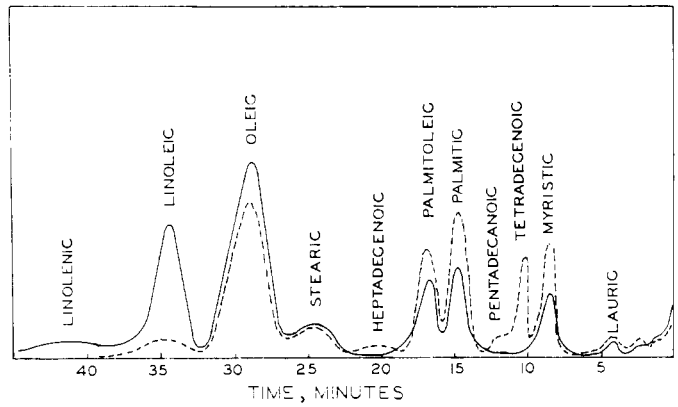

Figure 2. Gas chromatographic separation of methyl esters of free fatty acids isolated from beef and pork fat

– – – Beef
—— Pork

For a homologous series of straight-chain fatty acid methyl esters, the saturated acids fall along one straight line; those with one double bond fall along another line; and fatty acids with two double bonds fall along a third line. On the poly(vinyl acetate) partitioning column used in this work, for a given chain length, the acids emerge from the column in the order of increasing unsaturation as exemplified by the series stearic, oleic, linoleic, and linolenic (*6*). On the basis of this information and a knowledge of the retention volumes for the methyl esters of lauric, tridecanoic, myristic, palmitic, heptadecanoic, oleic, linoleic, and linolenic acids, tentative assignments of chain length and degree of unsaturation were given to the unknown peaks. In Figure 3, the log of the retention volumes is plotted against the number of carbon atoms. The points fall along two distinct straight lines, rendering probable the assignment made.

Carbonyl compounds were isolated from samples of beef and pork fat taken from the same batches of fat used in the fatty acid studies and carried through the same heat treatment procedures. The amount of carbonyl found before and after heating in vacuum was extremely low. Heating the fat samples in air increased the amount of carbonyl compounds. Table II shows the amounts of carbonyl compounds isolated from beef and pork fat by heating at 100° C. in air. The increase in the carbonyl concentration must be due to oxidation; therefore, the exclusion of air as in vacuum heating minimizes the formation of carbonyl compounds, while heating the fat in air increases carbonyl formation.

Heating the fats produced considerably more variation in odor than heating the lean meats. In addition, these odors were not as reproducible from batch to batch as were the odors obtained from lean meat. The odor of

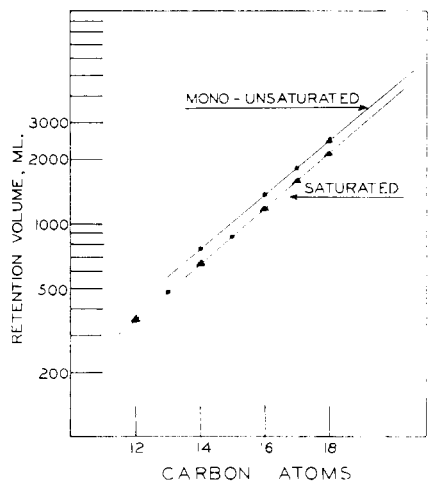

Figure 3. Log of retention volume *vs.* number of carbon atoms for monosaturated and saturated fatty acid methyl esters

▲ Known acids ● Postulated acids

the volatiles produced by heating the fats may be tabulated as follows:

Heat Treatment, 100° C.	*Beef*	*Pork*
Vacuum	Sweet and applelike	Pungent and cheeselike
Air	Fried-fat	Bacon

Beef and pork fat are more homogeneous materials than the lean portions of these meats. There are, therefore, a greater number of possible precursor systems for the aroma of cooked lean meats than for the aroma of heated fats. Some effort was made to identify the precursor systems in lean meat.

The possibility that vacuum pyrolysis of a water-soluble animal protein *per se* might yield results similar to those obtained by heating the lyophilized meat extracts was first examined. Gelatin was carried through the vacuum pyrolysis procedure for the treatment of the lean meat powder extracts. No fraction corresponding to I was isolated, and no "ammoniacal" or "sulfurous"

odor detected. Reducing sugars, present in muscle, could react with protein and possibly produce the observed aromas. A mixture of gelatin plus glucose was vacuum-pyrolyzed; the results were negative. Gelatin is, however, low in sulfur-containing amino acids, and so the same experiments were repeated with soluble egg albumin powder and with egg albumin plus glucose. The results were again negative. Since lactic acid was isolated in appreciable quantity and pH could have a profound effect on the results of heat treatment (pH of an initial cold water extract on ground beef was approximately 5.5), a model system, consisting of 10 grams of egg albumin, 2 grams of glucose, and 100 mg. of lactic acid, was prepared and treated in the same fashion as the lyophilized lean meat extracts. Some odor was obtained, indicating the lactic acid may be important in developing flavor in cooked meat. However, this odor did not resemble the odor "profile" obtained from the meat powders. Relaxation of rigor with time results in the production of a more desirable flavor than that associated with fresh beef. It may be that as glycolysis continues the increase in lactic acid concentrations may result in this better flavor.

Because the protein systems heated were apparently not the flavor precursors sought, attention was directed to the low molecular weight fraction present in the water extract of lean meat. Dialysis experiments indicated that the material passing through the membrane contained some of the flavor precursors of lean beef. When the white, fluffy powder obtained from this dialyzate by freeze-drying was carried through the vacuum pyrolysis and fractionation procedure applied to the lyophilized water extracts of lean beef and lean pork, a fraction very similar to beef I and pork I was isolated. However, when the free amino acids were separated from a similar dialyzate and heated alone, at both pH 5.5 and pH 8.0, no recognizable meaty odors were obtained. The odors were, in fact, classified as unpleasant, indicating that the free amino acids as such were not flavor precursors.

Conclusions

The identity of the compounds isolated from lean beef and lean pork, as well as the marked resemblance in chromatographic and spectrophotometric behavior of fractions not completely characterized, leads to the conclusion that a similar basic meaty flavor is obtained on heating the lean of beef and pork.

The flavor differences that exist in pork and beef may have their origins in the fat portions of these meats. The fat may not only produce different flavor compounds in different ratios, but perhaps also act as a storage depot for lipide-soluble foreign compounds that, on heating, also contribute to flavor.

Examination of possible precursor systems for the origin of lean meat flavor indicated that the flavor precursors may well be the low molecular weight, water-soluble fractions of lean meats. These produce the characteristic flavor of lean meat, presumably by some kind of interaction between amino acids and the low molecular weight carbohydrates and polypeptides present, and no single compound or class of compounds is responsible for cooked "meat" flavor.

Acknowledgment

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MEAT FLAVOR CHEMISTRY

Precursors of Beef Flavor

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Attempts were made to isolate and characterize some of the constituents of beef muscle tissue which evolve the distinctive odor and flavor associated with cooked beef. Beef muscle tissue was fractionated into a number of water-soluble and water-insoluble fractions. Dialysis of the water extract of uncooked ground beef gave a diffusate which, after lyophilization, was capable of producing an odor similar to that of broiled steak when heated with fat, and a beef broth odor and flavor when boiled in water. The components of this fraction were separated by column chromatography and gel filtration. They are of relatively low molecular weight and contain peptides, carbohydrates, and phosphates as yet unidentified.

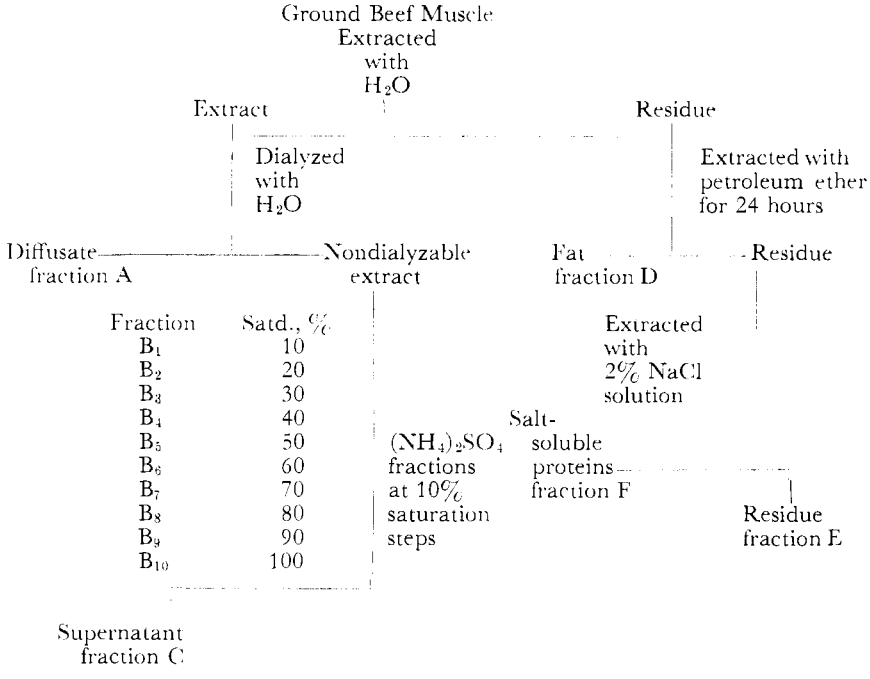
Their results indicated that press fluids of raw meat had a highly concentrated flavor on cooking. Cooking prior to extraction increased the flavor, indicating that full flavor development may be due to heating the juice and the fiber together.

Results of studies on chicken flavor by Bouthilet (*4*) suggested that the flavor was composed of at least two parts—a sulfur-containing material and a fatty acid–type material. The sulfur compound was highly labile and gave off hydrogen sulfide upon standing. Pippin and Eyring (*9*) found that the bulk of the volatile nitrogen components in chicken flavor volatiles was ammonia, which did not constitute part of the characteristic chicken flavor. Volatile flavors were associated with neutral or acidic compounds. The volatile sulfur constituents consisted mainly of hydrogen sulfide.

The complexity of cooked extracts was elegantly demonstrated by Wood and Bender (*11*), who isolated and identified a large number of volatile and nonvolatile compounds in commercial ox muscle extract. Hornstein, Crowe, and Sulzbacher (*6*) heated, in vacuum, a freeze-dried powder obtained from a cold water extract of raw ground beef and trapped the volatiles. Components of the volatile fraction identified were acetone, acetaldehyde, formaldehyde, hydrogen sulfide, ammonia, and methylamine. These authors also reported that the flavor precursors were cold water–extractable.

In the present studies substances have been isolated from raw beef muscle, which, when boiled with water, gave a characteristic beef broth odor and flavor, and when heated with fat, gave an odor similar to broiled steak. The initial observation was made on ground beef which had been serially extracted five times with acetone (1000 grams of beef, 1200 ml. of acetone). The fat obtained from the third acetone extract, when heated, gave the broiled steak type of odor. Analysis of the fat indicated that only normal saturated triglycerides were present. Attempts at duplication of this procedure were not always successful. In some instances, the material from the third acetone extract merely gave the odor of heated fat.

Diagram I. Procedure Used for Fractionation of Beef Muscle



These and other observations led to consideration of the possibility that other substances being carried over in the fat were responsible for the odor produced. On further investigation, the precursor substances were found in the dialyzable portion of a water extract of raw beef.

Procedure

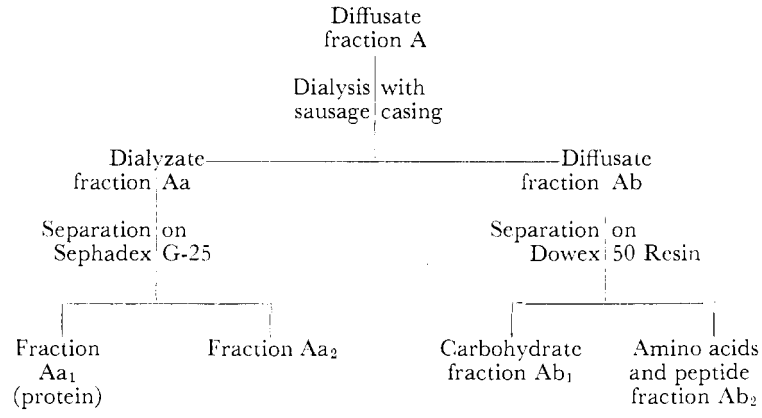
Round of beef (1000 grams), freed from all extraneous fat, was fractionated as outlined in Diagram I. With the exceptions of fractions A and D, all others were dialyzed against running water until a negative sulfate or chloride test was obtained, then lyophilized. Fraction A was lyophilized as the diffusate. Fraction A was obtained by filling cellulose dialysis tubing (Visking) with 200 ml. of distilled water and rotating the tubing in the water extract of beef (2 × 1200 ml. per 1000 grams of meat) for 16 hours in the cold, ~3° C.

Further efforts at separation of fraction A into its constituents were successful when cellulose sausage casing (Visking) was inadvertently substituted for dialysis tubing in one of the experiments. The broiled steak type of odor was not obtained in this case. Thus, by using dialysis tubing which would allow the desired material to enter, and then transferring this diffusate to sausage casing, two more fractions were obtained (Diagram II). The material that remained in the sausage casing (Aa) was primarily protein in nature, while the material that diffused through the sausage casing (Ab) was composed primarily of compounds of small molecular weight such as sugars, amino acids, and small peptides.

Fraction Aa was further separated by using gel filtration on Sephadex G-25 (cross-linked dextran), using the procedure of Porath and Flodin (*10*). Two distinct peaks were obtained by this procedure (Figure 1).

Fraction Ab was further separated by passing the diffusate through Dowex 50 H^+ form 200-400 mesh, 8× linkage. The material that passed through the column (ninhydrin-negative) appeared to be carbohydrate in nature (Ab_1). By passing 2*N* ammonium hydroxide

Diagram II. Separation Procedure for Fraction A



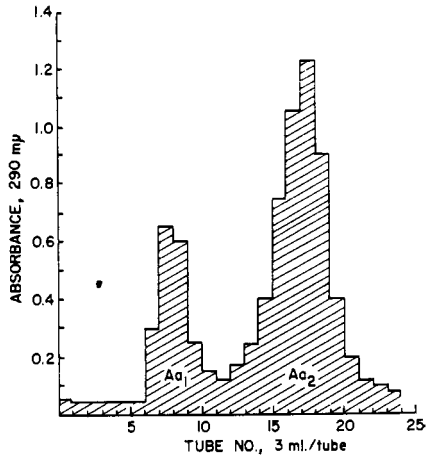


Figure 1. Separation of fraction Aa on Sephadex G-25

through the column, amino acids and small peptides were obtained (Ab_2).

Results and Discussion

When fresh ground beef muscle was fractionated by the procedure outlined in Diagram I and the individual fractions were either boiled in water for 20 minutes or heated in fat, only fraction A produced any characteristic odor or taste. The remaining fractions on boiling produced no odor and were practically tasteless. When heated with fat, they gave a burned protein odor. If ground beef were cooked to an internal temperature of 77° C. and then fractionated, fraction A had a characteristic beef bouillon odor, and fraction E (residue) had a flavor and odor more nearly that of cooked beef. However, the flavor was considerably less intense than that of fraction A. Again, the remaining fractions had no appreciable odor or taste.

Fraction A, as obtained from fresh ground beef muscle, proved very unstable. The material changed from a white granular material to a brown tarry mass, even when kept in an evacuated desiccator. This change was apparent a short while after removal from the lyophilizer and reached the brown tarry stage in about 3 to 7 days. During this period, ammonia and/or amines were given off, and at the end, the material had a stale, old meat odor.

After separation into fractions Aa and Ab by the second dialysis, fraction Aa, which contained the protein material, was fairly stable, but fraction Ab, containing the low molecular weight sugars and amino acids, turned brown rapidly. When fraction Ab was passed through Dowex 50, the eluent (Ab_1) was ninhydrin-negative, but was strongly positive for carbohydrates by either anthrone, triphenyltetrazolium chloride, or aniline acetate tests. On eluting the column with 2*N* ammonium hydroxide, an amino acid fraction was obtained (Ab_2). Both fractions were stable.

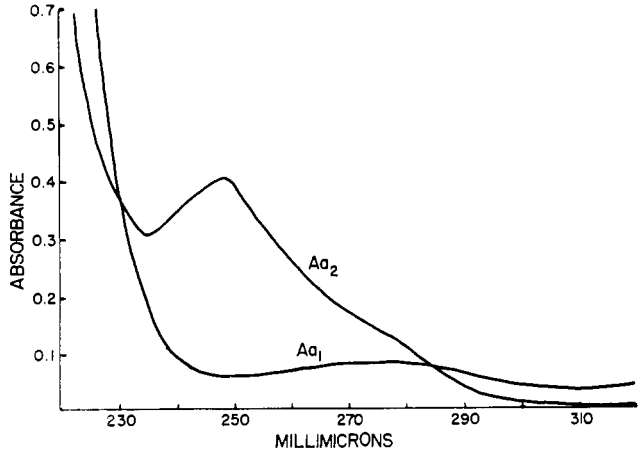


Figure 2. Ultraviolet spectra of fractions Aa_1 and Aa_2

Fraction Aa_1 (~5 to 10 mg. per 1000 grams of beef), as obtained by gel filtration, was a light tan material. Positive ninhydrin and negative carbohydrate tests were obtained. In the ultracentrifuge (60,000 r.p.m.) a 2% solution of this material in water formed a single peak, which, after 3 hours' running time, diffused into a low flat curve in the center of the cell. A spectrum taken in the ultraviolet indicated a normal protein curve (Figure 2). On paper electrophoresis in veronal buffer (pH 8.7, 400 volts, 3 amperes, and 6 hours), the spotted material divided into two spots (one about 1 cm. and the second about 2 cm. toward the positive pole). In acetate buffer at pH 4.0, only one spot appeared toward the negative pole.

Fraction Aa_2 (~10 mg. per 1000 grams of beef) was a white fluffy powder and ninhydrin-positive. A strong positive carbohydrate reaction was obtained. An estimation, using anthrone reagent and glucose as a standard, indicated a carbohydrate content of approximately 16%. However, because of lack of knowledge of the characteristics of the compound, the possible presence of interfering substances, and the exact nature of the carbohydrates present, the analysis is considered, at present, to be an estimate. The material was hydrolyzed on Dowex 50 and analyzed for hexuronic acids, hexosamines, and hydroxyproline, according to the procedure of Anastassiadis and Common (*1*). Negative results were obtained for all three tests. However, when water was added to the test for hexuronic acids (carbazole method), a positive test developed, indicating the presence of hexoses. Readings taken on a Beckman DU spectrophotometer at 520 and 420 mμ gave a 520/420 ratio of 1.06, indicating that the material was not just glucose, which has a ratio of 2.65 with this reagent. Hydrolysis of the material with 6*N* hydrochloric acid for 3 hours, and paper chromatography indicated a minimum of eight ninhydrin spots. The substances represented by these spots have not yet been identified.

The unhydrolyzed fraction Aa_2 gave a faint positive test for phosphate ion, but on hydrolysis with perchloric acid for 1 hour at 175° C., gave a very strong positive test. Estimations indicated a phosphate content of 7 to 7.5%.

The spectrum of the unhydrolyzed material taken in the ultraviolet (Figure 2) indicates a peak at 248 mμ. The unhydrolyzed material was also subjected to ultracentrifugation at 60,000 r.p.m., but no peak was discernible, indicating that the material is of low molecular weight.

On paper electrophoresis in veronal buffer at 8.7 under the same conditions as for fraction Aa_1, the bulk of the ninhydrin-positive material moved toward the negative pole. A heavy spot appeared at 2.5 cm. from the origin and another at 1 cm. A very faint ninhydrin-positive spot moved 9 cm. toward the positive pole. When a duplicate strip was checked for carbohydrate, the bulk of the material also moved toward the negative pole in the same position as the ninhydrin-positive material. One faint sugar spot was located at 12.2 cm. toward the positive pole. When a duplicate strip was checked for phosphorus, the bulk of the material remained at the base line, but some of the material streaked toward the positive pole with a definite spot at around 13 cm.

When this fraction (Aa_2) was run in 0.1*M* sodium carbonate at pH 11, the ninhydrin-positive material moved toward the positive pole. Two spots appeared, one at 4.5 cm. and another at 7 cm. The bulk of the carbohydrate material again moved toward the negative pole in the same position as it did at pH 8.7, but two spots moved toward the positive pole, one at 5.5 cm. and another again at 13 cm. The phosphorus-containing material moved essentially the same as it did at pH 8.7. The fact that a pH of 11 was necessary to cause the ninhydrin-positive material to move to the positive pole, suggested a high content of basic amino acids. However, a Sakaguchi test for arginine was only faintly positive.

Fraction Aa_2, when heated with fat, produced an odor similar to that of broiled steak. However, when small aliquots of fractions Aa_1 (protein) and Ab_1 (carbohydrate) were added to Aa_2 and then heated, the odor, based on subjective evaluation, was equivalent to the odor observed for the original fraction A. At the present stage of this work it appears that fraction Aa_2 contains the material necessary for the "basic" odor of broiled steak, but other constituents—i.e., protein (fraction Aa_1) and carbohydrate (fraction Ab_1)—are necessary for the development of the cooked meat flavor and odor. Natural fat and hydrogenated fat, which were used interchangeably in heating the isolated material, also contribute something to the odor. The same basic odor can be produced if the three fractions are heated in mineral oil, but it is distinguishable from that when natural or hydrogenated fats are used.

The fractionation procedure was tried on chicken and pork loin. The material (fraction Aa_2) isolated in these experiments had the same basic odor as the material isolated from beef. When heated with fat, the odor was similar to that of broiled steak. However there were enough distinguishing characteristics to indicate which material had been isolated from chicken and which came from pork. The 248-mμ peak was present in both materials.

At present, attempts are in progress to identify the various components present in fraction Aa_2, and to attempt to determine the role of these constituents in the production of odor and flavor in meat.

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FEED ADDITIVES MEASUREMENT

Ultraviolet Method for Determination of Streptomycin in Feeds

STANLEY E. KATZ

Department of Agricultural Chemistry, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

THE USE OF ANTIBIOTICS in animal feeds is constantly increasing and state feed control laboratories face problems in determining these drugs. As most feed control laboratories do not have facilities for biological assay procedures, the development of chemical methods for determining antibiotics in feeds is an urgent problem.

All current AOAC methods for antibiotics are microbiological assay procedures. When these AOAC procedures for antibiotics were being studied (*12*), streptomycin was not considered because it was not widely used. Now that its use in poultry and swine feeds is increasing, it is imperative that a suitable chemical method be devised.

The streptomycin molecule can be determined by any one of three basic ways: analysis for the streptose moiety by determining the alkaline degradation product, maltol; for the combined guanidine groups; and for the *N*-methyl-1-glucosamine fraction.

Schenck and Spielman (*14*) showed that heating streptomycin at 100° C. for 3 minutes with 1*N* sodium hydroxide converted to streptose moiety of the streptomycin molecule quantitatively to maltol (3-hydroxy-2-methyl-4-pyrone).

Eisenman and Bricker (*6*) reported the use of this reaction in a spectrophotometric method, in which the color reaction between maltol and ferric iron was employed to estimate the concentration of streptomycin. Boxer, Jelinek, and Leghorn (*4*) used the color reaction between maltol and the Folin-Ciocalteu phenol reagent, as well as the ferric iron–maltol complex, for streptomycin determinations. St. John, Flick, and Tepe (*13*) and Angeles (*1*) adapted the colorimetric maltol procedure for the assay of streptomycin in fermentation broths.

Various investigators determined streptomycin by means of the combined guanidine groups. The two general procedures used were colorimetric estimation with sodium nitroprusside and potassium ferrocyanide (*10*) and colorimetric estimation of the condensation product with biacetyl and 1-naphthol (*2, 3, 7*).

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The ultraviolet method of determining streptomycin in feeds involves a dilute sulfuric acid extraction of the streptomycin from a ground feed, separation of the aqueous extract from the feed solids, neutralization of the extract, and further separation of the neutralized extract from the solids. This is followed by concentration and purification of the streptomycin with an ion exchange resin, elution of the streptomycin from the resin, conversion of the streptose moiety of the molecule to maltol, and the quantitative determination of the streptomycin by means of the ultraviolet absorbance of maltol. Recoveries at the recommended level averaged 98.4%, with a coefficient of variation of 4.8%.

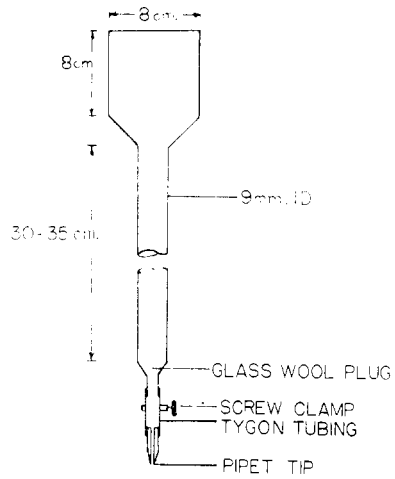

Figure 1. Amberlite IRC-50 Na form column

Determination of streptomycin using the *N*-methyl-1-glucosamine fraction of the molecule has not been widely employed. Kamata *et al.* (*8*) used the reaction between the *N*-methyl-1-glucosamine and acetylacetone, followed by the colorimetric reaction with *p*-dimethylaminobenzaldehyde, to estimate the streptomycin.

The separation of streptomycin from fermentation broths using ion exchange resins has been the subject of much discussion. Doery, Mason, and Weiss (*5*) as well as St. John, Flick, and Tepe (*13*) used Amberlite IRC-50 in their analytical procedures for the chemical assay of the streptomycin content of fermentation broths. Kotula (*9*) in his investigation of the adsorption and elution of streptomycin with five different cation exchangers, showed Amberlite IRC-50 to be the most suitable, with recoveries in the order of 93%.

Procedure

Preparation of Calibration Curve. Prepare a calibration curve by transferring 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml. of a standard streptomycin solution containing 100 μg. of streptomycin per ml. into 10-ml. volumetric flasks. Add 1 ml. of 1*N* sodium hydroxide and bring to volume with distilled water. This will yield standards from 50 to 600 μg. Place the 10-ml. volumetric flasks in a 90° C. hot water bath for 5 minutes. Cool the flasks by placing them in an ice water bath. Determine the absorbance of each standard with a Beckman DU spectrophotometer at 324 mμ in 1.0-cm. silica cells. Plot a standard curve from the observed absorbance values.

Standardization of Amberlite IRC-50 Resin. To minimize resin problems due to variations in available exchange resin batches, the following procedure was used.

Place 100 grams of Amberlite IRC-50, H form, 16 to 50 mesh, in a 1-liter Erlenmeyer flask with 250 ml. of 5% sodium hydroxide and shake mechanically for 1 hour. Wash the resin into a Salvarsan tube, or similar tube, plugged with glass wool at the constricted end to retain the resin. Wash the resin until the effluent is neutral to litmus.

Air-dry the Amberlite IRC-50, Na form, for 24 hours and store the resin in a closed bottle. Prepare an ion exchange column containing 8 inches of resin. The dimensions of a convenient form of the column are shown in Figure 1. Backwash the resin and allow it to settle without a flow. Drain the column to within 1 cm. of the resin bed and wash the column with 100 ml. of distilled water. Never allow the liquid level to drain into the resin bed. After the column wash water has drained to within 1 cm. of the resin bed, add 500 μg. of streptomycin in 250 ml. of water. Pass the solution through the resin at the volumetric rate of 3 to 5 ml. per minute. Drain the liquid level to within 1 cm. of the resin bed. Wash the column with two 25-ml. portions of distilled water and drain to within 0.5 cm. of the resin bed. Elute the column with 2% (by volume) sulfuric acid at the volumetric rate of 1 ml. per minute. Collect 5-ml. fractions of the eluate in 10-ml. volumetric flasks containing 5 ml. of 1*N* NaOH. Twelve fractions are usually sufficient. Place the volumetric flasks in a 90° C. hot water bath and proceed as described under Preparation of Calibration Curve.

Determine the recovery and elution curve for the batch of resin. If the recoveries of streptomycin are 95% or greater, the resin is suitable for use. If recoveries are less than 95%, repeat this complete procedure.

Determination of Streptomycin in Feeds. Place a quantity of ground feed containing approximately 500 μg. of streptomycin in a 500-ml. Erlenmeyer flask, fitted with a ground-glass stopper. Add 200 ml. of 0.02*N* sulfuric acid and shake mechanically for 1 hour. Place the contents of the flask in a 250-ml. centrifuge bottle and centrifuge for 5 minutes at 2000 r.p.m. Filter the turbid solution through H. Reeve Angel No. 711 filter paper, or its equivalent. Measure the quantity of filtrate. This will yield the aliquot size for the calculations later in the procedure.

Neutralize the filtrate with 1*N* sodium hydroxide to a pH value of 7.0 ± 0.2. Place the filtrate in another 250-ml. centrifuge bottle and centrifuge for 5 minutes. Filter the clear supernatant through H. Reeve Angel No. 711 filter paper. Transfer the gelatinous precipitate onto the filter paper and wash several times with water. Discard the precipitate.

Pass the filtrate and washings through the Amberlite IRC-50, Na form, column at the volumetric rate of 3 to 5 ml. per minute. Permit the filtrate to flow to within 1 cm. of the resin bed. Wash the column with four 50-ml. portions of distilled water and drain each to within 1 cm. of the column before addition of the next wash. Drain the final wash to within 0.5 cm. of the column.

Elute with 2% (by volume) sulfuric acid at the volumetric rate of 1 ml. per minute. Discard the eluate before the appearance of the streptomycin and collect the fraction containing the streptomycin (determined from elution curve).

Pipet 9 ml. of the well-mixed eluate into two 10-ml. volumetric flasks. Bring to volume with 7*N* sodium hydroxide and shake well. Place one flask (I) directly in an ice water bath. Place the other flask (II) in a 90° C. hot water bath for 5 minutes and then cool in the ice water bath. Centrifuge the contents of each volumetric flask before determining the absorbance values of each in 1.0-cm. silica cells with water as a blank.

Subtract the absorbance of flask I from that of flask II and determine the quantity of streptomycin from a stand-

ard curve. Determine the total amount of streptomycin present from the ratio of the total volume of extracting liquid to the aliquot taken and from the ratio of the total volume of streptomycin-containing eluate to the 9-ml. aliquot. Total micrograms of streptomycin in the sample can be converted to grams per ton by using the appropriate factors.

Results

Determination of Streptomycin by Combined Guanidine Groups. SODIUM NITROPRUSSIDE–POTASSIUM FERROCYANIDE METHOD. The Monastero procedure (*10*) for the determination of combined guanidine groups, although sufficiently sensitive, proved to be too dependent upon pH for consistent results. In the elution of streptomycin from the resin, it was extremely difficult to adjust the pH of the eluate precisely to a value of 7.0 with no appreciable change in volume.

Biacetyl - Naphthol Condensation Method. The low sensitivity as well as the relatively high blank made the determination by the biacetyl-naphthol method (*2, 3, 7*) unsuitable for streptomycin determination in feeds.

Analysis by *N*-Methyl-1-glucosamine Fraction. Determining streptomycin by means of the *N*-methyl-1-glucosamine fraction was not attempted because the intensity of the color developed in the condensation with acetylacetone and *p*-dimethylaminobenzaldehyde was grossly insensitive (*8*) at the levels necessary in feed determinations.

Analysis by the Alkaline Degradation Product, Maltol. COLORIMETRIC METHOD. With the procedure of St. John, Flick, and Tepe (*13*) (a colorimetric reaction between iron and maltol), the smallest quantity determinable was approximately 1 mg. Although extremely reproducible and relatively sensitive, this method was still insufficiently sensitive for determining streptomycin in feeds.

ULTRAVIOLET METHOD. This procedure is basically similar to that used in the colorimetric iron determination of maltol (*13*). Measurement of the ultraviolet absorbance of maltol rather than of the color complex proved to be a very sensitive, reproducible method of determining the streptomycin content in feeds.

A calibration curve in the range of 50 to 600 μg. was established according to the previously described procedure. Twenty-six determinations, all at different levels, from five separately prepared standard solutions were made. The coefficient of determination, r^2 (*11*), indicates the percentage of explainable variation in the concentration as determined by absorbance readings. The calculated r^2 value of 0.9918 indicates that 99.18% of the variation is accounted for and 0.82% is not. The 0.82% unaccounted for is considered the per cent error in pure solution.

Recoveries of Streptomycin from Feed. An unmedicated poultry feed, Rutgers' Ration 15, was blended with streptomycin at a level of 20 μg. per gram of feed (approximately 20 grams per ton of feed). The blended sample was ground prior to analysis. Recoveries in the 300- to 600-μg. level (Table I) ranged from 91.6 to 108.0% with an overall average of 96.5%. Six replications at the same level, 500 μg., yielded an average recovery of 98.4%. The coefficient of variation between replications at the 500-μg. level was 4.8%.

Because aliquots taken from the eluate containing streptomycin are approximately 1/3 to 1/4 of the total volume, a factor of 3 to 4 is necessary. This means at the 200-μg. level an aliquot would contain 50 to 65 μg. of streptomycin. These concentrations approach the lower limit of the calibration curve and are subject to greater errors. The aliquot of a 500-μg. sample would contain 125 to 165 μg., a more accurately determinable quantity. Sample size is strictly governed by the amount expected in the aliquot taken from the eluate fraction containing the streptomycin.

Several attempts to eliminate the need to aliquot the eluate containing the streptomycin were unsuccessful. Converting the streptose moiety to maltol and steam-distilling the maltol were not satisfactory; concentrating the acid eluate failed, and chloroform extraction of the maltol proved to be short of quantitative.

The measurement of a sample's absorbance before and after its conversion to maltol eliminates any interfering substances absorbing at 324 mμ. The increase in absorbance after heating is attributable to streptomycin alone. Determinations on the same feed with no streptomycin added indicated that other substances may absorb at 324 mμ, but heating does not alter these absorbance values. Some drugs commonly used with streptomycin, such as penicillin, furizolidone, sulfaquinoxaline, nicarbazine, and glycarbylamide, did not interfere. Other drugs were not studied for possible interference.

The binding of streptomycin by some of the feed components initially caused a problem. Preliminary studies of extraction showed that streptomycin was not quantitatively removed from the feed, and, as time progressed, less and less streptomycin was removed from the feed by extraction. By using a dilute aqueous acid extraction, the binding was broken and the streptomycin quantitatively recovered from the feed samples. It appears that grinding and/or storing the sample for a time caused the sulfate salt to revert to the free base, which is insoluble in water. The dilute acid extracted the free base as the soluble sulfate with a quantitative recovery.

Table I. Recovery of Streptomycin from a Laboratory-Prepared Feed

Feed Sample, Grams	Streptomycin, μg. Present	Streptomycin, μg. Found	Recovery, %
10	200	150	75.0
		170	85.0
		155	77.5
		160	80.0
		150	75.0
15[a]	300	275	92.1[a]
		280	93.6[a]
25[a,b]	500	490	98.0[a,b]
		480	96.0[a,b]
		472	94.4[a,b]
		510	102.0[a,b]
		463	92.6[a,b]
		540	108.0[a,b]
30[a]	600	580	96.6[a]
		550	91.6[a]
		Av.[a]	96.5
		Av.[b]	98.4

[a] Range in which quantitative recovery is possible.
[b] Recommended range.

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Effect of Feeding Butylated Hydroxyanisole to Dogs

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Groups of weanling Cocker Spaniel pups were fed BHA at levels of 0.0, 5.0, 50.0, and 250.0 mg. per kg. of body weight per day for a 15-month feeding period to see what effect, if any, these subacute levels of BHA might have on the dog. General health and weight gains were good throughout the period. Hemoglobin and blood cell counts were not appreciably affected by feeding BHA at the levels used here. Urine from dogs fed BHA contained higher levels of glucuronates and a higher ratio of total to inorganic sulfates, indicating that BHA excretion was by this route. Microscopic examination of tissue sections at the time of autopsy showed no changes beyond normal variation, except in three animals which had received the highest dosage, in which liver injury had occurred. The results indicate that dogs can ingest BHA for a long period without harm, at a level at least 220 times the maximum allowable level for this antioxidant in lard.

THE use of antioxidants to prevent the development of oxidative rancidity in animal fats used in food and feed has become very widespread. Permission for the use of butylated hydroxyanisole (a mixture of 2- and 3-*tert*-butyl-4-methoxyphenols, BHA) alone and in combination with other antioxidants and synergists in animal fats and shortenings containing animal fats at specified levels was granted in 1948 (*2*). Since that time, a large proportion of the edible animal fats produced have been treated with BHA as a preservative. The use of such materials in foods makes it desirable to find out more about the effects of the antioxidant when ingested at high levels over a long period of time. The present study was initiated to study the effects of feeding high levels of BHA to dogs.

Previous and concurrent work using rats to study the acute and chronic toxicity of BHA (*4*) indicated that levels of BHA up to 2% of the diet had no demonstrable effect over a 6-month period, and that the LD_{50} for rats was of the order of 4.1 to 5.0 grams per kg. of body weight. This information served as a guide in selecting levels of BHA to feed to dogs in a study of the chronic toxicity of the antioxidant in this species.

Procedure

Sixteen Cocker Spaniel pups were weaned at 6 weeks of age and within 1 to 2 months after weaning, litter mates were assigned to groups with four pups in each group as follows:

1. Control group, basal ration only.
2. Basal ration plus a low level of BHA which probably would produce no adverse effect. This group received 5 mg. of BHA per kg. of body weight per day.
3. Basal ration plus an intermediate level of BHA which might or might not produce injury. This group received 50 mg. of BHA per kg. of body weight per day.
4. Basal ration plus a high level of BHA which approached the highest level tolerated in the diet. This group received 250 mg. of BHA per kg. of body weight per day.

Each group consisted of one male and three females, except group 4 which had two males and two females.

Dosages were adjusted to changes in feed consumption and body weight to maintain the above levels. The basal ration was composed of toasted corn flakes, ground, 24.60%; toasted wheat flakes, ground, 24.56%; soybean oil meal (50% protein), 17.58%; meat and bone meal (50% protein), 13.80%; lard, 8.00%; fish meal (menhaden), 2.76%; wheat germ meal, 4.60%; dried skim milk powder, 2.30%; vitamins A and D oil (2250 U.S.P. units A and 300 I.C. units D per gram), 0.46%; iodized salt, 0.24%; dried brewer's yeast, 0.46%; and riboflavin supplement (BY-500), 0.74%. The BHA levels necessary to obtain the desired dosages were computed and the necessary amounts dissolved in the lard portion before mixing the diets. The dogs were housed in metal cages with expanded metal floors in a heated room and given their food allotment and fresh water twice daily. Body weight and food consumption records were kept for a 14- to 15-month period. Blood and urine analyses were made three times for each dog — twice during the test and again at the time the dogs were sacrificed for autopsy and histological examination of their tissues. The first two blood samples were drawn from a leg vein, and the third sample was collected by heart puncture at the time the dogs were sacrificed.

Results

Weight Gain and Feed Consumption. Weaning weight, final weight at 16 to 18 months of age, and average daily feed consumption are shown in Table I. No appreciable differences in weight gains in the different groups were noted except in the group 4 receiving the highest level of BHA (250 mg. BHA per kg. of body weight daily). The dogs in this group gained less weight and consumed less feed per day. The lowered feed consumption was due to the fact that BHA has a hot, peppery taste which the dogs do not like and it was difficult to disguise the taste of that amount of BHA in the feed. At lower levels (groups 2 and 3), the dogs consumed the feed without difficulty after building up gradually to those levels over an acclimatization period of a few days. During the course of the feeding period, the level of BHA in the diet had no influence on the age at which maximum weight was reached. Some dogs reached their peak weight at 7 months of age, while others continued to gain until they were sacrificed. In all dogs, minor fluctuations in body weight were noted, particularly after about 7 months of age. At the time of autopsy, dog number 11 in group 3 contained a large amount of adipose tissue in the body, and dog number 15 in group 4 was very thin. However, all dogs in the different groups appeared to be normal in general appearance.

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Table I. Effect of BHA Ingestion by Dogs

Group No.	Dog No.	Litter Source No.	Sex	Weaning Wt., G.	Final Wt., G.	Age at Sacrifice, Mo.	Av. Daily Feed Consumption, G.	Hb, %	Red Blood Cells, Blood 1000	Hemoglobin and Blood Cell Counts at Time of Sacrifice: White P.M.N., cells, no.	Differential White Cells[a]: P.M.N. no.	Lympho. no.	Mono. no.	P.M.E. no.	P.M.B. no.
1	1	IV	M	1500	10,000	16	225	17.5	5640	7,200	68	27	4	0	1
	2	II	F	1250	7,900	18	280	17.7	4960	4,500	81	19	0	0	0
	3	III	F	1150	10,800	18	280	17.3	6180	4,250	65	35	0	0	0
	4	I	F	1100	6,900	18	225	15.0	4870	7,000	75	23	2	0	0
							Av. 252	16.9	5412	5,737	72	26			
2	5	I	M	1450	9,000	18	240	17.0	5520	5,050	79	21	0	0	0
	6	II	F	1350	9,400	18	225	16.0	6200	8,900	69	30	1	0	0
	7	III	F	1200	10,300	18	270	16.7	4930	7,800	74	25	1	0	0
	8	IV	F	1200	6,450	16	255	16.5	6040	8,100	64	36	0	0	0
							Av. 245	16.6	5672	7,462	71.5	28			
3	9	II	M	1750	9,350	18	285	15.0	6760	3,250	83	23	4	0	0
	10	I	F	1450	6,150	18	240	15.2	5920	7,300	82	17	1	0	0
	11	III	F	1300	11,150	18	255	14.5	5530	10,900	72	26	2	0	0
	12	IV	F	1400	5,200	16	240	14.5	7070	13,200	79	18	3	0	0
							Av. 255	14.8	6213	8,681	79	21			
4	13	III	M	900	6,600	18	195	15.0	6670	5,450	78	18	3	1	0
	14	II	F	1400	6,500	18	210	15.0	6456	11,800	75	22	3	0	0
	15	I	F	1175	4,325	18	165	15.5	6310	10,900	79	20	1	0	0
	16	IV	M	1450	6,050	16	240	16.0	7510	7,450	62	33	3	2	0
							Av. 202	15.4	6736	8,900	73.5	25.7			

[a] Differential white cell counts. P.M.N. = polymorphonuclear neutrophils. Lympho. = lymphocytes. Mono. = monocytes. P.M.E. = polymorphonuclear eosinophils. P.M.B. = polymorphonuclear basophils.

Table II. Urinalyses of Dogs Fed BHA

(Mg./100 Ml.)

Group No.	Dog No.	Color	pH Reaction to Nitrazine Paper	Albumin	Sugar	Color Test for BHA in Urine	Glucuronic Acid in Urine[a]	Sulfates/100 Ml. Urine: Total sulfates	Inorganic sulfates	Ethereal sulfates	Ratio of Total to Inorganic Sulfates
1	1	Pale yellow	Alkaline, high	−	±		151.3	10.55	8.95	1.60	1.18:1
	2	Pale yellow	Alkaline	±	−		62.5	9.77	8.16	1.61	1.20:1
	3	Pale yellow	Alkaline, high	−	−		215.0	6.06	4.36	2.30	1.39:1
	4	Pale yellow	Alkaline, high	−	±						
							Av. 142.9	8.79	7.15	1.84	1.26:1
2	5	Yellow	Alkaline, high	±	−		240.0	9.41	7.31	2.10	1.29:1
	6	Pale yellow	Acid, slight	−	+		470.0	10.87	9.23	1.64	1.18:1
	7	Pale yellow	Alkaline	−	−	+	370.0	7.42	6.14	1.28	1.21:1
	8	Yellow	Alkaline, high	−	+	+					
							Av. 360.0	9.23	7.56	1.67	1.33:1
3	9	Pale yellow	Alkaline, high	−	−		117.5	4.45	3.19	1.26	1.40:1
	10	Pale yellow	Alkaline, high	−	±	+	437.5	7.04	4.11	2.93	1.71:1
	11	Pale yellow	Alkaline, high	−	−	+	187.5	6.75	4.34	2.41	1.56:1
	12	Pale yellow	Alkaline, high	−	±	+					
							Av. 247.5	6.08	3.88	2.20	1.56:1
4	13	Yellow amber	Alkaline, high	±	+	+	460.0	6.10	0.59	5.51	10.41:1
	14	Yellow amber	Alkaline, high	−	+		430.0	4.13	0.46	3.67	9.06:1
	15	Pale yellow	Neutral	−	+	+	427.5	5.91	2.22	3.69	2.66:1
	16	Yellow amber	Acid, slight	−	+	−					
							Av. 439.1	5.38	1.42	4.29	7.37:1

[a] Glucuronic acid in 1-day collection.
Microscopic appearance of urine was normal for all dogs except that urine of dog No. 16 contained white blood cells and bile.

Blood Studies. Results of blood studies are shown in Table I. Blood studies were made at intervals of approximately 5, 8, and 13 months after start of the feeding test. Hemoglobin, red cell counts, white cell counts, and differential white cells shown in Table I are values obtained at 13 months or when the dogs were sacrificed. While there was a tendency toward a slight increase in red cells and white cell counts as the level of BHA in the diet was increased, the values are within the normal range. The feeding of BHA at the levels used here appeared to have no appreciable effect upon hemoglobin level or upon cell counts.

Urinalysis. Routine urinalyses were run on each dog several times during the feeding test. Values obtained at the end of the feeding period when the dogs were sacrificed are shown in Table II. Positive sugar tests were obtained on some of the dogs that received both the control diets and the diets containing BHA, although there were only two dogs in groups 1, 2, and 3 whose urine showed only a faint positive sugar test while urine from all four dogs in group 4 was strongly positive for sugar. Average glucuronate levels were increased in the urine from dogs receiving BHA in their diet.

Samples of urine collected from the dogs at time of sacrifice were assayed qualitatively for BHA, using a color reaction based on the reagent *N*-2,6-trichloro-*p*-benzoquinoneimine. It was observed that all of the dog urine samples reacted with the reagent to give shades of blue and green, indicating that the urine contained small amounts of ma-

terials that react similarly to BHA to produce colored products. Absorbance curves were plotted, and indicated that BHA or other compounds giving a similar color reaction were present in the urine of dogs receiving BHA in their diet at the levels fed.

Quantitative assays of urine for total sulfates and inorganic sulfates were run on composite samples consisting of three or more urine samples from three dogs in each lot. Results show that dogs on the control diet, and on a low level of BHA (5 mg. of BHA per kg. of body weight) had normal urinary sulfate ratios. Dogs on the intermediate level (50 mg. of BHA per kg. of body weight) had a slight increase in organic sulfate, while dogs fed the higher level of BHA (250 mg. of BHA per kg. of body weight) had a considerably increased proportion of organic sulfates in the urine.

General Appearance of Dogs. All of the dogs were alert and active, had healthy hair coats and mild dispositions, and appeared to be healthy and contented throughout the course of the feeding period. The feeding of BHA at the levels here used appeared to have no effect upon the outward appearance or general health of the dogs.

Autopsy Findings and Histological Studies. Gross examination of the organs at autopsy revealed one animal (dog No. 3 in control group) with focal endocardial calcification of the left atrium without other lesions. The remainder of the animals showed no gross lesions except for one dog in the group receiving the highest dosage (dog No. 16, group 4) which had bile in the urine.

There was no obstruction of the common, cystic, or hepatic ducts in this dog, but the liver was bile stained and the capsular surface was finely granular.

Microscopic examination of representative sections from heart, liver, lung, pancreas, spleen, kidney, intestine, lymph node, stomach, thyroid, parathyroid, adrenal, gonad, and bone marrow revealed no changes beyond normal variation except in group 4 receiving the highest level of BHA in the diet. Abnormal variations in this group were all associated with the liver. Three of the four animals of group 4 had a liver cell degeneration and a diffuse granulocytic infiltration. The lobular structure of the liver was normal and there was no excessive connective tissue proliferation. There was diffuse parenchymal degeneration, with marked narrowing of the hepatic sinusoids. The Kupffer cells contained an increased amount of hemosiderin and there were focal accumulations of bile pigment in the periportal areas.

Functional and structural changes occurring during any hepatic disorder are usually more manifest in animals with insufficient dietary intake. The average daily feed intake of dogs 13, 14, and 15 in group 4 was lower than the average intake of dogs in other lots, but dog No. 15 with the lowest daily feed intake did not show signs of liver degeneration. However, during the course of the experiment it became apparent that this dog showed a definite dislike for feed containing the 250 mg. level of BHA. Because of this dog's dislike for BHA, it was decided to reduce the level of BHA in its feed, but to maintain as high a level as possible and yet allow proper feed intake for maintenance and growth. As a result, the average daily intake of BHA for dog No. 15 amounted to only 183 mg. of BHA per kg. of body weight, which may have accounted for the absence of liver degeneration in this dog.

Discussion and Conclusions

To all outward appearances, the dogs were not affected by the BHA in their diet except insofar as the BHA reduced palatability of the diet. There was a tendency toward reduced feed intake, particularly in group 4 receiving the highest level of BHA. Reduced feed intake was probably a contributing factor toward lower weight gains by the dogs in this group.

BHA in the concentrations here fed had no effect on the blood picture of the dog. Hemoglobin levels and cell counts did not seem to vary appreciably among the groups.

Results of urinalysis indicate increased levels of glucuronates in the dogs fed BHA, and a changed ratio of ethereal to inorganic sulfate. Positive sugar tests in urine from dogs fed BHA may have been due to the high levels of glucuronates in the urine. It would appear that the dog excretes BHA in the form of glucuronates and sulfate conjugates. Similar BHA excretion pathways have been reported for the rabbit (*1*).

At the time the dogs were sacrificed, they all appeared vigorous and well nourished, and of the treated animals, only the one animal in the group receiving the highest dosage of BHA appeared to have any gross pathological condition.

Liver injury was found by microscopic examination in three of the four animals in the group receiving the highest dosage of BHA. All other organs and tissues examined appeared to be normal.

It would appear from these studies that the dog is able to ingest BHA at the two levels here fed (5.0 mg. and 50.0 mg. per kg. of body weight per day) with no deleterious effects. However, when the dog is fed 250 mg. of BHA per kg. of body weight per day, the dog is unable to cope with this large amount without damage to the liver. Because three of the four dogs in group 4 weighed over 6000 grams at the end of the feeding period, these three dogs were each receiving more than 1.5 grams of BHA per day. The other dog in group 4, dog No. 15, did not consume as much food per day, did not gain as much weight, and refused to consume BHA at a level of 250 mg. per kg. of body weight. It did consume an average of 183 mg. of BHA per kg. per day, so that at the end of the test period this dog was consuming approximately 786 mg. of BHA per day, or roughly half of the amount consumed by the other three dogs in the group. This dog showed no sign of liver injury at the time of sacrifice.

Present regulations (*3*) allow the use of BHA in lard at a level not to exceed 0.01%. The lowest level of BHA fed here to dogs was 5 mg. per kg. of body weight per day, which amounted to approximately 0.22% of BHA in the lard of the diet or 22 times the maximum allowed in lard for human consumption. Group 3 received approximately 2.24% of BHA in the lard, or 220 times the maximum amount allowed in lard. At 22 and 220 times the maximum allowable levels of BHA in the lard, no evidence of injury was observed. It was only in group 4 where three of the dogs received 250 mg. of BHA per kg. of body weight per day, or approximately 11.0% of BHA in the lard of the diet and 1100 times the maximum level allowed in lard, that any liver injury was observed. The one dog in group 4 (dog No. 15) that actually consumed about half of this amount, or approximately 550 times the level allowed by Meat Inspection Division, Bureau of Animal Industry regulations, did not show liver injury.

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Brix Factors for Determining the Solids in Starch Hydrolyzates

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The Brix hydrometer is widely used in the food industry for the measurement of solids in sugar sirups, but the readings obtained on corn sirups overestimate the solids. The amount of overestimation increases as the dextrose equivalent decreases. In the present work factors were obtained to correct Brix to actual solids. The ash of the usual starch hydrolyzates is largely sodium chloride, which affects the Brix reading more than an equal weight of sirup solids. This salt effect is compensated for by the results of a silver nitrate titration. A new, rapid, and accurate procedure for the determination of the dextrose equivalent is based on the dry substance value obtained by the Brix hydrometer, which has a precision comparable to the value based on a dry substance determination by the vacuum oven.

THE CONVENIENCE of the per cent by weight scale has prompted use of the Brix hydrometer in corn sirup produced in a wide range of dextrose equivalents (D.E., per cent reducing sugars calculated as dextrose and expressed on a dry substance basis). Fetzer and Evans (*2*) took advantage of this convenience to obtain correction factors to convert the Brix readings to actual solids.

Although the decision of Fetzer and Evans to base their factors on the assumption that the various products have a constant ash was justified at the time, conditions have changed. At present corn sirup and other starch hydrolyzates are often deionized. On the other hand, certain residual molasses resulting from the refining of dextrose and containing appreciable quantities of ash are commercial products. For this reason, it seemed highly desirable to redetermine the factors, basing them on the actual carbohydrate solids. Because the ash in these products is largely sodium chloride, its presence in the sirup can be determined volumetrically and corrected for its effect upon the observed Brix. A table for D.E. based on the two variables—Brix and Lane-Eynon titration—would be of great value not only within the laboratories of the wet milling industry but to the users of corn sirups.

The factors to correct the Brix reading to actual solids are based on the following considerations:

Determining the factors by specific gravity and actual solids, thus eliminating the reducing sugar determination.

Basing the factors for the various D.E.'s on ash-free hydrolyzates and determining the effect of the ash on the hydrometer by a volumetric determination of salt, with tables to convert the titration directly to the Brix correction necessary for the amount of salt present.

Corn Sirups and Corn Sugars

The starch hydrolyzates used in this research were, with one exception, commercial products produced by the acid hydrolysis of starch. The products in the D.E. range of 30 to about 58 are noncrystallizing and are termed corn sirups; those in the D.E. range of about 80 to 93 crystallize to a firm solid and are termed crude corn sugars (Nos. 70 and 80). No commercial acid-hydrolyzed product is produced in the range between corn sirups and corn sugars. To provide such a product, a special factory conversion was made, employing the hydrolytic conditions for corn sirup. This product had a D.E. of 68.3 and is designated as a "special sirup." The usual analyses were made on hydrolyzates (Table I).

Effect of Ash on Brix

The effect of salt on the specific gravity at three Brix levels of sucrose was determined in the following manner:

Table I. Analysis of Starch Hydrolyzates

Type	Moisture, %	Dry Substance, %	Ash, %	N × 6.25, %	$[\alpha]_D^{20}$	Reducing Sugars, %	D.E.	Ash-Free D.E.
Corn sirup	22.35	77.65	0.248	0.0245	164.3	24.85	32.0	32.3
	18.58	81.42	0.334	0.0280	146.9	34.52	42.4	42.6
	18.79	81.21	0.410	0.0330	123.4	46.13	56.8	57.0
Special sirup	21.92	78.08	0.258	0.0440	103.3	53.33	68.3	68.6
Corn sugar	17.34	82.66	0.537	0.0530	65.7	69.68	84.3	84.9
	12.37	87.63	1.262	0.0742	53.7	80.00	91.3	92.6

Table II. Effect of Sodium Chloride on Specific Gravity

Grams/250 Ml. Sucrose	Grams/250 Ml. NaCl	NaCl, %	20°/20° C.	Increase	Increase per 1.00% NaCl
			17.00 Brix		
45.338	0.000	0.000	1.06973	0.00000	0.00000
43.524	1.814	4.000	1.07195	0.00223	0.00056
42.618	2.720	6.000	1.07313	0.00341	0.00057
40.804	4.534	10.000	1.07521	0.00549	0.00055
			20.00 Brix		
53.996	0.000	0.000	1.08302	0.00000	0.00000
53.456	0.540	1.000	1.08362	0.00060	0.00060
52.916	1.080	2.000	1.08431	0.00129	0.00065
51.836	2.160	4.000	1.08550	0.00248	0.00062
49.676	4.320	8.000	1.08805	0.00503	0.00063
48.596	5.400	10.000	1.08930	0.00628	0.00063
			23.00 Brix		
62.869	0.000	0.000	1.09653	0.00000	0.00000
59.097	3.772	6.000	1.10093	0.00440	0.00073
56.582	6.287	10.000	1.10383	0.00730	0.00073

The quantity of sucrose (reagent grade) needed to produce, for example, 20.00 Brix was weighed, transferred to a 250-ml. Manufacturing Chemists' Association volumetric flask, dissolved in about 200 ml. of water, cooled to 20° C., and made to volume. The apparent specific gravity at 20° 20° C. was obtained in quadruplicate and the results were averaged. This value, zero ash, was checked against the published values to establish the accuracy of the procedure.

Then 99.00, 98.00, 96.00, 92.00, and 90.00% portions of the above weight of sucrose, with the corresponding weight of sodium chloride to assure 100.00% of the original solids, were transferred to the volumetric flask, dissolved in about 200 ml. of water, cooled, and made to volume, and the apparent specific gravity (20°/20° C.) was determined as before.

The increase in specific gravity indicates the effect of replacing a given weight of sucrose by sodium chloride; the increase divided by the per cent of salt used gives the increase in terms of 1.00% salt. The detailed results for levels of sucrose, 17.00, 20.00, and 23.00 Brix, are shown in Table II.

The data indicate that the effect of sodium chloride on the specific gravity is linear in amounts up to 10.00%. These data enable further calculations to be made in terms of Brix (Table III).

Table III. Brix Corrections for Salt

	Salt, %		Increase	
Brix	Dry basis	As is	Brix	For 1.00% salt
17.00	10.00	1.700	1.21	0.121
20.00	10.00	2.000	1.41	0.141
23.00	10.00	2.300	1.61	0.161

On the basis of the sucrose replaced by sodium chloride, the effect on the specific gravity is much greater. This is shown in Table IV, which is based on the experimental data given in Table II. The values in this table for 1.00% sodium chloride have been used for the calculations for determining Brix factors.

Because the ash in starch hyrolyzates is essentially sodium chloride, the amount present may be determined volumetrically. For this purpose, 0.0855N silver nitrate (1.00 ml. equals 0.00500 gram of sodium chloride) was employed for titrating 5.00 ml. of the sirup containing the salt. The milliliters of silver nitrate required for 10.00% salt, dry basis, for 20.00 Brix were obtained by substituting in the following equation:

$$\frac{\text{Ml. of } 0.0855N \text{ silver nitrate} \times 0.00500}{5.00 \times 1.07991\ (5)\ \text{Table 114}} \times 100 = 2.000\%\ \text{salt}$$

Ml. = 21.60

Because the increase in Brix for this amount of salt is 1.41, the amount per milliliter of 0.0855N silver nitrate is 0.0653 Brix. Thus, the observed Brix may be corrected to true solids by subtracting the correction obtained by multiplying the milliliters of silver nitrate used for the titration by the factor of 0.0653. The above procedure was applied to the data obtained for the two other Brix values cited with results as follows:

17.00 Brix	18.18 ml.	0.0666 per ml.
23.00 Brix	25.15 ml.	0.0640 per ml.

Table V has been computed for the Brix range of 17.00 to 23.00 and for sufficient range in titration to cover all starch hydrolyzates and the usual residual sirups obtained in the manufacture of dextrose.

Table IV. Effect of Salt on Specific Gravity in Terms of Sucrose Replaced

	Grams/250 Ml.		Sp. Gr., 20°/20° C.		Increase in Sp. Gr.	
Brix, 20° C.	Sucrose	Sucrose used, 90.00%	Calcd., Table 114 (5)	Found	10.00% salt	1.00% salt
17.00	45.338	40.805	1.06682	1.07521	0.00839	0.00084
20.00	53.996	48.995	1.08047	1.08930	0.00883	0.00088
23.00	62.869	56.658	1.09494	1.10383	0.00889	0.00089

Table V. Brix Subtractions for Effect of Salt Based on Silver Nitrate Titration

5.00-ml. sample. 0.0855N $AgNO_3$
20.0° C. 1.00 ml. = 0.00500 gram NaCl

Titn., Ml.	Observed Brix						
	17.00	18.00	19.00	20.00	21.00	22.00	23.00
1.00	0.07	0.07	0.07	0.07	0.06	0.06	0.06
1.50	0.10	0.10	0.10	0.10	0.10	0.10	0.09
2.00	0.13	0.13	0.13	0.13	0.13	0.13	0.13
2.50	0.17	0.17	0.16	0.16	0.16	0.16	0.16
3.00	0.20	0.20	0.20	0.20	0.19	0.19	0.19
3.50	0.23	0.23	0.23	0.23	0.23	0.22	0.22
4.00	0.27	0.26	0.26	0.26	0.26	0.26	0.25
4.50	0.30	0.30	0.30	0.29	0.29	0.29	0.28
5.00	0.33	0.33	0.33	0.33	0.32	0.32	0.32
5.50	0.37	0.36	0.36	0.36	0.36	0.35	0.35
6.00	0.40	0.40	0.39	0.39	0.39	0.38	0.37
6.50	0.43	0.43	0.42	0.42	0.42	0.42	0.41
7.00	0.47	0.46	0.46	0.46	0.45	0.45	0.44
7.50	0.50	0.50	0.49	0.49	0.48	0.48	0.47
8.00	0.53	0.53	0.53	0.52	0.52	0.51	0.51
8.50	0.57	0.56	0.56	0.55	0.55	0.54	0.54
9.00	0.60	0.60	0.59	0.59	0.58	0.58	0.57
9.50	0.63	0.63	0.62	0.62	0.61	0.61	0.60
10.00	0.67	0.66	0.66	0.65	0.64	0.64	0.63
10.50	0.70	0.69	0.69	0.69	0.68	0.67	0.66
11.00	0.73	0.73	0.72	0.72	0.71	0.70	0.70
11.50	0.77	0.76	0.76	0.75	0.74	0.74	0.73
12.00	0.80	0.79	0.79	0.78	0.78	0.77	0.76
12.50	0.83	0.83	0.82	0.82	0.81	0.80	0.79
13.00	0.87	0.86	0.85	0.85	0.84	0.83	0.82
13.50	0.90	0.89	0.89	0.88	0.87	0.86	0.85
14.00	0.93	0.93	0.92	0.91	0.90	0.90	0.89
14.50	0.97	0.96	0.95	0.95	0.94	0.93	0.92
15.00	1.00	0.99	0.99	0.98	0.97	0.96	0.95
15.50	1.03	1.03	1.02	1.01	1.00	0.99	0.98
16.00	1.06	1.06	1.05	1.04	1.03	1.02	1.01
16.50	1.10	1.09	1.08	1.08	1.07	1.06	1.05
17.00	1.13	1.12	1.12	1.11	1.10	1.09	1.08
17.50	1.16	1.16	1.15	1.14	1.13	1.12	1.11
18.00	1.20	1.19	1.18	1.17	1.16	1.15	1.14
18.50	1.23	1.22	1.22	1.21	1.20	1.18	1.17
19.00	1.26	1.26	1.25	1.24	1.23	1.22	1.21
19.50	1.30	1.29	1.28	1.27	1.26	1.25	1.24

Brix Factors for Starch Hydrolyzates

The Brix reading can be converted to corn sirup or corn sugar solids by obtaining the ratio from the data obtained from two independent determinations on a given sirup:

$$\frac{A}{B} = \frac{\text{actual solids} - \text{vacuum oven moisture}}{\text{Brix solids} - \text{specific gravity}}$$

Ash-free products would have been ideal for this procedure, but they were not available. It is possible to place the results obtained on an ash-free basis from the data which have been reported.

Table VI. Brix Factors to Convert Brix Solids to Actual Solids

D.E.	Factor	D.E.	Factor
30.0	0.9685	69.0	0.9910
36.0	0.9720	75.0	0.9930
43.0	0.9760	79.0	0.9940
50.0	0.9800	85.0	0.9950
64.0	0.9880	93.0	0.9960

All of the six hydrolyzates shown in Table I were diluted to approximately 17.0, 20.0, and 23.0% solids and analyzed as follows:

A. Actual solids by the Filter Cel-vacuum oven procedure (*1*).
B. Brix solids from specific gravity obtained by a pycnometer, 20°/20° C.

The actual data obtained on one of the dilutions are shown in detail:

Corn Sirup. 56.8 D.E.; ash 0.41% D.E.: 57.0 ash-free D.E.
A. Dry substance on sirup. 19.658, 19.677, 19.677, 19.659%. Average 19.668% (each value shown average of duplicates).

Ash-free dry substance — 19.668 × 0.9959 = 19.587%

B. Specific gravity 20°/20° C. 1.08290; 1.08293; 1.08291; 1.08291. Average 1.08291 (each value shown average of duplicates).

Ash-free specific gravity. 1.08291 — (0.00088 × 0.41) = 1.08255

Corresponding Brix 20°/20° C. = 19.908

Ratio $\frac{19.587}{19.908} = 0.984$

This factor shows that if a corn sirup 56.8 D.E. were diluted to 20.00 Brix, the actual solids would be obtained by multiplying 20.00 by 0.984 or 19.68%.

At least two sets of data, as shown above, were obtained on each sirup; however, four sets were obtained on sirups with D.E.'s of 68.3, 84.3, and 91.3. The emphasis on the latter sirups was deemed necessary, as the plot of factor *vs.* ash-free D.E. showed a marked change in the slope of the curve in this area. The curve between 30 and 69 D.E. is essentially a straight line, which is in accord with unpublished chromatographic data on the composition of acid-converted starch hydrolyzates. Above 69 D.E. the curve levels off, showing little increase in slope above 90 D.E. This condition would be expected from our present knowledge of the hydrolysis of starch at this level of D.E. Essential points on the graph are shown in Table VI.

Table VII. Additions in D.E. to Compensate for Overestimation of Solids by Brix Hydrometer

D.E.	D.E. Addition	D.E.	D.E. Addition
30.0	0.98	70.0	0.63
35.0	1.02	72.0	0.57
40.0	1.06	74.0	0.54
45.0	1.05	76.0	0.51
50.0	1.02	78.0	0.49
55.0	0.96	80.0	0.47
60.0	0.86	82.0	0.45
62.0	0.82	85.0	0.43
64.0	0.77	87.0	0.41
66.0	0.72	90.0	0.40
68.0	0.66	93.0	0.37

Determination of Dextrose Equivalents

The Lane and Eynon (*3, 4*) procedure for reducing sugars is the accepted method in the wet milling industry. The method for determining D.E., employing this procedure, the Brix hydrometer for the solids, and the factor for correction of the solids as developed here,

Table VIII. D.E. of Corn Sirups, Lane and Eynon Titration

(Brix 20° C., corrected for salt, sirup diluted 50.00 ml. to 500.0 ml.)

Titn., Ml.	Brix 17.00	18.00	19.00	20.00	21.00	22.00	23.00
10.0	67.0	63.2	59.7	56.6	53.8	51.2	48.8
10.5	63.9	60.3	56.9	54.0	51.3	48.8	46.5
11.0	61.1	52.6	54.4	51.6	49.0	46.6	44.4
11.5	58.6	55.2	52.2	49.4	46.9	44.6	42.6
12.0	56.2	53.0	50.0	47.4	45.0	42.8	40.8
12.5	54.0	50.9	48.1	45.5	43.2	41.1	39.2
13.0	52.0	49.0	46.2	43.8	41.6	39.6	37.7
13.5	50.1	47.2	44.6	42.2	40.1	38.2	36.4
14.0	48.3	45.6	43.0	40.8	38.7	36.8	35.1
14.5	46.7	44.1	41.6	39.4	37.4	35.6	33.9
15.0	45.2	42.7	40.2	38.1	36.2	34.5	32.8
15.5	43.8	41.3	39.0	36.9	35.1	33.4	31.8
16.0	42.5	40.1	37.8	35.8	34.0	32.4	30.8
16.5	41.3	38.9	36.7	34.7	33.0	31.4	29.9
17.0	40.1	37.7	35.6	33.7	32.1	30.5	29.1
17.5	39.0	36.6	34.6	32.8	31.2	29.7	28.3
18.0	37.8	35.6	33.7	31.9	30.3	28.9	27.6
18.5	36.8	34.7	32.8	31.1	29.5	28.1	26.8
19.0	35.9	33.8	32.0	30.3	28.8	27.4	26.1
19.5	35.0	33.0	31.2	29.5	28.1	26.7	25.5
20.0	34.1	32.0	30.4	28.8	27.4	26.1	24.8

Table IX. D.E. of Corn Sugar Sirups, Lane and Eynon Titration

(Brix 20° C., corrected for salt, sirup diluted 25.0 ml. to 500.0 ml.)

Titn., Ml.	Brix 17.00	18.00	19.00	20.00	21.00	22.00	23.00
10.2	...	...	...	...	...	...	94.1
10.4	...	...	...	...	...	...	92.3
10.6	...	...	...	...	...	...	90.6
10.8	...	...	...	...	...	93.3	88.9
11.0	...	...	...	...	...	91.6	87.3
11.2	...	...	...	...	...	90.0	85.8
11.4	...	...	...	...	93.0	88.4	84.3
11.6	...	...	...	...	91.4	86.9	82.9
11.8	...	...	...	94.7	89.9	85.5	81.6
12.0	...	...	...	93.1	88.4	84.1	80.2
12.2	...	...	...	91.6	86.9	82.7	78.9
12.4	...	...	...	90.2	85.6	81.4	77.6
12.6	...	...	93.8	88.7	84.3	80.1	76.4
12.8	...	...	92.3	87.4	83.0	78.9	75.2
13.0	...	...	90.9	86.1	81.7	77.7	74.1
13.2	...	94.9	89.5	84.8	80.5	76.5	73.0
13.4	...	93.4	88.2	83.5	79.3	75.4	71.9
13.6	...	91.9	86.9	82.3	78.1	74.3	70.9
13.8	...	90.7	85.6	81.1	77.0	73.3	69.9
14.0	...	89.5	84.5	80.0	75.9	72.3	68.9
14.2	93.7	88.2	83.3	78.9	74.9	71.3	68.0
14.4	92.4	87.0	82.2	77.8	73.9	70.3	67.1
14.6	91.2	85.8	81.0	76.7	72.8	69.4	66.7
14.8	90.0	84.7	80.0	75.7	71.9	68.5	65.3
15.0	88.8	83.6	78.9	74.7	71.0	67.6	64.5
15.5	86.8	80.9	76.4	72.4	68.8	65.5	62.5
16.0	82.3	78.4	74.1	70.2	66.7	63.5	60.6
16.5	80.8	76.1	71.9	68.1	64.8	61.7	58.8
17.0	78.5	73.9	69.8	66.2	62.9	59.9	57.2
17.5	76.2	71.8	67.9	64.4	61.2	58.3	55.6
18.0	74.3	69.5	66.1	62.7	59.6	56.7	54.1
18.5	72.2	68.1	64.4	61.0	58.0	55.2	52.7
19.0	70.4	66.3	62.8	59.5	56.6	53.8	51.4
19.5	68.6	64.7	61.2	58.0	55.1	52.5	50.0
20.0	67.0	63.1	59.7	56.6	53.8	51.2	48.8

is given in the equation which follows for corn sirup of 20 Brix with a dilution of the sirup, 50.0 ml. to 500.0 ml.

$$\frac{12.02/\text{ml. titration}}{50.0/500.0 \times 20.00 \times 1.08096 \times \text{factor}} \times 100$$

This calculation requires the use of a factor, which is specific for the D.E. before D.E. is known. This impasse is avoided by obtaining D.E. additions in advance and applying the required addition to a given calculation based on Brix solids. The additions to be applied were derived from the data which appear in Table VI and are shown in Table VII.

The additions of D.E. required to correct for the overestimation of solids in corn sirup and corn sugar by the Brix hydrometer are surprising and unexpectedly simple. The data are particularly useful in the analysis of such sirups as first and second greens obtained in the manufacture of dextrose. Hydrol, corn sugar molasses, or second greens will vary in D.E. within the range of 68 to 75, and the salt within the range of 6 to 10% dry basis. Thus, the D.E. of the sugar itself with range from 75 to 88 D.E. obtained by the vacuum procedure for solids and the arbitrary addition of 0.5 D.E. to the D.E. obtained by the above method of analyses will be within 0.1 D.E., well within the tolerance of the procedure for the reducing sugar determination.

Laboratories in the corn wet milling industry determine a very large number of D.E.'s daily, particularly if refined corn sugar or dextrose is manufactured. To save time and expedite results to the plant, most laboratories have large tables with coordinates of Brix and "milliliters of titration," which give the D.E. for the specific condition involved. In the tables, the Brix values, 20° C., have been adjusted for the effect of salt and the D.E. value obtained carries the correction for the overestimation of solids by the Brix hydrometer. Abbreviated forms of the two tables are shown (Table VIII for corn sirup and Table IX for corn sugar sirups).

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PLANT PECTIN ANALYSIS

Determination of Pectic Substances by Paper Chromatography

R. M. McCREADY and MILDRED GEE

Western Regional Research Laboratory, U. S. Department of Agriculture, Albany 10, Calif.

Physical methods of purifying several fruit and vegetable pectinic acids resulted in polymers containing nonuronide sugar units. The sugars, α-D-galactose, β-L-arabinose, α-L-rhamnose, and α-D-xylose, were separated from the hydrolysis products of apricot pectin by thick-paper chromatography and identified by comparing their x-ray powder diagrams with those of authentic specimens. Two widely different molecular species of galacturonic acid–containing polymers were separated from apricot polysaccharides. Attempts to resolve copper-purified apricot pectinic acid by further copper precipitation, dialysis, paper electrophoresis, and fractionation of the acetate into different molecular species were unsuccessful. Nonuronide sugars appear to be incorporated into the galacturonan molecule of pectic substances from most plant materials.

THE PECTIC POLYSACCHARIDES exist in plants in close physical union with araban and galactan. This association is so firm that upon extraction of polysaccharides from plants, a triad containing galacturonan, araban, and galactan is obtained. Because of the facility of paper chromatography for detection of small amounts of sugars in complex polymers, researches on composition of pectic substances have been stimulated in recent years.

Reports of composition of most fruit and vegetable pectins indicate that they are mixtures of polysaccharides or molecules apparently containing sugar residues other than galacturonic acid (*1, 4, 5, 7, 13, 16, 20, 24*). Many attempts to isolate a pure galacturonan from a wide variety of plant materials were unsuccessful unless strong chemical procedures were applied, which were suspected of hydrolyzing glycosidic linkages and degrading one or more of the carbohydrate polymers.

The question of composition and structure of undegraded pectic substances appears to be open to further investigation. Mainly, the unanswered question is whether pectin is a pure galacturonan admixed with the carbohydrates araban and galactan, or whether the so-called galacturonan is a complex carbohydrate containing some nonuronide sugars as part of the molecule.

The experiments reported here indicate the difficulties involved in attempts to prepare pure galacturonan from some natural materials and suggest that families of soluble complex galacturonic acid–containing polysaccharides occur in some plants.

Experimental

Analyses. Pectic substances isolated by precipitation from aqueous solution by ethyl alcohol were dried in vacuo at 60° C., ground to pass 60-mesh, humidified to an equilibrium moisture content of about 10%, and analyzed. Methods of analyses used were mainly those described by Owens *et al.* (*21*). Carbomethoxy (ester methoxyl) analyses were made by saponification, moisture analyses by oven drying in vacuo, ash analyses by incineration at 600° C., anhydrouronic acid analyses by a colorimetric carbazole method (*14*), and acetyl analyses by a hydroxamic acid color reaction (*15*). Rotations are specific rotations at 25° C. using the *D*-line of sodium. Intrinsic viscosities are those extrapolated to infinite dilution. Quantitative sugar analyses were done from reflection densities of colored spots on paper chromatograms (*17*).

Qualitative Chromatography. Polyuronides (1.0 gram in 50 ml. of 1*N* sulfuric acid) were boiled under reflux

is given in the equation which follows for corn sirup of 20 Brix with a dilution of the sirup, 50.0 ml. to 500.0 ml.

$$\frac{12.02/\text{ml. titration}}{50.0/500.0 \times 20.00 \times 1.08096 \times \text{factor}} \times 100$$

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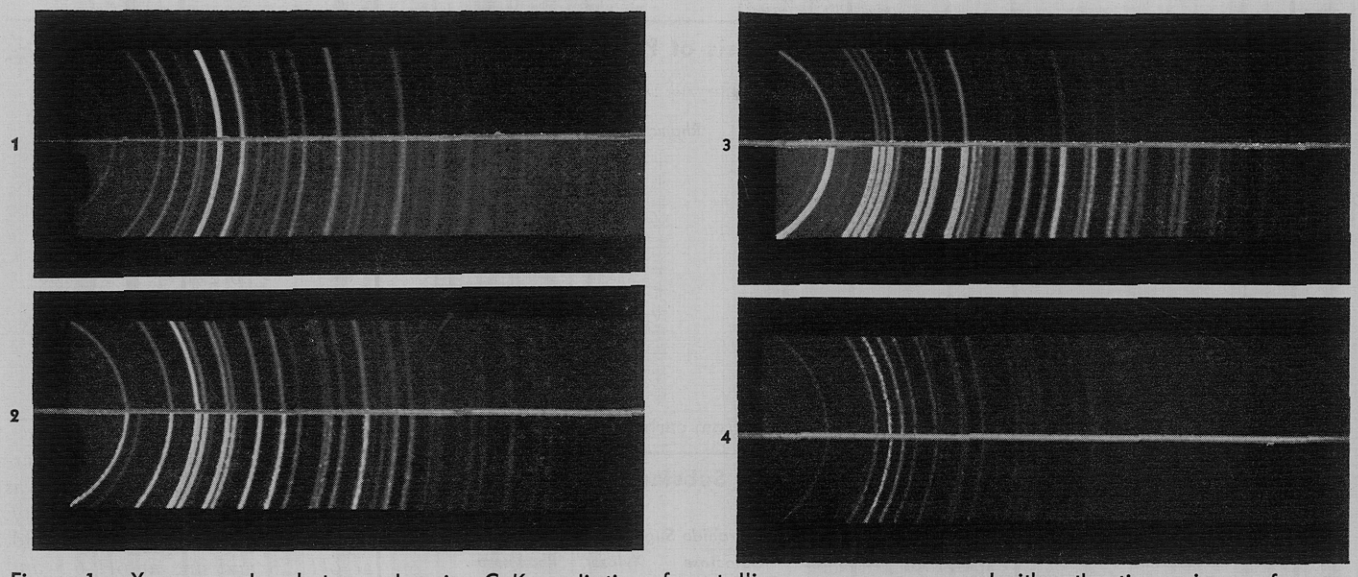

Figure 1. X-ray powder photographs using CuK_α radiation of crystalline sugars as compared with authentic specimens of sugars
Upper halves: unknowns. Lower halves: β-L-arabinose, α-D-galactose, α-L-rhamnose hydrate, and α-D-xylose, respectively

for 2.5 hours. The cooled solution containing the partial hydrolysis products of the polymer was neutralized with barium carbonate and the barium sulfate removed by filtration. The filtrate was concentrated to 10% solids for quantitative chromatography. When testing for sugars alone, 3 volumes of ethyl alcohol were added to precipitate the insoluble barium salts. The filtered solution was evaporated to a sirup for qualitative chromatography. Five-microliter spots of sirup were placed on the starting line of a sheet of Whatman No. 1 filter paper, and the chromatogram was developed in the ascending direction with a miscible solvent of ethyl acetate, pyridine, and water—5, 2.5, and 3 volumes, respectively. Sugars and galacturonic acid were revealed as brown or red spots on the dried chromatogram after dipping in ethyl acetate solution containing 2% each of aniline and trichloroacetic acid, followed by heating for 5 minutes at 85° C.

Extraction and Isolation of Pectin. Two hundred and fifty grams of fresh sliced pectin-containing material, 10 grams of paper pulp, 1500 ml. of water, and sulfuric acid to pH 2 were heated together at boiling for 20 minutes. The disintegrated mass was filtered with suction on canvas precoated with a diatomaceous silica filter-aid. The cooled filtrate was poured into 2 volumes of 95% ethyl alcohol and left standing for 15 minutes, after which the precipitated pectin was strained and pressed on a nylon cloth. The pressed cake was shredded by hand, stirred in 5 volumes of 60% ethyl alcohol, pressed, shredded, washed with 95% ethyl alcohol, and dried in vacuo. The dried product was ground to pass 60-mesh for analysis.

Purification by Copper Precipitation. Pectic substances are quantitatively precipitated from aqueous solution by cupric ion and thus freed from copper-soluble polysaccharides (*19*). Pectinic acid (5 liters of 0.5% solution adjusted to pH 5.2) was precipitated with 250 ml. of 15% cupric sulfate solution. The precipitate was centrifuged, washed twice by suspending it in water, and recentrifuged. The precipitate was dissolved in 1 liter of water, when the pH was adjusted to 1.5 with hydrochloric acid. The pectinic acid was precipitated from this solution by pouring it slowly into 2 volumes of 95% ethyl alcohol. The gelatinous precipitate was strained from the solution on nylon cloth, pressed by hand, shredded in 10 volumes of 70% ethyl alcohol–1% hydrochloric acid, and pressed. This was repeated twice to remove copper ions, followed by washing three times in 70% ethyl alcohol to remove mineral acid. A final washing in 95% ethyl alcohol further dehydrated the pectic materials so that they dried in a fluffy condition.

Identification of Sugars. The sirup containing the partial hydrolysis products of 10 grams of purified apricot pectinic acid was applied to thick papers (Whatman No. 103, 0.03 inch thick) for large-scale separations of sugars for identification purposes. The technique employed has been described (*18*). The areas occupied by the sugars were excised in strips and eluted with water by capillary flow. The sugar solutions were treated batchwise with charcoal and ion exchange resins. Upon slow evaporation the sugars crystallized. The crystals were identified by comparing their x-ray powder diagrams with those of authentic specimens (Figure 1). No attempt was made here to crystallize and identify galacturonic acid as the acidic constituent of these pectinic acids. Qualitative paper chromatography of acid hydrolysis products has demonstrated that the sole acidic constituent was galacturonic acid (*8*). All of the polymers examined here contained only galacturonic acid as the acidic constituent.

Analyses of several copper-purified pectinic acids from various sources are shown in Table I. Pectinic acid showing 90% anhydrouronic acid and containing 10% carbomethoxyl, when corrected for 4.5% methylene (the oxygen of the carbomethoxyl is measured in the anhydrouronic acid), would show a corrected anhydrouronic acid content of about 94.5%. Several reports in the literature expressed analyses as galacturonic acid and as methoxyl, apparently not taking into account the anhydrous character of polymerized sugars or that only the methylene of the methoxyl should be used to correct for anhydrouronic acid.

The citrus pectins are those showing the highest anhydrouronic acid content and the highest accounted-for material. The other fruit and vegetable pectins are lower in anhydrouronic acid. Sugar beet pectin contains 4.5% acetyl, apricot pectin 2.3%, and the citrus pectins only 0.2%. Corrections in the anhydrouronic acid of these pectinic acids shown in Table I for acetyl were not made, but they would not be changed by more than a few per cent.

Purified pectinic acids from several sources are similar in nonuronide sugar composition. Some of these pectinic acids showed traces of several other sugars not identified, but a large group showed mainly the four sugars positively identified. Coleman *et al.* (*7*) and Carrao (*4*, *5*) demonstrated the presence of arabinose, galactose, xylose, and rhamnose among the hydrolysis products of pectic substances from coffee, grapes, and persimmons, respectively. In sisal pectic acid Aspinall and Canas-Rodrigues (*1*) found the above sugars plus D-glucose, 2-*O*-methyl-L-fucose, and 2-*O*-methyl-D-xylose. Newbold and Josyln (*20*) and McCready, Jeung, and Maclay (*16*) demonstrated the presence of rhamnose, galactose, and arabinose among the hydrolysis products of highly purified

Table I. Sugars Liberated by Partial Acid Hydrolysis of Purified Pectinic Acids

Source	Anhydrouronic Acid[a]	Arabinose	Galactose	Rhamnose	Xylose
Orange	92.1	+	+	+	0
Grapefruit	91.7	+	+	+	0
Lemon	90.4	+	+	+	0
Fig	87.1	+	+	+	0
Carrot	76.7	+	+	+	0
Apple	88.0	+	+	+	+
Peach	86.8	+	+	+	+
Pea pod	84.8	+	+	+	+
Apricot	83.1	+	+	+	+
Pear	82.6	+	+	+	+
Sugar beet	82.3	+	+	+	+
Avocado	79.0	+	+	+	+

[a] Content corrected for water, ash, and methylene impurity from carbomethoxyl.

Table II. Composition of Apricot Pectic Substances

Treatment	Anhydro-uronic Acid,[a] %	Nonuronide Sugars, %			
		Arabinose	Galactose	Xylose	Rhamnose
Unpurified	47.2	10.0	1.7	1.0	1.0
Oxalate extracted	...	17.0	3.5	1.0	1.0
Acid–ethyl alcohol ppt.	62.0	14.4	1.9	1.1	1.0
Copper ppt.					
1X	75.5	9.2	2.3	1.1	1.1
2X	76.0	9.0	2.0	1.0	1.0
Copper ppt. 1X and dialyzed	78.0	6.0	2.0	1.0	1.0
Pectic acid	...	7.2	2.5	0.9	0.9
Polygalacturonase-treated, dialyzed	24.0	8.0	13.0	2.0	2.0
Copper-soluble fraction	18.6	8.0	32.2	6.9	2.3

[a] Water- and ash-free basis.

Table III. Fractionation of Acetylated Apricot Pectinic Acid

Fraction	Weight, Grams	Rotation
1	0.028	
2	0.031	
3	0.211	+134
4	0.278	
5	0.325	
6	0.271	+166
7	0.330	
8	2.38	
9	2.28	+132
10	0.88	
Residue	0.45	+124

citrus pectic acids. Researches on tomato fruits (*24*), passion fruits (*13*), and other fruit and vegetable materials showed the presence of nonuronide sugars. Some of the reports have included the four sugars positively identified here plus glucose, mannose, and unidentified sugars from some pectinic acids.

Solms, Büchi, and Deuel (*22*) and Büchi and Deuel (*3*) isolated soluble galacturonic acid–containing carbohydrates from grapes that yielded an aldobiuronic acid, 2-α-D-galacturonopyranosido-L-rhamnopyranose. Hostettler and Deuel (*9*) showed that plantago mucilage contains a polymer which yields upon hydrolysis an aldobiuronic acid, 4-α D - galacturonopyranosido - D - xylose. Several gums and mucilages have been reported to contain galacturonic acid, galactose, arabinose, xylose, rhamnose, and other sugars. Jones and Smith (*11*) have suggested that gums might be regarded as carbohydrate polymers which contain D-glucuronic acid as the acidic constituent, and mucilages as those which contain D-galacturonic acid as the acidic moiety. An inspection of these definitions and the ones for pectic substances (*12*, *23*) reveals that none is adequate for the known carbohydrates in plants. There are many known exceptions to suggested classifications and it is likely that families of plant carbohydrates containing less than 10% and up to 100% of galacturonic acid and many kinds of nonuronide sugars may occur in plants.

The composition of apricot pectinic acid was of particular interest because of its low anhydrouronic acid content and the presence of four nonuronide sugars. Pectinic acid extracted from commercial Blenheim apricot purée with boiling water was isolated as described and purified by dissolving in water and precipitating in acidified ethyl alcohol.

Blenheim apricot purée was also extracted with ammonium oxalate–oxalic acid at pH 3.6 described by Bishop for sunflower heads (*2*) and isolated as described.

The partially purified apricot pectinic acid was dissolved in 0.5% solution, adjusted to pH 5.2, and precipitated with copper sulfate as described. A part was prepared for analysis and a part was dissolved, filtered, and precipitated a second time with copper and purified for analysis (Table II).

The copper-containing aqueous solution from the once copper-precipitated apricot pectinate was adjusted to pH 7. A precipitate of insoluble copper salts was removed by filtration. The solution was passed through a Dowex 50(H) ion exchange column to remove cupric ions, concentrated to 5% solids, and precipitated with 4 volumes of 95% ethyl alcohol. The product was washed with 95% ethyl alcohol and worked up as described. The compositions of the pectinic acid precipitated by cupric ion and that soluble as a copper salt are shown in Table II.

The insoluble fraction of copper-precipitated apricot pectinic acid was subjected to further tests for homogeneity. Two solutions of 0.5% pectinic acid, one at pH 2.8 and one at pH 6.5 and both containing phenyl mercuric nitrate as a preservative, were dialyzed in Visking cellulose sausage casings against distilled water for 12 days. The contents of the cellulose sausage casings were precipitated with ethyl alcohol; the pectinic acid was hydrolyzed with acid and sugars were determined in the products by qualitative chromatography. Both preparations contained like amounts and kinds of the four sugars previously described. Upon hydrolysis the material outside of the bags showed traces of arabinose. Analyses of the insoluble fraction are shown in Table II.

Purified apricot pectinic acid was converted to pectic acid by de-esterification at 25° C. with sodium hydroxide at pH 12. The pectic acid was isolated by precipitation with sulfuric acid at pH 1.5, and the precipitate was pressed and washed with water and then with ethyl alcohol (Table II).

The purified apricot pectic acid was dissolved in water and hydrolyzed with 0.01% of purified fungal polygalacturonase (*10*) at pH 4.5 and 25° C. for 2 days. After hydrolysis the solution was dialyzed in a cellulose sausage casing for 5 days. The content of the casing was concentrated and a polymer was precipitated with 3 volumes of ethyl alcohol. The analyses of the polygalacturonase-treated, dialyzed polymer are shown in Table II.

The results of the fractionation and purification of apricot pectinic acid shown in Table II indicate that pectinic acid mixtures were easily purified from an anhydrouronic acid content of 47% to 76% with copper precipitation. Further attempts to increase the anhydrouronic acid value by a second copper precipitation did not change the pattern of the sugar components. Most of the arabinose can be hydrolyzed from apricot pectinic acid by boiling in 0.02*N* oxalic acid for 2.5 hours. Because the arabinose moiety appears to be a furanoside and very labile to acid hydrolysis,

quantitative changes in amounts readily occur whenever acid conditions are used to extract or purify pectic substances. For these reasons the composition of pectic substances as they exist in apricots may not be accurately represented as shown in Table II.

Analyses of the copper-insoluble and copper-soluble materials as shown in Table II show that at least two groups of galacturonic acid–containing carbohydrates occur in apricot pectic substances. The copper-soluble fraction showed 18.6% of anhydrouronic acid and a low intrinsic viscosity of 0.86. The low molecular weight and the insufficient number of acid groups permit it to remain soluble in the presence of cupric ion. This fraction may not be homogeneous but may be a mixture of several molecular species that have similar solubility properties.

Analysis of the copper-insoluble apricot pectinic acid showed 76% anhydrouronic acid and an intrinsic viscosity of 2.66. The copper-precipitated lemon pectinic acid yielded 85% anhydrouronic acid, and a molecular weight of 35,000 based on an intrinsic viscosity of 5.5. These materials both have high molecular weights and high anhydrouronic acid contents and precipitate quantitatively from aqueous solution with cupric ion.

Under the conditions of this experiment, citrus polygalacturonic acid hydrolyzed completely to dialyzable fragments, whereas the polygalacturonase-treated apricot pectic acid yielded an undialyzable residue containing 24% anhydrouronic acid. If the nonuronide sugars were not part of the apricot pectic acid molecule, it would have been hydrolyzed to dialyzable galacturonic and oligouronic acids, leaving only the nonuronide carbohydrate residues.

Copper-purified apricot acid was subjected to paper electrophoresis in an attempt to show the presence of polymers such as araban and galactan. A 1% solution was applied on a sheet of Whatman 3MM filter paper as a line in the center of a 40-cm. filter paper strip, which had been previously immersed in buffer solution and blotted. The moist paper strip was mounted between glass plates and subjected to a potential of 300 volts for 6 hours. The carbohydrate materials moved in a broad band 10 cm. in width and showed no separation. Attempts to make a separation in several different buffer solutions gave similar results.

The apricot pectinic acid was further tested for homogeneity by attempted fractionation of the acetate. Eight grams of apricot pectinic acid yielded 9 grams of acetate by the method of Carson and Maclay (*6*). Fractionation was done by using 7.5 grams of acetate dissolved in 500 ml. of chloroform. Skellysolve B was added in 50-ml. increments, and the precipitate when formed was centrifuged. The solvent was decanted and the residue dried in vacuo. Fractions were collected after addition of ten 50-ml. increments of solvent and a residue of 0.45 gram remained dissolved. Results are summarized in Table III. Hydrolysis and chromatography of these fractions failed to show that a fractionation of molecular species had been made. Galacturonic acid and the nonuronide sugars occurred in the same proportions as in the original acetate. The optical rotation of combined fractions 2, 3, and 4 was +134; fractions 5 and 6, +166; fractions 8 and 9, +132. The residue gave a rotation of +124. The very small changes in rotation and the fact that there was no trend in the rotation changes indicated that no major fractionation of molecular species was accomplished. It appears that the fractionation was made on the basis of molecular weight.

Discussion

There is little doubt that pectic substances examined here and elsewhere contain polymerized nonuronide sugars. Determination of their mode of linkage has not yet been established, because pectic substances are difficult to purify and labile to alkali, resist exhaustive methylation, and are associated with araban, galactan, and other polysaccharides. An exception was the pectin from sunflower heads, which was extracted with oxalate and proved to be a pure galacturonan (*2*). The oxalate procedure was applied to apricots, but the resulting pectic substances contained nonuronide sugars in proportions comparable to those extracted by other means. Studies of polymers such as the copper-soluble fraction high in nonuronide sugars may permit the characterization of these materials.

Many researches reported nonuronide sugars among the hydrolysis products of pectic substances other than the four identified in this work. The composition of pectic substances from various plants appears to be more complex than results of analysis made on the materials shown here. There were traces of unidentified reducing substances among the hydrolysis products of several of the pectinic acids shown in Table I and the nonuronide composition of these selected pectinic acids as they occur in plants is by no means settled.

Mucilages contain all of the constituents found in these pectic substances and traces of a number of other sugars. Some of the so-called pectic substances might be inseparable from mucilages, and the reverse may also be true. The definitions do not describe the carbohydrate polymers as they occur in plants, and polymers containing from high to low contents of galacturonic acid occur.

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