DIFFERENCES IN THE NATURE OF NITROGEN PRECIPITATED BY VARIOUS METHODS FROM WHEAT LEAF EXTRACTS

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SUMMARY

1. Four methods of precipitating proteins from leaf extracts were compared.

2. During slow heat coagulation, much nucleic acid is lost, probably from the activity of leaf RNase during heating.

3. After rapid heat coagulation some nucleic acid remains uncoagulated.

4. TCA and perchloric acid behave similarly and precipitate N which consists of protein N as well as nucleic acid N.

5. Uranyl TCA precipitates some TCA soluble polynucleotides and also renders some lipoprotein–polynucleotide complex insoluble in fat solvents.

INTRODUCTION

ROUELLE[1] first demonstrated the presence of proteins in leaves by separating them from a juice of hemlock by heat coagulation, but systematic work on leaf proteins began about 1920 when OSBORNE AND WAKEMAN[2] and CHIBNALL AND SCHRYVER[3] separated crude protein preparations from extracts of spinach and cabbage leaves, respectively. For the next 20 years flocculation by heat, ethanol or by acids like HCl and acetic acid was commonly used to separate proteins from leaf extracts, but of various precipitating reagents tried later, TCA was preferred and heat coagulation and TCA precipitation are now most used.

CHIBNALL AND GROVER[4] used HCl to adjust the pH of leaf extracts and showed that leaf proteins are least soluble between pH 4.0 and 5.0, although they are again precipitated when the pH is taken below 2.5. Their observations have been confirmed with many species of plants. When acids like HCl and acetic acid are used to precipitate leaf proteins, the pH of extracts has to be adjusted accurately, whereas this is not necessary with TCA. Proteins are precipitated from leaf extracts within the wide range of 1–15 % (w/v) final TCA concentration.

The proteins precipitated from leaf extracts by different methods are not necessarily the same. SLADE AND BIRKINSHAW[5] and PIRIE (unpublished) found that precipitates of some leaf extracts by TCA contained 10–20 % more nitrogen than did heat coagula of similar extracts. I have also observed 6–15 % more N in TCA precipitates from leaf extracts of several species of plants than in heat coagula. The

Abbreviations: TCA, trichloroacetic acid; NA-P, nucleic acid-Phosphorus; NP, nucleoprotein; RNase, ribonuclease.

observations do not suggest that age of leaves affects this difference, but the point has not been studied systematically.

As the different methods of precipitating proteins from leaf extracts seem not to have been studied in detail, the difference in the nature of N precipitated from leaf extracts of some species of plants was studied by four methods, viz. heat coagulation and precipitation by TCA, perchloric acid and a TCA reagent containing uranyl ions. The last two reagents were chosen for study because NEUBERG AND STRAUSS[6] suggested that perchloric acid was a better reagent than TCA and because a Uranyl TCA reagent[7,8] was preferred for studies on nucleic acid and nucleoprotein.

MATERIALS AND METHODS

Leaves (about 200 g) from field-grown wheat (*Triticum vulgare*) were minced in a domestic meat mincer and the sap was squeezed out by hand through a cloth. The residue was reminced and again squeezed. The two lots of sap were mixed and either used at once or stored at 4°. The mixture (extract) from leaves was diluted with 3 volumes of water for all experiments.

Fractionation of extract

The diluted extract was fractionated by differential centrifugation in a cold room. It was first centrifuged at 1,000 × *g* for 7 min and the supernatant fluid was re-centrifuged at 10,000 × *g* for 30 min. These values for centrifugation were chosen because previous work on tobacco leaf extracts made with buffered sucrose[9] showed that starch and intact chloroplasts are sedimented at 1,000 × *g* and chloroplast fragments and mitochondria at 10,000 × *g* leaving an almost clear supernatant fluid.

The sediments from 1,000 × *g* and 10,000 × *g* suspended in water (final volume 20 ml) are referred to as fractions I and II and the supernatant fluid as fraction III.

Precipitation methods

2.0-ml samples of a diluted extract or its fractions were used for precipitation of proteins. The precipitates and the coagulum were sedimented at 1,500 × *g* (10 min), washed with an appropriate medium and again centrifuged at 1,500 × *g* (10 min).

The samples were heat coagulated by leaving them in a boiling water-bath for 5 min, then they were rapidly cooled, centrifuged and washed with water. The precipitation with acid reagents was done by adding to the samples (a) 1.0 ml of 15 % (w/v) TCA, (b) 0.5 ml of 1 *N* $HClO_4$ and (c) 2.0 ml of a Uranyl TCA reagent containing 0.5 % (w/v) uranyl nitrate in 2.5 % (w/v) TCA solution. The precipitated samples were left at 4° for about 1 h and then centrifuged and washed respectively with (a) 5 % TCA, (b) 0.2 *N* $HClO_4$ and (c) the Uranyl TCA reagent diluted with an equal volume of water.

Fractionation of phosphorus compounds of extract

The method, HOLDEN[10] described for fractionating phosphorus compounds of leaf tissues was used with certain modifications.

After precipitation of the extract, the supernatant fluid and the fluid used for washing were pooled, diluted to a suitable volume with 2 *N* H_2SO_4 and a part was taken to estimate total P which is referred to as "soluble P".

Before proceeding with ethanol–ether extraction, the heat coagulum was extracted with 2.0 ml of cold 0.2 N $HClO_4$ and the whole of this extract was used to estimate what will be called "acid soluble P". This cold acid treatment facilitates complete extraction of phospholipids with ethanol–ether[11] and also eliminates interference by non-nucleic acid contaminants when total nucleic acid-phosphorus is estimated spectrophotometrically[12].

Ethanol–ether extraction was done with four successive 2.5-ml lots of ethanol–ether (3:1, v/v), which were pooled and the whole used to estimate total P, referred to as "ethanol–ether soluble P".

This was followed by a single step extraction of total nucleic acid by boiling the residue with 5.0 ml of 1 N $HClO_4$ in a water-bath for 20 min[12]. After separating the insoluble residue by centrifugation, it was washed with 5.0 ml of 1 N $HClO_4$ and again centrifuged. The extract and wash were pooled and used for spectrophotometric estimation of nucleic acid-phosphorus, referred to as "total NA-P".

Chemical estimations

Protein N was determined by a micro-kjeldahl method.

Total P was estimated by the method of HOLDEN AND PIRIE[13] in the precipitates obtained from the extract by various methods. Similarly it was estimated in all the fractions of phosphorus compounds of the extract, except the nucleic acid fraction in which NA-P was measured spectrophotometrically by the method of SPIRIN[12].

RESULTS

Precipitates produced from the extract of wheat leaves by TCA contained 10–15 % more N than did precipitates produced by heat coagulation. The extract was fractionated by differential centrifugation to find which fraction contained most of the "extra N" precipitated by TCA. The fractions and samples of original extract were incubated at 37° for 2 h when their N content was measured and compared with that of unincubated samples and Table I gives the results of one such experiment.

TCA precipitated more N than did heat coagulation from all the fractions but the difference was greatest with fraction III. Less N was precipitated by both techniques from each fraction after incubation than before, but the dimunition was greater with the TCA precipitation than with heat coagulation. Hence the two forms of precipitation gave precipitates that differed less after incubation than before. This suggests that heat coagulation promotes an enzyme action that proceeds slowly during incubation. The two most obvious possibilities are nuclease action and protease action.

PIRIE[14] isolated a nucleoprotein from the supernatant fluid of a tobacco leaf extract and as most of the "extra N" of a TCA precipitate of the wheat-leaf extract also occurs in a similar supernatant fluid, so the total nucleic acid in the two types of precipitate was studied. Table II shows the total NA-P in the fresh and incubated samples both of the unfractionated extract and its fractions.

TCA precipitates contained more NA-P than heat coagula and the difference was again greatest with fraction III. After incubation of the samples, less NA-P was precipitated by both techniques and again the loss was greater in the precipitates produced by TCA.

TABLE I

N-CONTENT OF HEAT AND TCA PRECIPITATES OF WHEAT LEAF EXTRACT AND ITS FRACTIONS

N was determined in a heat coagulum and a TCA precipitate of 2.0-ml samples of wheat leaf extract, diluted four fold, and its fractions and also in similar precipitates of samples incubated at 37°. The values given in the table have been calculated for undiluted extract.

Sample	*Period of incubation at 37° (h)*	*N content of*		*Extra N in TCA precipitate (%)*	*Loss of N during incubation*	
		Heat coagulum (g/l)	*TCA precipitate (g/l)*		*Heat coagulable (%)*	*TCA precipitable (%)*
nfractionated extract	0	2.00	2.25	11	—	—
	2	1.75	1.83	4	9	19
Fraction I	0	0.29	0.32	8	—	—
	2	0.26	0.27	4	11	15
Fraction II	0	0.6	0.65	8	—	—
	2	0.54	0.56	4	11	14
Fraction III	0	1.12	1.29	15	—	—
	2	1.02	1.1	7	9	15

TABLE II

TOTAL NUCLEIC ACID PHOSPHORUS CONTENT OF HEAT AND TCA PRECIPITATES OF WHEAT LEAF EXTRACT AND ITS FRACTIONS

Total NA-P was measured in a heat coagulum and a TCA precipitate of 2.0-ml samples of wheat leaf extract, diluted four fold, and its fractions and also in similar precipitates of samples incubated at 37°. The values given in the table have been calculated for undiluted extract.

	Period of incubation at 37° (h)	*NA-P content of*		*Extra NA-P in TCA precipitate (%)*	*Loss of NA-P during incubation*	
		Heat coagulum (g/l)	*TCA precipitate (g/l)*		*Heat coagulable (%)*	*TCA precipitable (%)*
ıfractionated extract	0	0.037	0.148	300	—	—
	2	0.015	0.018	20	60	88
Fraction I	0	0.01	0.017	70	—	—
	2	0.006	0.0065	8	40	62
Fraction II	0	0.0045	0.012	166	—	—
	2	0.004	0.0045	12	11	62
Fraction III	0	0.0225	0.12	433	—	—
	2	0.0095	0.0105	10	53	91

A comparison of Tables I and II shows that the loss of 0.42 g of N/l during incubation is accompanied by a loss of 0.13 g of nucleic acid-P. Taking N:P ratio in nucleic acid as 15.3:8.0 (see ref. 15), this loss of nucleic acid-P accounts for 0.24 g of N *i.e.* about half of the N lost when the extract is incubated. Evidently N compounds other than nucleic acid are also lost and loss by protease action is an obvious possibility. TRACEY[16] found more protease in wheat leaves than in the leaves of most other species of plants; I shall discuss in a later paper the autolysis that this brings about.

Fractionation of phosphorus compounds of the extract after precipitation

Table III shows the P content of various fractions of phosphorus compounds of the extract, when samples were precipitated by heat and TCA. There was more than

TABLE III

FRACTIONATION OF PHOSPHORUS COMPOUNDS OF EXTRACTS PRECIPITATED BY HEAT AND TCA

Fractionation of phosphorus compounds of 2.0-ml samples of wheat leaf extract, diluted four fold, after precipitation by heat and TCA was done by the method described in the experimental section. The values given in the table have been calculated for undiluted extract.

Fractions	*Phosphorus in various fractions after precipitation*	
	by heat (g/l)	*by TCA (g/l)*
Soluble P	0.59	0.5
Total P in precipitate	0.076	0.165
Acid soluble P	0.004	—
Ethanol–ether soluble P	0.02	0.025
Nucleic acid-phosphorus (NA-P)	0.053	0.138

twice as much P in a TCA precipitate as in a heat coagulum and almost all of this "extra P" was present as "extra NA-P". This "extra P" shows as an increase in "soluble P" after coagulation of the extract by heat, but after heating it was no longer in the form of nucleic acid precipitable by TCA.

Enzyme inactivation by boiling

The enzymic digestion of nucleoprotein during heating was kept to a minimum by using the technique of heat coagulation described by PIRIE[14] to make stable nucleoprotein. The extract was added directly to boiling water in such a way that the boiling was continuously maintained during the addition and for 2 min afterwards. Total NA-P was measured in the coagulum produced in this way and in a TCA precipitate of the supernatant fluid after separating the coagulum. Table IV gives the results of one such experiment with values for a TCA precipitate and an ordinary heat coagulum, for comparison.

TABLE IV

TOTAL NUCLEIC ACID-PHOSPHORUS CONTENT OF A COAGULUM OF EXTRACT OBTAINED BY BOILING COMPARED WITH THAT OF A TCA PRECIPITATE AND AN ORDINARY HEAT COAGULUM

2.0-ml samples of wheat leaf extract, diluted four fold, were precipitated (a) by TCA, (b) by adding the extract direct to boiling water and (c) by heating the sample in a water-bath. Total NA-P was measured in the precipitates thus obtained and also in the TCA precipitates of the supernatant fluids got from the two types of heat coagulation. The values given in the table have been calculated for undiluted extract.

Methods of precipitation	*Total NA-P in*	
	precipitate (g/l)	*supernatant fluid (g/l)*
TCA precipitation	0.183	—
Coagulation by boiling	0.108	0.06
Coagulation by heating in a water-bath	0.063	0.006

The coagulum obtained by immediate boiling not only contained 70 % more NA-P than the ordinary heat coagulum, but the supernatant fluid from it also contained nucleic acid in a form precipitable by TCA, and in an amount comparable to that present in the ordinary heat coagulum. The total NA-P in the boiled coagulum and the supernatant fluid together equals about 90 % of that precipitable by TCA.

Precipitation by perchloric acid and Uranyl TCA

Perchloric acid and Uranyl TCA precipitated as much protein N as did TCA. The amount and nature of P precipitated by perchloric acid and TCA were almost similar, but the Uranyl TCA precipitate contained about 12 % more total P and about 25 % more NA-P than did a TCA precipitate. There was also 40 % less ethanol–ether soluble P in the Uranyl TCA precipitate than in a TCA precipitate.

DISCUSSION

The facts that a TCA precipitate contains more N than a heat coagulum and that more TCA precipitable N is lost when extracts are incubated, are indications that the two precipitates are of a different nature. As much of the "extra N" of TCA precipitates from the extract occurred in the supernatant fluid after removing the chloroplast fraction and as PIRIE[14] isolated a NP from a similar supernatant fluid of tobacco leaf extract, it seemed likely that more NP was precipitated by TCA than by heat. This expectation was confirmed by studying the nucleic acid content and more conclusively, by studying the phosphorus compounds of the two types of the precipitate.

This posed the question: Why is less NP precipitated by heat? TCA precipitation was done at 4° and the pH of the extract shifted from about 6 to that between 0.5 and 1.5, and under these conditions enzymic activity is presumably impossible. HOLDEN AND PIRIE[13] found no leaf RNase activity below pH 2.0 and PIRIE[17] showed that, at 0°, TCA precipitates of nucleoproteins do not show leaf RNase activity even in presence of citrate ions which are the most effective activators of this enzyme. But leaf RNase is not inactive when the leaf extract is coagulated by heating in a water-bath.

Nucleases are closely associated with leaf NP (see ref. 14) and leaf RNase survives heating at 53° for 3 min, complete inactivation occurring only at 88° (see ref. 13). Therefore the leaf RNase could hydrolyse considerable amounts of nucleic acid while the extract was being heated in the water-bath.

Pancreatic RNase[8] leaves a "resistant core" of nucleic acid precipitable by TCA, but leaf RNase renders all nucleic acid unprecipitable by TCA[14,18]. No nucleic acid precipitable by TCA was, therefore, released in a soluble form when the extract was coagulated in a boiling water-bath. However, when the extract was added directly to boiling water, the enzyme was quickly inactivated and some TCA precipitable nucleic acid was released in a soluble form because nucleoprotein was partly disrupted by heat[17,19].

TCA and perchloric acid behave similarly as precipitants of leaf proteins, but Uranyl TCA seems to precipitate some of the TCA soluble polynucleotides and to render some lipoprotein–polynucleotide complex insoluble in ethanol–ether. VENKATARAMAN AND LOWE[20] suggested that a lipoprotein–polynucleotide complex passes

into ethanol phase because rat liver NP becomes partially soluble after precipitation by cold TCA. Probably some leaf NP may also dissolve in ethanol–ether after it is precipitated by cold TCA, but not after precipitation is done by uranyl TCA.

TCA-precipitated N consists not only of protein N, but also of nucleic acid N and the amount of N precipitated by TCA varies with the interval elapsing between making the leaf extract and doing the precipitation.

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