

# On Citromycetin, a New Yellow Colouring Matter Produced from Glucose by Species of Citromyces

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*Studies in the Biochemistry of Micro-organisms.*PART XI.—*On citromycetin, a new yellow colouring matter produced from glucose by species of Citromyces.*

By ARTHUR CLEMENT HETHERINGTON and HAROLD RAISTRICK.

The genus *Citromyces* WEHMER comprises a number of species which were considered by WEHMER to be unique in the fact that they produce considerable quantities of citric acid by the fermentation of sugar. Because of this, WEHMER gave to the genus the name of *Citromyces*. The genus is closely related to *Penicillium* and *Aspergillus*, and has in fact been dropped by THOM in his recent book on the genus *Penicillium* (1929). THOM prefers to regard all species previously regarded as *Citromyces*, as species of *Penicillium* (see Part IV).

Physiologically the genus *Citromyces* was considered to be distinguished from other genera by the production of citric acid, but more recent work has shown that not only is the production of citric acid a fairly common characteristic of other genera, particularly of *Aspergillus* and *Penicillium*, but that, quantitatively, certain species which are not *Citromyces* give much larger yields of citric acid.

During the work of preparation of carbon balance sheets for a number of species of moulds, balance sheets were prepared for a few species of *Citromyces* and seemed to have a certain amount of interest (see Part IV). The history of the species used in the work described in this paper is as follows :—

1. *Citromyces* sp. Catalogue No. Ad. 6. Obtained from the National Collection of Type Cultures, Lister Institute, via Mr. F. T. BROOKS, Cambridge.
2. *Citromyces* sp. Catalogue No. Ad. 7. Isolated at Ardeer.
3. *Citromyces* B. WEHMER, Catalogue No. Ad. 67. Purchased from the National Collection of Type Cultures, Lister Institute, No. 606.
4. *Citromyces glaber* WEHMER. Catalogue No. Ad. 68. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.
5. *Citromyces Pfefferianus* WEHMER. Catalogue No. Ad. 69, also purchased from Baarn.

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In view of the fact that an interesting and new biochemical product was obtained from all the above five species we considered it desirable to obtain expert opinion as to the authenticity of these cultures. For this reason we sent sub-cultures to Professor CARL WEHMER, of the Technical High School, Hanover, the originator of the genus *Citromyces*, and to Professor PHILLIP BIOURGE, of the University, Louvain.

Professor WEHMER, to whom we sent each of the above cultures, together with three other cultures of *Citromyces*, i.e., Catalogue Nos. Ad. 21, Ad. 71 and Ad. 73, wrote :

“ Ihre *Citromyces*-Formen sind hier microscopiert, auch auf Zuckerlösung mit Mineral-salzen, einige ebenfalls auf Gelatine, Agar und Kartoffeln cultiviert.

“ Das Resultat ist wenig ermutigend, die Masse für Sporen, Köpfchen, Sporenträger mit Sterigmen sind nahezu oder ganz übereinstimmend, sodass ich mich nicht getraue, den einzelnen Formen bestimmte Speciesnamen zu geben. Es mag sein, dass man bei detailliertem Studium gewisse feine morphologische Unterschiede feststellen kann, dazu bedarf es aber langer Arbeit. In wie weit sich culturelle und chemisch-physiologische Differenzen verwerten lassen, muss ich dahingestellt lassen, ob sie zu einer Species-Trennung in dieser Gattung berechtigen, wäre eine weitere Frage. Man müsste auch das Verhalten bei höherer Temperatur, bei Luftmangel, etc., feststellen bez. kennen. Beim Wachstum auf Zuckerlösung ergeben sich 2 Gruppen, die einen verfärben diese auch nach längerer Zeit kaum (7, 21, 68 u.a.), die anderen bilden gelbliches *Pigment* ; Deckenunterseite jener bleibt farblos. Die Säurebildung kann bei ein und derselben Form erheblich variiren, sodass bei Kreidegegenwart auf 15 Prozentiger Saccharose-Lösung mit Nährsalzen bald wenig, bald reichlich Calciumcitrat erscheint.

“ Ich bin der Meinung, dass die geschickten 8 Culturen mehrfach identisch sind und sich vielleicht auf 2 verschiedene Formen oder Arten verteilen lassen ; natürlich ist das nur eine *Schätzung* auf Grund des von mir Gesehenen, sie bedürfte genauerer Prüfung.

“ Im übrigen handelt es sich bei allen 8 um notorische *Citromyces*-Formen, auch Ad. 7 und Ad. 21 sind solche ; um diese hatten Sie speciell angefragt.”

Professor BIOURGE, to whom we sent each of the above five cultures together with *Citromyces* cultures Ad. 71 and Ad. 73 wrote :—“ Ad. 7 est identique à l'espèce reçue de Prof. Pollacci, Istituto di Botanica della R. Università di Pavia!cette année même sous l'étiquette *Citromyces Pfefferianus*.

“ Votre *Citromyces Pfefferianus* Ad. 73 est identique à mon *Citromyces Pfefferianus*, No. 162, reçu de Pribram en 1911, très peu après la mort de Kral qui le tenait de Wehmer.

“ Le *Citromyces* B. WEHMER Ad. 67 est probablement le véritable. Il se caractérise par la formation de grains bruns insolubles. La saveur du liquide est citrique pure, pas amère comme celle de Ad. 6.

“ *Citromyces glaber* Ad. 68 fait très peu d'acide, mais le liquide n'est pas amer. L'exactitude de l'étiquette est simplement possible.

“ Ad. 71 *Citromyces lacticus* MAZÉ et PERRIER est très rapproché de *Citromyces Pfefferianus* (Kral-Pribram). Il est moins actif. Le revers de thalle sur liquide sucré est d'un

orangé plus marqué dans les replis. Ce sont des différences de détail, paraissant négligeables.

“ En résumé :—

Ad. 6 = Voisin de *Citromyces Pfefferianus* de Pavie.

Ad. 7 = *Citromyces Pfefferianus* de Pavie non de Wehmer.

Ad. 67 *Citromyces* B. WEHMER = probably genuine.

Ad. 68 *Citromyces glaber* WEHMER = *Citromyces glaber* WEHMER probably.

Ad. 69 *Citromyces Pfefferianus* Baarn = *Citromyces Pfefferianus* de Pavie pas de Wehmer.

Ad. 71 *Citromyces lacticus* = *Citromyces Pfefferianus* WEHMER (?).

Ad. 73 = *Citromyces Pfefferianus* WEHMER !

“ Votre citromycétine est donc tirée de tous *Citromyces*. Soyez en paix sur ce point.”

We wish to express our indebtedness to both Professor WEHMER and Professor BOURGE for their kindness in examining our cultures.

The salient features of the balance sheets of the five species Ad. 6, Ad. 7, Ad. 67, Ad. 68 and Ad. 69, which are given in detail in Part IV, are the following :—

TABLE I.—Principal data in carbon balance sheets of five species of *Citromyces*.

Species.	Catalogue No.	Experiment No.	Carbon unaccounted for.	Carbon in non-volatile acids.	Carbon in Volatile Neutral Compounds.	Carbon in Synthetic Compounds.	Increase in Acidity, c.c. N/1 per 250 c.c.
			gm.	gm.	gm.	gm.	c.c.
<i>Citromyces</i> sp. ...	Ad. 6	134	0·620	0·345	0·126	0·191	8·4
<i>Citromyces</i> sp. ...	Ad. 7	F 5	0·566	0·402	0·101	0·218	6·6
<i>Citromyces</i> B. ...	Ad. 67	F 74	0·686	0·304	0·208	0·036	4·1
<i>Citromyces glaber</i> WEHMER	Ad. 68	F 75	0·393	0·341	0·059	0·093	7·6
<i>Citromyces Pfefferianus</i>	Ad. 69	F 76	0·567	0·315	0·071	0·090	7·9

NOTE.—The values given for the different classes of carbon in columns 4, 5, 6 and 7 are expressed in the same manner as in the carbon balance sheets given in Part IV, i.e., in gm. of carbon per 250 c.c. of medium.

Consideration of the balance sheets shows that all these species produce considerable amounts of acid and of “ carbon unaccounted for,” and in two cases, at any rate, of “ synthetic carbon.” They are distinguished biochemically, however, by the very remarkable reaction that culture solutions, on which any of these species have grown, give with ferric chloride solution. On the addition of a few drops of ferric chloride to the culture fluid from a mature culture on CZAPEK-DOX 5 per cent. glucose solution, a very intense greenish-black coloration is obtained, and provided that the culture has been sufficiently

active, there is a copious deposition, on standing, of a dark brown, amorphous, iron precipitate. This reaction is so intense that solutions from an active culture will still give a very strong reaction even after diluting to 100 times the original volume.

The work described in this paper deals with the isolation of the fermentation product responsible for this coloration and with its chemical investigation. This product has been isolated in the form of a yellow crystalline compound to which it is proposed to give the name "citromycetin," and it may be said safely that this product is more typical of, and specific for, the genus *Citromyces* than is citric acid, since, of several hundred cultures of different species from different genera, only a few species of *Citromyces* produce citromycetin, or give the colour reaction which is typical of it. The colour reaction is not given by all species of *Citromyces*, and it varies in intensity in those species that give a positive reaction.

*Influence of different factors on the yield of citromycetin.*

No prolonged effort has been made to define optimum conditions for the production of citromycetin. It is important, therefore, to note that the yields given are not by any means as large as are probably obtainable.

From time to time, however, various isolated observations have been made, and these, while necessarily incomplete, are given as indications of the lines on which work might be carried out if it were desired to increase the yield of material.

(a) *Influence of aeration.*—A copious supply of air is necessary for the production of citromycetin. It was first noticed in the metabolism experiments, where aeration was strictly controlled and was very limited, that the colour reaction with ferric chloride, of any of the *Citromyces* examined, was very much less intense than with the same culture grown in flasks plugged with cotton wool. Analysis of the air contained in the respective flasks showed that, while in the metabolism flasks practically all the oxygen had disappeared on standing overnight, those flasks which were plugged with cotton wool had usually a copious supply of oxygen.

The effect of aeration was also shown in a very marked manner in some of the earlier large scale experiments. Erratic results were often obtained, and in the light of later experience it seems probable that these were due to unequal aeration of the twelve trays since, because of the method of aeration adopted, it was impossible to ensure uniform aeration of all the trays. Thus it was noticed several times that, of the twelve trays in the same tank (sterilizer-incubator), all of which contained equal amounts of culture medium and had been sown with equal amounts of the same culture, some gave satisfactory yields of citromycetin while others gave scarcely any. (See Table V.)

(b) *Effect of concentration of iron salts in the medium.*—The CZAPEK-DOX medium used throughout all the metabolism experiments contained 0.001 per cent. of ferrous sulphate. Comparative experiments with media identical in all respects except that they contained varying amounts of ferrous sulphate showed that the yield of citromycetin,

judged by its colour reaction with ferric chloride, increases with the amount of ferrous sulphate in the medium up to a maximum of 0.02 per cent. and after that decreases with higher concentrations of ferrous sulphate.

(c) *Influence of the nature of the containing vessel.*—While it is evident that the erratic results obtained with the different trays in one and the same tank were probably largely caused by inequality of aeration, it was felt that this was not the only explanation. The trays in which the mould was grown in the tank were made of iron covered with enamel. After being in use for some time these trays showed signs of pitting. The influence of the nature of the trays on the yield of citromycetin was shown conclusively by comparing the yields obtained in these enamelled trays with those given in fused quartz trays of the same size obtained from the Thermal Syndicate, Ltd. ("Vitreosil"). The results are summarized in Table V under tanks 11 and 12. In these experiments six enamelled trays were used and six silica trays. These were all enclosed in the same tank and every effort was made to keep all other conditions constant. Not only was the yield of citromycetin uniformly larger in the silica trays than in the iron trays but the quality of product obtained was very much superior.

(d) *Influence of temperature of incubation and source of carbon.*—In all the large scale experiments the tank was placed in a room the temperature of which fluctuated very considerably. The results of experiments about to be described show that this would have a marked effect on the yield of citromycetin obtained.

Five litres of medium were made up of the same composition as that described on p. 215 with the exception that no glucose was added to it at this stage. The medium was divided into two halves and to one half was added 125 gm. of pure glucose (5 per cent.) while to the other half was added 125 gm. of Price's pure glycerol. These two media were now tubed in 10 c.c. quantities and sown with a spore suspension of *Citromyces* sp. Ad. 7. Forty tubes of each medium were then incubated at a series of different temperatures. These temperatures which, with the exception of room temperature, fluctuated very little, were taken three times per day during the course of the experiment, and the following are the averages of these figures :—

- (1) Room temperature = 14.7 °C ; (2) 23.5 °C ; (3) 27.5 °C ; (4) 29.9 °C ; (5) 31.8 °C ;
- (6) 36.9 °C.

The average temperature of the room in which the tank was incubated over the same period was 23.8 °C., but this temperature fluctuated over a much wider range than any of the temperatures given above.

Four tubes of each medium, at each temperature, were examined at suitable intervals and the amount of citromycetin produced was estimated by the method given on p. 232. This method was applied to the glucose set of tubes exactly as is given on p. 232. With the glycerol set of tubes all that was found necessary was an iodine estimation, since alkaline iodine was shown to have no appreciable oxidising effect on pure glycerol. The results are summarized in the following tables.

TABLE II.—Production of citromycetin from a glycerol medium at different temperatures.

Average Temperature of Incubation.	Iodine Absorption Figure in c.c. of N/10 Iodine per 100 c.c. Medium after Number of Days' Incubation indicated at Head of each Column.									
	6 days.	8 days.	12 days.	14 days.	16 days.	20 days.	23 days.	26 days.	30 days.	35 days.
Room temp.	25.5	33.3	72.0	100.3	154.8	199.3	248.0	273.0	275.5	286.3
23.5° C.	26.3	51.5	128.8	152.3	175.5	207.3	208.3	204.5	194.0	207.3
27.5° C.	29.8	47.3	96.3	96.8	103.0	112.8	120.0	119.8	108.5	107.3
29.9° C.	32.3	55.5	89.8	96.5	116.5	124.3	119.3	114.0	115.3	124.5
31.8° C.	27.5	38.3	84.8	73.3	77.8	89.3	71.3	66.8	72.0	82.5
36.9° C.	—	39.8	33.0	36.3	37.0	36.8	37.3	38.5	34.3	48.8

TABLE III.—Production of citromycetin on glucose medium at different temperatures.

Average Temperature of Incubation.	Iodine Absorption Figure in c.c. of N/10 Iodine per 100 c.c. Medium after Number of Days' Incubation indicated at Head of each Column.							
	8 days.	12 days.	14 days.	16 days.	20 days.	23 days.	28 days.	35 days.
Room temp.	26.1	51.9	53.7	55.1	46.2	63.9	78.6	87.7
23.5° C.	35.1	59.7	51.1	61.7	84.1	108.1	119.7	104.8
27.5° C.	23.6	62.1	57.8	86.0	88.4	101.0	89.2	} No material available for analysis.
29.9° C.	44.2	70.1	77.3	86.9	106.1	105.2	96.4	
31.8° C.	8.8	28.6	27.7	28.8	37.0	33.4	24.6	
36.9° C.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	

These results indicate that not only is the production of citromycetin influenced very considerably by the temperature of incubation but that the optimum temperature is very different on a glycerol medium from that on a glucose medium. Thus with a glycerol medium the yield of citromycetin is at a maximum when the mould is cultivated at about 14.7° C. As the temperature increases the yield of citromycetin steadily falls. On the other hand, with a glucose medium the optimum temperature seems to lie between 23.5° C. and 29.9° C., and the yield decreases on either side of this range. It also appears that better yields of citromycetin may be expected from a 5 per cent. glycerol medium than from a 5 per cent. glucose medium. Hence in the later large scale experiments 5 per cent. glycerol was used as the medium.

Ferric chloride tests and acidities determined by titration to phenolphthalein were carried out at the same time as the estimations of citromycetin, and it was found that the three different estimations invariably ran parallel, *i.e.*, the larger the amount



This medium was made by dissolving all the constituents in the water, except the potassium phosphate, which was dissolved in a separate small portion of water and finally mixed with the main bulk. Five litres of this medium were distributed in each of the twelve incubating trays of the combined incubator-sterilizer, and during distribution, the liquid was kept stirred by a current of air in order to ensure uniform mixing of the precipitated phosphate. The apparatus was then closed and sterilized by steaming for an hour on each of three consecutive days. After cooling, each tray was sown with a suspension of spores from a beer wort agar Roux bottle culture of *Citromyces* sp. Ad. 7, which was found most suitable for the purpose. The spore suspension was made in sterile distilled water and introduced by means of a sterile pipette through the inoculation openings of the tank. The spores were then distributed over the surface of the trays by means of a sterile bent aluminium rod. After sowing, the mould was not further disturbed, and was incubated at an average temperature of 22°–24° C. The mould grew very quickly, and in successful experiments a dark green, heavily sporing, felt was established on all trays. During the course of incubation a continuous stream of sterile air was passed over the surface of each tray as it had been found to be essential in order to ensure good yields of material that the mould should be thoroughly aerated. From time to time samples were withdrawn and a measure of the quantity of citromycetin was obtained by the method given on p. 232. This figure slowly increased until it reached a maximum when practically all the glucose had disappeared, which usually happened in from 10 to 12 days.

At the end of this time the culture solution was filtered from the mycelium which was then thoroughly extracted by grinding with small amounts of water. The filtrate and filtered washings were then acidified with sulphuric acid (50 per cent. by volume), 100 c.c. of this concentration being added with vigorous stirring to the contents of one tank. On acidification, the culture solution, which was initially dark brown in colour, immediately became lighter and deposited a considerable quantity of a brownish black amorphous precipitate, the nature of which has not been investigated except to prove that it contains practically no citromycetin. This precipitate was filtered off and the clear orange yellow filtrate evaporated *in vacuo* at a low temperature. During the evaporation crude citromycetin separated in yellowish brown crystalline crusts. The whole solution was evaporated to about 3 to 5 litres and allowed to stand. Almost all the citromycetin separated out on standing and was filtered off, carefully washed and dried. This constituted the crude citromycetin. The mother liquors contain considerable quantities of citric acid which may be isolated as a calcium salt after removal of the sulphuric acid.

The results obtained in twelve tanks are summarized in Table V.

#### *Purification of crude citromycetin.*

The crude product contains, in addition to citromycetin, varying amounts of a brownish black tarry material which interferes considerably with the crystallisation.

TABLE V.—Details of “Tank” experiments for preparation of Citromycetin

Carbon Compound Fermented.	Period of Incubation.			Location of Tank.	Type of Tray used.	Residual Glucose by Polarimeter.	Estimated Citromycetin as c.c. N/10 Iodine per 100 c.c. Medium.
	Start.	Finish.	Duration in Days.				
Glucose ...	21.12.25	Tray 6 taken off 11.1.26 Remainder 18.1.26	21 28	Tank incubated in sterilizing room of old Biochemical Lab.	Enamelled iron	Per cent. 0·13	—
Glucose in 9 trays. Maltose in 3 trays.	29.1.26	3 trays on 9.2.26 5 trays on 10.2.26 4 trays on 11.2.26	11 12 13	Ditto	Ditto	0·2–0·6	—
Glucose ...	22.4.26	4 trays on 3.5.26 5 trays on 4.5.26 3 trays on 5.5.26	11 12 13	Ditto	Ditto	0·14–0·58	—
Glucose ...	8.5.26	3 trays on 18.5.26 4 trays on 19.5.26 5 trays on 20.5.26	10 11 12	Ditto	Ditto	0·11–0·35	—
Glucose ...	22.5.26	6 trays on 1.6.26 6 trays on 2.6.26	10 11	Ditto	Ditto	0·15–0·4	223·3
Glucose ...	24.12.26	2 trays on 6.1.27 3 trays on 8.1.27 1 tray on 10.1.27 3 trays on	13 15 17 19	Ditto	Ditto	nil–0·180 (average 0·095)	107–236 (average 164·3)

mycetin. Organism used—*Citromyces* sp. Ad. 7.

Estimated Citro-mycetin as c. N/10 dine per 100 c.c. medium.	Yield of Crude Citro-mycetin.	Remarks.
—	3.8 gm.	Tray 6 was the only tray of the whole 12 giving a reasonable ferric chloride reaction, and it was from the contents of this tray that the 3.8 gm. were obtained. None of the other trays gave a good reaction with ferric chloride and no citromycetin could be isolated from any of them. The temperature of the sterilizing room in which Tanks 1–10 were incubated fluctuated very widely ranging between 10°–26° C. in the earlier tanks and 20°–26° C. in the later tanks where an attempt was made to lessen the fluctuations.
—	125.7 gm. from 9 glucose trays. 50.5 gm. from 3 maltose trays.	The yield of citromycetin judged from the ferric chloride reaction varied considerably from tray to tray and although a considerable amount of crude material was isolated subsequent investigations showed that the content of citromycetin was not very large. No advantage was evident in using maltose as compared with glucose.
—	116.4 gm.	In this tank 0.02 per cent. of ferrous sulphate was used in the medium for the first time, Tanks 1 and 2 having only contained 0.001 per cent. It was noticed that the mycelium in all trays was covered with spore heads and was of a fairly uniform dark greenish colour. The results in different trays were still erratic and investigation showed that aeration had not been uniform. The quality of the crude citromycetin was very much superior to that obtained in Tank 2.
—	131.3 gm.	The yield of citromycetin in the different trays still varied very considerably although in this tank an attempt was made to secure uniform and ample aeration. The quality of crude citromycetin was quite good.
223.3	142.2 gm.	In this tank each tray was sown with the spores from two Roux bottles instead of the customary one. Even as early as the fourth day the mycelium of all trays visible was this time seen to be green. The trays were more uniform than previously. It was definitely shown by analysis that very little citromycetin is left in the acidified and evaporated solution after standing for some time.
07–236 (average 164.3)	84.6 gm.	Sowing consisted of one Roux bottle per tray. In this tank for the first and only time aluminium grids were placed in each of the trays to support the mycelial felt. The poor results and small yields of citromycetin appear to be due in part at any rate to the presence of aluminium grids, since considerable quantities of potash alum crystals separated from the con-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY OF BIOLOGICAL SCIENCES			8.1.27 1 tray on 10.1.27 3 trays on 12.1.27 3 trays on 14.1.27	17 19 21				
	Glucose ...	12.2.27	6 trays on 22.2.27 5 trays on 23.2.27 1 tray on 24.2.27	10 11 12	Ditto	Ditto	0.11 (average of 12 trays)	247.1 (average of (12 trays)
	Glucose ...	19.3.27	1 tray on 31.3.27 11 trays on 2.4.27	12 14	Ditto	Ditto	0.17 (average sample of all 12 trays)	120.0 (average of 12 trays)
	Glucose ...	2.4.27	12 trays on 18.4.27	16	Ditto	Ditto	See remarks	See remarks
	Glucose ...	29.4.27	12 trays on 10.5.27	11	Ditto (average tem- peratures 24°-26° C.)	Ditto	0.018 (average sample)	289.8 (average sample)
	Glycerol (Price's pure)	21.11.27	6 trays on 15.12.27  6 trays on 17.12.27	24  26	New Biochemical Lab. (Aver- age tempera- ture about 18° C.) Ditto	6 silica trays.  6 enamelled iron trays	—  —	400.2  186.9

		<p>cit. The poor results and small yields of citromycetin appear to be due in part at any rate to the presence of aluminium grids, since considerable quantities of potash alum crystals separated from the concentrated mother liquors on standing indicating that considerable erosion of the aluminium grids must have taken place.</p>
247.1 (average of 2 trays)	125.7 gm.	<p>The different trays gave more uniform results than previously with the exception of one tray in which growth was not quite so good as in the remainder. The quality of citromycetin obtained was quite good.</p>
120.0 (average of 2 trays)	70 gm.	<p>This tank gave entirely unsatisfactory results for which no explanation has been forthcoming, but the unsatisfactory nature of it is evidenced not only by the low yield of crude citromycetin isolated but also by the estimated figure with alkaline iodine.</p>
See remarks	25.5 gm. See remarks.	<p>In experiments 9-12 the new aluminium tank was used whereas in experiments 1-8 the old iron tank constructed in the factory had been used throughout. On opening the tank on 18.4.27 it was seen that only one tray had given a really typical growth with a characteristic colour reaction. Three others were fairly satisfactory but by no means good. These four trays were worked up in the usual way and gave a yield of 25.5 gm. of crude citromycetin. All the other trays gave little or no ferric chloride reaction and microscopic examination revealed the fact that they were all infected by extraneous organisms. These eight trays were therefore rejected and were not worked up. The cause of infection seems to have been splashing of the wash water from the air wash bottle into the trays.</p>
289.8 (average sample)	187 gm.	<p>This tank was uniformly good throughout and gave the best yields and best quality of material so far obtained. The method of aeration adopted was to leave the tank for three days after inoculation with cotton wool plugs in the sampling holes. Aeration was carried out simultaneously through the main perforated pipe and through the twelve side tubes. At no time were any of the trays aerated separately and aeration of all trays was continuous from 2.5.27 to 10.5.27. The rate of aeration was about 50 cubic feet per hour. The sowing consisted of a suspension of spores from six Roux bottle cultures on beer wort agar. These were half of twelve sown on 25.3.27, the other six having been used for Tank 9. The six cultures used for Tank 10 had been stored from 2.4.27 till the date of using in an unused incubator at room temperature with the necks of the Roux bottles plugged but uncovered.</p>
400.2	117.0 gm.	<p>In this tank six fused quartz trays referred to on p. 213 were used for the first time and in the other half of the tank six of the old enamelled iron trays were used. The results obtained with the silica trays were very much better than those obtained with the enamelled iron trays. The yield of citromycetin was almost twice as large, amounting for a full tank to the equivalent of 234 gm. The quality of the crude citromycetin was very much superior and the colour of the metabolism solution in the silica trays was a dark orange compared with a brownish black in the iron trays. This indicates that the mould is attacking and dissolving the enamel and the metal of this type of tray with deleterious results to the yield and quality of citromycetin. The appearance of the mycelium</p>
186.9	58 gm.	

2	Glycerol (Price's pure)	22.12.27	6 trays on 17.1.28 6 trays on 18.1.28	26  27	Ditto  Ditto	6 silica trays  6 enamelled iron trays	—  —	326·3  251·2

		trays. This indicates that the mould is attacking and dissolving the enamel and the metal of this type of tray with deleterious results to the yield and quality of citromycetin. The appearance of the mycelium at the end of the experiment is quite different from that usually obtained on a glucose medium. On glucose this mould spores freely giving a dark green felt, whereas in a glycerol medium the mycelium is practically white at the end of the experiment.
326·3	89·4 gm.	The results obtained with Tank 12 were very similar to those from Tank 11 except that the difference in yield between the silica and enamelled iron trays was not quite so marked.
251·2	70·9 gm.	

(Facing page 216.)

Various methods were tried to remove this tarry product and the two most suitable are described in the following methods of purification.

(a) 97 gm. of crude material from Tank 5, Fraction 5*b*, were dissolved in 1 litre of boiling 66 O.P. rectified spirit. The solution was cooled and while still slightly warm 3 litres of ether were added. This gave rise to a copious amorphous precipitate, consisting of the tarry material, which was filtered off after a few minutes. Weight of precipitate, 3.20 gm. The clear ether-alcohol solution was evaporated to about 500 c.c. when an equal volume of boiling water was added to the boiling solution and the mixture allowed to stand for two days. About 70 gm. of material separated in the form of dark yellow rhomboidal crystals. These were fractionally crystallised from 50 per cent. aqueous alcohol after treatment with a small quantity of charcoal. Crystallisation of the citromycetin was now very rapid, as it separated immediately on cooling in fine yellow needles. No other crystalline product was isolated, and the material was separated into four fractions of slightly decreasing purity:—

							Gm.
Fraction 1	..	..	..	..	..	..	38.8
Fraction 2	..	..	..	..	..	..	7.2
Fraction 3	..	..	..	..	..	..	14.6
Fraction 4	..	..	..	..	..	..	3.3

The final mother-liquors on evaporation gave an alcohol-soluble tar weighing 24.8 gm. from which further quantities of citromycetin could be extracted by dissolving it in alcohol and precipitating with a large volume of ether.

(b) The tarry matter may also be separated somewhat less thoroughly by taking advantage of the fact that it is more soluble in 50 per cent. aqueous alcohol than is citromycetin. This method can only be applied efficiently, however, if the proportion of tar in the crude citromycetin is not high. With very impure specimens of crude citromycetin, the whole product is soluble in small amounts of aqueous alcohol and, for purification, is better treated by method (a). Method (b) is carried out as follows:—

A quantity of crude citromycetin is ground in a mortar with sufficient hot 50 per cent. aqueous alcohol to convert it into a thin paste. This is then quickly filtered on a Buchner funnel and washed with small quantities of the same solvent. The filtrate is brownish black in colour while the colour of the insoluble material is changed from the original dark brown to a much lighter yellowish brown. If the colour of the product is not satisfactory it is again extracted by grinding with hot aqueous alcohol. Finally, it is recrystallised several times from 50 per cent. aqueous alcohol, from which it separates quickly, on cooling, in yellow needles.

It should be noted that the use of animal or blood charcoal for purification of citromycetin solutions should be avoided whenever possible since citromycetin is so easily adsorbed by all samples of charcoal that excessive loss is inevitable if this material is used.

*General properties of citromycetin.*

Citromycetin crystallises from 50 per cent. aqueous alcohol in lemon-yellow needles. It is fairly soluble in cold absolute alcohol, hot glacial acetic acid, is less soluble in acetone, very slightly soluble in boiling water, and almost insoluble in cold water. It does not contain nitrogen.

It does not give a very definite melting point. On heating it begins to darken in colour at 263° C., and it becomes progressively darker in colour until it is almost black and then melts and decomposes at about 283°–285° C.

It is readily soluble in aqueous solutions of sodium carbonate or bicarbonate, with evolution of CO<sub>2</sub> and the formation of a deep yellow solution. It is also soluble in aqueous solutions of potassium acetate. On acidifying an aqueous solution of citromycetin in carbonate or acetate a yellow gel is formed which, on standing, slowly crystallises in yellow needles. It is immediately soluble in sodium hydroxide, giving a yellow to brown coloured solution which darkens on exposure to air, obvious decomposition taking place.

Its aqueous or alcoholic solutions give a very intense olive green colour with ferric chloride, which changes on standing to a dark brown. It reduces ammoniacal silver nitrate in the cold, and BENEDICT'S copper solution on boiling. It yields a green fluorescent solution with concentrated sulphuric acid.

It is optically inactive. 0.6484 gm. of air-dried citromycetin (C<sub>14</sub>H<sub>10</sub>O<sub>7</sub>·2H<sub>2</sub>O) dissolved in the theoretical amount of N/10 sodium hydroxide to give a monosodium salt and made up to 100 c.c. gave no rotation in a 40 cm. tube.

It does not contain any methoxyl groups, as shown by a ZEISEL estimation.

Citromycetin does not appear to contain a normal-reacting ketonic group, as several attempts to prepare one or other of the usual ketonic derivatives failed. The only product which could be isolated from the action of phenylhydrazine on citromycetin was a salt of phenylhydrazine with citromycetin which immediately decomposed with excess of either alkali or acid. In order to avoid the acidity of citromycetin itself attempts were made to prepare derivatives of the methyl ester of *O*-dimethylcitromycetin (see p. 225) with (*a*) phenylhydrazine, (*b*) semicarbazide, (*c*) hydroxylamine, (*d*) hydrazine. Varied conditions and durations of boiling were tried, but in no case were any derivatives recovered except in one experiment where the ester was boiled with alcoholic hydrazine for 20 hours. In this case a small amount of reaction product was isolated but this was definitely proved to be neither a hydrazone of the methyl ester of *O*-dimethylcitromycetin nor of *O*-dimethylcitromycetin itself.

Citromycetin, when crystallised from 50 per cent. aqueous alcohol, contains two molecules of water of crystallisation. This water of crystallisation is not easily removed. Long continued drying *in vacuo* over concentrated sulphuric acid or phosphorus pentoxide only removes a portion of it, and in order to ensure complete removal it is necessary to dry the material for some hours at a temperature of 150° C. Figures obtained on drying are summarized along with the combustion results (*q.v.*).

Considerable difficulty was met with for some time in obtaining consistent combustion results. The reasons for this were :—

- (a) Difficulty of removing all the water of crystallisation from citromycetin.
- (b) Difficulty of freeing citromycetin from traces of the decarboxylated product.

Acid solutions of citromycetin on heating readily lose  $\text{CO}_2$  (see pp. 227 and 230) and this applies even to solutions to which acid has not been added, since the acidity of citromycetin itself is sufficient to cause a considerable amount of auto-decomposition. In the following combustion results these two sources of error have been eliminated as far as possible.

Three different samples were analysed and were purified as follows :—

(a) *By treatment with absolute alcohol.*—A sample of citromycetin which had been recrystallised several times from 50 per cent. alcohol was boiled with insufficient absolute alcohol to dissolve all of it. It was then filtered, refluxed for a few moments with charcoal, an equal volume of boiling water added, refiltered and the solution cooled quickly. The crystals which separated were again treated in a similar manner and were finally air-dried for analysis.

(b) *Via barium salt.*—A sample of citromycetin which had been several times crystallised from 50 per cent. alcohol was suspended in cold water and N/4 barium hydroxide added, with constant shaking, until about two-thirds of the material had dissolved. It was then filtered quickly and the clear filtrate immediately acidified with N/1 hydrochloric acid. Citromycetin separated quickly, first as a partial gel, which then crystallised completely in fine yellow needles. These were well washed and air-dried for analysis.

(c) *Via potassium salt.*—A quantity of the potassium salt of citromycetin was prepared according to the method given on p. 222. It was thoroughly extracted with boiling absolute alcohol, and 2.38 gm. were dissolved in the cold in 50 c.c. of water. This solution was filtered and to it were added 50 c.c. of cold alcohol containing the equivalent of 5 c.c. of N/1 hydrochloric acid. Citromycetin quickly crystallised, and was well washed and air-dried for analysis.

Before combustion all samples were dried to constant weight in a stream of dry, oxygen-free nitrogen at  $150^\circ \text{C}$ . By this means there was very little darkening in colour, whereas if the substance were dried in air at this temperature considerable darkening took place.

The following results were obtained on combustion of these dried samples. They agree with an empirical formula for citromycetin of  $\text{C}_{14}\text{H}_{10}\text{O}_7 \cdot 2\text{H}_2\text{O}$ . (Table VI, p. 220.)

The following attempts were made to determine the molecular weight of citromycetin :—

(a) *By the elevation of the boiling point of alcohol.*—0.2205 gm. of air-dried citromycetin was dissolved in 14.98 c.c. of absolute alcohol. Observed rise in boiling point of alcohol

TABLE VI.—Results of combustion of citromycetin.

Sample.	Weight of Air Dried Material taken.	Loss in Weight at 150° C.	Percent- age of H <sub>2</sub> O of cryst.	Weight of Sample (dried at 150° C.) analysed.	Weight, CO <sub>2</sub> .	Weight, H <sub>2</sub> O.	Percent- age, C.	Percent- age, H.
	gm.	gm.		gm.	gm.	gm.		
Purified according to method (a) ...	0·1637	0·0178	10·87	0·1459	0·3091	0·0468	57·78	3·59
Purified according to method (b) ...	0·1494	0·0174	11·64	0·1320	0·2810	0·0420	58·02	3·56
Purified according to method (c) ...	0·1503	0·0161	10·71	0·1342	0·2846	0·0420	57·83	3·59
Theoretical for C <sub>14</sub> H <sub>10</sub> O <sub>7</sub> ·2H <sub>2</sub> O	—	—	11·04	—	—	—	—	—
C <sub>14</sub> H <sub>10</sub> O <sub>7</sub> ...	—	—	—	—	—	—	57·92	3·47

= 0·107° C., corresponding to a molecular weight of 199·5 (theoretical for C<sub>14</sub>H<sub>10</sub>O<sub>7</sub>·2H<sub>2</sub>O = 326).

(b) *By Barger's capillary tube method*, BARGER (1904).—A solution of citromycetin in alcohol was compared by the above method against (1) a solution of vanillin in alcohol, and (2) a solution of dichloroaniline in alcohol. In each case a molecular weight of about 240 was observed.

The low figure for the molecular weight of citromycetin as obtained by methods (a) and (b) is probably caused by the ionisation of citromycetin in alcohol, since it is a strong acid.

(c) *By titration against sodium hydroxide*.—0·4728 gm. of air-dried citromycetin was suspended in a little water and titrated with N/10 sodium hydroxide to phenolphthalein. The sodium hydroxide was run in, with constant shaking, and the citromycetin slowly dissolved giving a bright yellow solution. The first colour change from yellow to orange was noticed after 28·33 c.c. of N/10 sodium hydroxide solution had been added, and a definite pink colour, confirmed by external indicator, after the addition of 28·78 c.c. These figures correspond to an equivalent for citromycetin of 166·8 and 164·3 respectively, and if one assumes that citromycetin is dibasic they give a molecular weight of 333·6 and 328·6 respectively. (Theoretical for C<sub>14</sub>H<sub>10</sub>O<sub>7</sub>·2H<sub>2</sub>O = 326.)

It is interesting to note that on the addition of N/10 hydrochloric acid to the titrated solution nothing visible occurred until after the addition of more than 14·4 c.c. of N/10 acid, indicating that the monosodium salt of citromycetin is readily soluble in water. On the addition of further N/10 acid citromycetin began to be precipitated and finally separated as a yellow gel which on standing shrank to a mass of yellow needles.

In a similar experiment with 0·4259 gm. of citromycetin dried to constant weight in

nitrogen at 150°C, part of the fraction purified via the barium salt (see p. 219) gave titration figures of 29·93 c.c. of N/10 sodium hydroxide to the first change and 30·01 c.c. to a permanent pink, corresponding to molecular weights of 284·6 and 283·9. (Theoretical for  $C_{14}H_{10}O_7 = 290$ .)

*Derivatives of citromycetin.*

(1) *Diacetyl compound*  $C_{14}H_8O_5$   $(O.CO.CH_3)_2$ .—The diacetyl derivative has been prepared by two different methods.

(a) By treatment of the potassium salt of citromycetin with acetic anhydride.

10·01 gm. of the dried potassium salt prepared from 10 gm. of citromycetin by the method described on p. 222 were mixed with 50 c.c. of acetic anhydride and allowed to stand overnight at room temperature. In the morning the potassium salt had completely dissolved. (On long standing in a sealed tube typical crystals of the acetyl derivative are deposited.) The solution was evaporated to complete dryness in a vacuum desiccator. The dried residue dissolved completely in 50 c.c. of water. Addition of dilute hydrochloric acid to this produced a heavy precipitate almost white in colour, which was filtered off, washed and dried. Weight = 10·18 gm. It was recrystallised from absolute alcohol, from which it separates in white prismatic crystals.

(b) By the usual method with acetic anhydride and sodium acetate.

5 gm. of citromycetin were heated with 5 gm. of anhydrous sodium acetate and 15 c.c. of acetic anhydride for one hour. The cooled residue was completely soluble in cold water, and this solution, on acidification with hydrochloric acid, gave a heavy white precipitate. This was dried and recrystallised from absolute alcohol. Yield of recrystallised material = 3·3 gm. of an acetyl compound identical with that prepared from the potassium salt. This substance had a melting point of 223°–224° C. (with decomposition). Although insoluble in water it is a strong acid which is readily soluble in aqueous solutions of sodium acetate or of potassium bicarbonate with evolution of carbon dioxide. Its alcoholic solution gives a pale yellow colour with ferric chloride.

The diacetyl compound shows a curious and so far unexplained behaviour on recrystallisation. It crystallises from absolute alcohol in typical “coffin lid” shaped crystals having a melting point of 223°–224° C. (with decomposition). On the other hand it separates from its solution in aqueous potassium acetate on acidification with dilute hydrochloric acid in needles having a melting point of 235°–236° C. (with decomposition). Neither of these forms contains any water of crystallisation and they are readily interconvertible, so that needle-shaped crystals (melting point 236° C.) on crystallising from absolute alcohol separate as “coffin lid” crystals with a melting point of 223° C., and these can be reconverted into the needle-shaped crystals by dissolving in aqueous potassium acetate and precipitating with dilute hydrochloric acid.

Combustions agree with the formula  $C_{14}H_8O_5$   $(O.CO.CH_3)_2$ , i.e.,  $C_{18}H_{14}O_9$ .

This material was dried to constant weight at 100° C. in air before combustion.

## Results of combustion of the diacetyl derivative of citromycetin.

Weight of Sample.	Weight of CO <sub>2</sub> .	Weight of Water.	Percentage Carbon.	Percentage Hydrogen.
0.1678	0.3556	0.0593	57.78	3.96
0.1555	0.3292	0.0548	57.74	3.94
Theoretical for C <sub>18</sub> H <sub>14</sub> O <sub>9</sub>	—	—	57.75	3.77

The compound was proved to be a diacetyl compound by determining the amount of acetic acid produced on hydrolysis. 0.4081 gm. was boiled for three hours under a reflux condenser with 30 c.c. of N/10 sulphuric acid and then heated for a further six hours on a steam bath. At the end of hydrolysis the acetic acid was distilled off by evaporation *in vacuo* until completely removed. The acetic acid produced was equivalent to 21.86 c.c. of N/10 sodium hydroxide. (Theoretical for C<sub>14</sub>H<sub>8</sub>O<sub>5</sub> (O.CO.CH<sub>3</sub>)<sub>2</sub> is 21.83 c.c.)

During hydrolysis the colour of the liquid rapidly became golden yellow, and a quantity of yellow needle crystals separated. These were composed of decarboxylated citromycetin, *i.e.*, of "citromycin" (see p. 227). At the same time carbon dioxide was evolved and was measured in one experiment as follows:—0.5040 gm. of diacetyl citromycetin was hydrolysed with 50 c.c. of 2N sulphuric acid on a boiling water bath for 17 hours. A constant stream of nitrogen was passed through the hydrolysis flask and reflux condenser and was bubbled through N/4 barium hydroxide, the excess of which was titrated at the end of the experiment with N/2 hydrochloric acid. Carbon dioxide equivalent to 5.21 c.c. of N/2 hydrochloric acid was formed. (Theoretical for 0.5040 gm. of diacetylcitromycetin — CO<sub>2</sub> ≡ 5.39 c.c. N/2 HCl). The products of hydrolysis of diacetylcitromycetin are thus two molecules of acetic acid, one molecule of carbon dioxide and one molecule of citromycin (see p. 227).

(2) *Potassium salt* (C<sub>14</sub>H<sub>9</sub>O<sub>7</sub>K.H<sub>2</sub>O).—The potassium salt is readily prepared by adding an alcoholic solution of citromycetin to an alcoholic solution of potassium acetate. 10 gm. of pure citromycetin were dissolved in 400 c.c. of boiling absolute alcohol, filtered, and to the boiling solution were added 100 c.c. of a filtered solution in absolute alcohol of 5 gm. of anhydrous potassium acetate. Crystallisation of the potassium salt commenced almost immediately. The potassium salt was filtered when cold, well washed with absolute alcohol and air-dried. Yield of potassium salt = 10.01 gm. The potassium salt crystallises in canary yellow needles, sometimes slightly curved, and showing a characteristic sprouting effect at the ends of the crystals.

0.6662 gm. of the air-dried potassium salt, dried to constant weight at 150° C. in nitrogen, lost 0.0356 gm. of water corresponding to 5.34 per cent. Calculated for C<sub>14</sub>H<sub>9</sub>O<sub>7</sub>K.H<sub>2</sub>O = 5.20 per cent. A sample of the potassium salt dried to constant weight in nitrogen at 150° C. gave the following combustion results.

Weight of Sample Analysed.	Total weight of CO <sub>2</sub>	Weight of Water.	Weight of Ash (K <sub>2</sub> CO <sub>3</sub> ).	Percentage Carbon.	Percentage Hydrogen.	Percentage Potassium.
0.1683 gm. ... ..	0.3178	0.0453	0.0351	51.50	3.01	11.79
0.1632 gm. ... ..	0.3087	0.0428	0.0344	51.60	2.93	11.93
Calculated for C <sub>14</sub> H <sub>9</sub> O <sub>7</sub> K. ... ..	—	—	—	51.20	2.76	11.91

0.6298 gm. dried to constant weight at 150° C. and ignited with concentrated H<sub>2</sub>SO<sub>4</sub> gave 0.1661 gm. K<sub>2</sub>SO<sub>4</sub> corresponding to 11.82 per cent. K.

(3) *Barium salt* (C<sub>14</sub>H<sub>9</sub>O<sub>7</sub>)<sub>2</sub>Ba.3H<sub>2</sub>O.—A weighed quantity (about 1 gm.) of citromycetin was suspended in 40 c.c. of cold water, and to this was added, with constant shaking, the calculated volume of N/4 barium hydroxide necessary to neutralise the weighed amount of citromycetin, on the assumption that this is a monobasic acid. Most of the citromycetin dissolved, and the solution was filtered as quickly as possible from the small residue of citromycin. On standing the barium salt crystallised from the clear filtrate in orange plates. These were filtered off, washed, dried and analysed.

0.1432 gm. of barium salt dried to constant weight in nitrogen at 150° C. lost 0.0102 gm. water, equivalent to 7.11 per cent. (Theoretical for (C<sub>14</sub>H<sub>9</sub>O<sub>7</sub>)<sub>2</sub>Ba.3H<sub>2</sub>O = 7.02 per cent.)

Samples of the barium salt dried to constant weight in nitrogen at 150° C. gave the following combustion results:—

Weight of Sample.	Total weight of CO <sub>2</sub>	Weight of Water.	Weight of Ash (BaCO <sub>3</sub> ).	Percentage Carbon.	Percentage Hydrogen.	Percentage Barium.
0.1326 gm. ... ..	0.2300	0.0340	0.0366	47.30	2.87	19.20
0.1473 gm. ... ..	0.2539	0.0379	0.0419	47.00	2.88	19.77
Calculated for (C <sub>14</sub> H <sub>9</sub> O <sub>7</sub> ) <sub>2</sub> Ba ... ..	—	—	—	46.96	2.53	19.20

(4) *Hydrobromide* C<sub>14</sub>H<sub>10</sub>O<sub>7</sub>.HBr.—1 gm. of citromycetin was dissolved in 40 to 50 c.c. of hot glacial acetic acid, the solution filtered, boiled, and to this was added 1–2 c.c. of fuming hydrobromic acid. The colour of the solution changed immediately from bright yellow to a dark orange and on standing the hydrobromide crystallised in dark orange prisms. These were washed with glacial acetic acid and dried *in vacuo* over sulphuric acid and potassium hydroxide. Weight = 1.01 gm. The hydrobromide is immediately decomposed by water giving citromycetin and hydrobromic acid.

0.5131 gm. of the hydrobromide was suspended in a little water and titrated with N/10 sodium hydroxide solution, using phenolphthalein as indicator. The titration was

carried out in the same manner as that described for the titration of citromycetin on p. 220. 41·82 c.c. of N/10 sodium hydroxide were required for neutralisation. (Theory for  $C_{14}H_{10}O_7 \cdot HBr$ , assuming this titrates as a tribasic acid, is 41·49 c.c.)

The percentage of hydrobromic acid in the hydrobromide was estimated by suspending a weighed amount in water, filtering from the precipitated citromycetin and estimating the HBr in the filtrate and washings by precipitation as AgBr after acidification with nitric acid.

Weight of Hydrobromide.	Weight of AgBr.	Percentage of HBr.
gm. 0·4877	gm. 0·2482	21·93
0·5131	0·2604	21·86
Theoretical for $C_{14}H_{10}O_7 \cdot HBr$	—	21·81

When citromycetin is dissolved in hot fuming *aqueous* hydrochloric, hydrobromic or hydriodic acid, and the solutions allowed to cool, citromycetin compounds separate in each case. A particularly promising compound is obtained with hydriodic acid and a quantity of this was prepared.

1 gm. of citromycetin was boiled with 100 c.c. of constant boiling point hydriodic acid solution until dissolved and then left to cool slowly. The hydriodide separates in beautiful, very fine orange needles about  $\frac{1}{4}$  in. long. These were filtered off on a Gooch crucible, washed with glacial acetic acid and then with dry ether. The yield of hydriodide was 1·15 gm. The crystals were dried *in vacuo* over phosphorus pentoxide and analysed in a similar manner to the hydrobromide. In duplicate experiments 0·2009 gm. and 0·2016 gm. gave 0·1224 gm. and 0·1231 gm. AgI respectively, corresponding to 33·20 per cent. and 33·26 per cent. HI. (Theoretical for  $C_{14}H_{10}O_7 \cdot HI = 30·62$  per cent.) This compound is obviously not a simple hydriodide of citromycetin, although it may be the hydriodide of citromycin (p. 229)  $C_{13}H_{10}O_5 \cdot HI$ . (Theoretical percentage of HI = 34·21.) It separates in the same typical orange crystals from the boiling hydriodic acid used in all the ZEISEL estimations subsequently described, *e.g.*, from the methyl ester of *O*-dimethylcitromycetin, *O*-dimethylcitromycetin itself, *O*-dimethylcitromycin (see p. 230) and *O*-monomethylcitromycin. It is immediately decomposed by treatment with water and the material regenerated is quite different in appearance from citromycetin. The regenerated material gives an instantaneous brown colour with ferric chloride in alcoholic solution (*cf.* citromycin), and crystallises from 50 per cent. aqueous alcohol in bunches of pale yellow feathery needles.

0·1579 gm. was dried in nitrogen at 150° C. There was no loss in weight (*cf.* citromycetin). On combustion this weight of material gave 0·3719 gm.  $CO_2$  and 0·0606 gm. water, corresponding to 64·23 per cent. carbon and 4·29 per cent. hydrogen.

(Theoretical for citromycin,  $C_{13}H_{10}O_5$ , carbon = 63·39 per cent., hydrogen = 4·09 per cent.)

(5) *The methyl ester of O-dimethylcitromycetin*  $C_{13}H_7O_3 (OCH_3)_2 \cdot COOCH_3$ .—20 gm. of citromycetin were suspended in 200 c.c. of water and treated with 100 c.c. of dimethyl sulphate. 10 per cent. sodium hydroxide was added with constant shaking, keeping the reaction mixture just alkaline. The temperature of the reacting mixture was regulated at about 40° C. The reaction was complete in about two hours. The methyl ester separated in a crystalline form towards the end of the methylation, and was separated and recrystallised several times from 50 per cent. alcohol, using charcoal to clear the solution. Yield of recrystallised material = 12 gm.

Large quantities of this compound were subsequently made for the investigation of the products of alkaline hydrolysis and were prepared by methylating the crude citromycetin as isolated from the metabolism solution. 64 gm. of crude citromycetin gave 31 gm. of pure methyl ester.

This compound (*O*-dimethylcitromycetin methyl ester) is also produced when citromycetin is methylated by treatment with diazomethane in ether solution.

The methyl ester crystallises in white silky needles having a melting point of 178° C. without decomposition. It is readily soluble in hot absolute or 50 per cent. alcohol but is almost insoluble in the cold. It is insoluble in aqueous solutions of potassium acetate, potassium bicarbonate or even of potassium hydroxide. Its alcoholic solution gives no colour with ferric chloride.

In view of the fact that this compound is very stable and can be readily purified by crystallisation, a sample was sent to SCHOELLER (Berlin) for analysis in order to obtain external confirmation of the empirical formula for citromycetin suggested by our own combustion results on this material.

The sample analysed was dried to constant weight over  $P_2O_5$  *in vacuo* and gave the following results :—

Weight of Substance.	Weight of $CO_2$ .	Weight of Water.	Percentage Carbon.	Percentage Hydrogen.
4·770 mg.	mg. 10·735	mg. 2·10	61·41	4·93
4·392 mg.	9·885	1·97	61·40	5·02
Theoretical for $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOCH_3$	—	—	61·43	4·86

ZEISEL estimations of the methoxyl content gave the following results :—0·3095 gm. of ester gave 0·6439 gm. AgI, corresponding to 27·47 per cent.  $OCH_3$  in the ester. In a duplicate estimation 0·2034 gm. ester gave 0·4321 gm. AgI, corresponding to 28·03 per cent.  $OCH_3$ . (Theoretical for  $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOCH_3 = 28·02$  per cent.)

(6) *O-Dimethylcitromycetin*  $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOH \cdot H_2O$ .—*O*-Dimethylcitromycetin

was prepared from the alkaline filtrate obtained when the crude methyl ester of *O*-dimethylcitromyccetin was filtered off from the methylation mixture. The details are as follows:—4 gm. of citromyccetin were shaken with 40 c.c. of water and dissolved by adding 24.5 c.c. of N/1 sodium hydroxide. 20 c.c. of methyl sulphate were then added and the methylation carried out as described above. About 160 c.c. of N/1 sodium hydroxide were added during the methylation. It was noticed that the crystalline precipitate of the methyl ester which appears towards the end of the methylation seems to decrease in amount as more sodium hydroxide is added, and if it is desired to prepare *O*-dimethylcitromyccetin the best yields of this material are obtained by keeping the reaction mixture alkaline for some considerable time. If, on the other hand, it is desired to prepare the methyl ester it is advisable to leave this in contact with excess of sodium hydroxide for as short a time as possible. In the present experiment the exposure to alkali was somewhat prolonged, 1.59 gm. of crude methyl ester were filtered off and the alkaline filtrate, on acidification, gave 2.46 gm. of crude *O*-dimethylcitromyccetin. This was repeatedly crystallised from 50 per cent. aqueous alcohol, from which *O*-dimethylcitromyccetin separates in white prisms which melt with decomposition at 217°–218° C. The substance is soluble in aqueous solutions of potassium acetate and potassium bicarbonate, and its alcoholic solution gives only a yellow colour with ferric chloride.

0.1497 gm. of the air-dried material, dried to constant weight in nitrogen at 140° to 150° C. loses 0.0079 gm. = 5.28 per cent. (Theoretical percentage of  $\text{H}_2\text{O}$  in  $\text{C}_{13}\text{H}_7\text{O}_3 \cdot (\text{OCH}_3)_2 \cdot \text{COOH} \cdot \text{H}_2\text{O} = 5.36$ .)

0.1418 gm. dried to constant weight at 150° C. in nitrogen gave in a ZEISEL estimation 0.2089 gm.  $\text{AgI} = 19.46$  per cent.  $\text{OCH}_3$ . (Theoretical percentage of  $\text{OCH}_3$  in  $\text{C}_{13}\text{H}_7\text{O}_3 \cdot (\text{OCH}_3)_2 \cdot \text{COOH} = 19.50$ .)

(7) *Action of bromine on citromyccetin*.—1 gm. of citromyccetin was dissolved in 5 c.c. of glacial acetic acid, cooled in ice, and 0.8 c.c. of bromine added. Orange red crystals very soon separated and there was considerable evolution of hydrobromic acid. The crystals were filtered off after standing overnight, washed with glacial acetic acid and dried *in vacuo* over potassium hydroxide. Weight of crystals = 1.60 gm. The bromine compound consists of orange red prisms which it was not found possible to recrystallise because of decomposition in all solvents tried. The substance was immediately decomposed by water, giving rise to a very dark brownish black powder. 0.2885 gm. of the bromine compound was treated with water and, after standing for half an hour, the insoluble material was filtered off and the amount of hydrobromic acid in combined filtrate and washings estimated as silver bromide. The weight of silver bromide was 0.2621 gm., which corresponds to 38.7 per cent. of bromine split off as  $\text{HBr}$  from the bromine compound. This figure was confirmed in a separate experiment by titration of the hydrobromic acid with sodium hydroxide, giving a figure of 39.1 per cent. On account of the unpromising nature of the compound it was not further investigated, but it appears that the action of bromine on citromyccetin first produces substitution and that the hydrobromic acid thus liberated then combines with the

brominated compound to give a hydrobromide. Treatment of this compound with water not only splits off the hydrobromic acid from the hydrobromide linking but must also produce hydrobromic acid from at least one of the substituted bromine atoms. This would explain the high percentage of hydrobromic acid split off on treatment with water.

(8) *Citromycin*.  $C_{13}H_{10}O_5$ .—On boiling citromycetin with dilute acid, carbon dioxide is slowly given off and a new compound, citromycin, is produced. The course of this reaction was investigated quantitatively as follows:—0.3115 gm. of citromycetin, dried in nitrogen at  $150^\circ C.$ , was boiled in an atmosphere of nitrogen with 50 c.c. of 2N sulphuric acid. A slow stream of purified nitrogen was bubbled through the reaction mixture and up through an upright condenser. The gaseous products were then bubbled through standard barium hydroxide. Hydrolysis was almost complete in 12 hours, but was continued for a total of 18 hours in all. The barium hydroxide was then titrated against N/2 hydrochloric acid to phenolphthalein. Carbon dioxide equivalent to 4.71 c.c. of N/2 hydrochloric acid had been produced. (Theoretical for  $C_{14}H_{10}O_7 \rightarrow C_{13}H_{10}O_5 + CO_2 \equiv 4.30$  c.c. N/2 HCl.) 0.27 gm. of citromycin was recovered from the hydrolysis mixture. (Theoretical yield = 0.26 gm.) From this it appears that on acid hydrolysis one molecule of citromycetin loses one molecule of carbon dioxide to form one molecule of citromycin.

For the preparation of citromycin in quantity 20 gm. of citromycetin were boiled with 1,500 c.c. of 2N sulphuric acid for two days. The yellow crystalline product, weighing 14.9 gm., was recrystallised from 50 per cent. alcohol, from which it separates in yellow needles containing no water of crystallisation. Citromycin is somewhat paler in colour than citromycetin and is also less soluble in all solvents. On heating it begins to darken at  $255^\circ$  to  $260^\circ C.$ , melting very indefinitely at  $285^\circ$  to  $290^\circ C.$  It is insoluble in aqueous solutions of sodium acetate or bicarbonate but is readily soluble in sodium hydroxide, giving an orange brown solution. Its solution in alcohol gives an intense brown colour with ferric chloride. It contains no methoxyl groups as was shown by a ZEISEL estimation.

It titrates as a monobasic acid to phenolphthalein. 0.2244 gm. was titrated with N/10 sodium hydroxide with phenolphthalein as indicator, and 9.17 c.c. of N/10 sodium hydroxide were required for neutralisation. (Theoretical for  $C_{13}H_{10}O_5$  titrated as a monobasic acid = 9.12 c.c. N/10 NaOH.)

Analyses of citromycin, dried to constant weight at  $150^\circ C.$  in nitrogen, agree with the formula  $C_{13}H_{10}O_5$ .

Weight of Sample.	Weight of $CO_2$ .	Weight of Water.	Percentage Carbon.	Percentage Hydrogen.
0.1685 gm.	0.3905	0.0621	63.20	4.12
0.1186 gm.	0.2761	0.0428	63.47	4.04
0.1039 gm.	0.2411	0.0392	63.30	4.22
0.1010 gm.	0.2345	0.0390	63.32	4.32
Theoretical for $C_{13}H_{10}O_5$	—	—	63.39	4.09

(9) *Diacetylcitromycin*.  $C_{13}H_8O_3 (O.CO.CH_3)_2$ .—2 gm. of citromycin were heated to boiling with 2 gm. of anhydrous sodium acetate and 6 c.c. of acetic anhydride in an oil bath for 30 minutes. The mixture was cooled and diluted with water. A quantity of crude acetyl compound separated and was filtered off and washed. Yield of crude material = 2.56 gm. (Note the fundamental difference between the behaviour of citromycetin and citromycin on acetylation. Diacetylcitromycetin remains in solution in the acetylation mixture, presumably because of the presence of a COOH group in citromycetin which is absent in citromycin.)

The crude acetyl compound was recrystallised from 50 per cent. aqueous alcohol from which it separated in fine white needles having a melting point of  $221^{\circ}$ – $222^{\circ}$  C. There was slight darkening at the melting point but no other obvious decomposition. It is insoluble in aqueous solutions of potassium acetate or potassium bicarbonate and gives no colour reaction in alcoholic solution with ferric chloride.

It was shown that the substance was a diacetyl compound by the following means: 0.4037 gm. was hydrolysed by boiling with 25 c.c. of N/1 sulphuric acid for 12 hours. The hydrolysed mixture was left at room temperature for a week and the regenerated citromycin was then filtered off on a sintered glass funnel, washed and dried at  $100^{\circ}$  C. Weight of regenerated citromycin = 0.2980 gm.  $\equiv$  73.8 per cent. (Theoretical for  $C_{13}H_8O_3 (O.CO.CH_3)_2 = 74.55$  per cent. and for  $C_{13}H_7O_2 (O.CO.CH_3)_3 = 66.10$  per cent.) The amount of acetic acid produced on hydrolysis was also estimated by the method given on p. 222. The acetic acid produced is equivalent to 23.59 c.c. of N/10 sodium hydroxide. (Theoretical for  $C_{13}H_8O_3 (O.CO.CH_3)_2 = 24.47$  c.c.)

Combustion results agree with the formula  $C_{13}H_8O_3 (O.CO.CH_3)_2$ .

Weight of Sample.	Weight of $CO_2$ .	Weight of Water.	Percentage Carbon.	Percentage Hydrogen.
0.1380 gm.	0.3133	0.0546	61.90	4.43
0.1305 gm.	0.2964	0.0552	61.95	4.73
Theoretical for $C_{13}H_8O_3(O.CO.CH_3)_2$	—	—	61.80	4.27

(10) *Potassium salt of citromycin*.  $C_{13}H_{10}O_5 \cdot C_{13}H_9O_5K$ .—1 gm. of citromycin was dissolved in 100 to 120 c.c. of boiling absolute alcohol and to this were added 10 c.c. of 5 per cent. alcoholic potassium acetate solution. The colour of the solution immediately darkened from pale yellow to orange. After boiling for a few minutes the solution was filtered and left to crystallise. The potassium salt crystallised out slowly in the cold in dense clusters of orange needles. Yield = 0.54 gm. It is much more soluble in alcohol than the potassium salt of citromycetin and, unlike this, does not lose weight on drying at  $150^{\circ}$  C. in nitrogen.

For analysis 0.1955 gm. of the salt was acidified with 10 c.c. of N/1 sulphuric acid,

left overnight in the cold and the precipitated citromycin weighed on a sintered glass funnel. Weight of citromycin regenerated = 0.1806 gm.  $\equiv$  92.39 per cent. The filtrate was evaporated to dryness and ashed in a platinum basin. Weight of  $K_2SO_4$  = 0.0348 gm.  $\equiv$  7.99 per cent. potassium. Since the theoretical values for citromycin and potassium in  $C_{13}H_9O_5K$  are 86.27 per cent. and 13.76 per cent. respectively it seemed that this estimation might be incorrect. It was, however, confirmed by ashing 0.3418 gm. of the potassium salt directly with sulphuric acid. Weight of ash = 0.0555 gm.  $\equiv$  7.29 per cent. K in potassium salt. The potassium salt of citromycin appears to be a compound containing two molecules of citromycin with only one hydrogen atom replaced by potassium. Such a compound  $C_{26}H_{19}O_{10}K$  gives 92.84 per cent. citromycin and 7.36 per cent. potassium.

Similar compounds have already been described by PERKIN (1899). In this paper PERKIN describes the preparation of the potassium salts of a number of phenolic colouring matters by the interaction of an alcoholic solution of the colouring matter with an alcoholic solution of potassium acetate. The majority of the colouring matters tested by PERKIN give normal potassium salts, but rhamnetin and rhamnazin, the methyl ethers of quercetin, form potassium salts analogous to those of citromycin. Since each consists of a monopotassium derivative of a double molecule of the colouring matter, this property goes hand in hand with the relatively feeble acid properties of citromycin.

(11) *Hydrobromide of citromycin*.  $C_{13}H_{10}O_5 \cdot HBr$ .—1 gm. of citromycin was dissolved in 75 c.c. of boiling glacial acetic acid and to this were added, while boiling, 2 c.c. of fuming hydrobromic acid. The original pale yellow colour changed to orange. The hydrobromide separated in orange yellow needles with irregular splintered ends. After washing with glacial acetic acid it was dried over sulphuric acid and potassium hydroxide for analysis. Weight of hydrobromide = 1.27 gm.

The hydrobromide was analysed by treating a weighed amount with water, which decomposed it immediately into citromycin and hydrobromic acid. The citromycin was filtered on a sintered glass funnel, dried and weighed, and the hydrobromic acid in the filtrate estimated as silver bromide.

Weight of Hydrobromide.	Weight of AgBr.	Weight of Regenerated Citromycin.	Percentage HBr.	Percentage Regenerated Citromycin.
0.2875 gm.	0.1591	0.2128	23.83	74.00
0.2901 gm.	0.1623	0.2151	24.10	74.15
Theoretical for $C_{13}H_{10}O_5 \cdot HBr$ .	—	—	24.75	75.25

A hydriodide was prepared by dissolving citromycin in boiling concentrated hydriodic acid. On cooling, glistening orange needles separated which were identical with those prepared in a similar manner from citromycetin and hydriodic acid (see p. 224).

(12) *O-Dimethylcitromycin*.  $C_{13}H_8O_3 \cdot (OCH_3)_2$ .—*O*-Dimethylcitromycin was prepared by methylating citromycin with dimethyl sulphate in presence of sodium hydroxide. 2 gm. of citromycin were dissolved in 8 to 10 c.c. of N/1 sodium hydroxide and 10 c.c. of dimethyl sulphate added. The whole was shaken vigorously and kept just alkaline by additions of N/1 sodium hydroxide. The *O*-dimethyl derivative separated during the course of methylation and was filtered off, washed and dried. Weight of crude product = 1.58 gm. It was recrystallised several times from 50 per cent. aqueous alcohol and when pure separated in pale yellow polyhedra, having a melting point of 225°–227° C. It was insoluble in carbonates or sodium hydroxide solution and its alcoholic solution gave a negative test with ferric chloride. It did not lose weight on drying in nitrogen at 150° C. and a sample so dried gave the following result in a ZEISEL estimation: 0.1933 gm. gave 0.3267 gm. of AgI, corresponding to 22.32 per cent.  $OCH_3$ . (Theoretical percentage  $OCH_3$  in  $C_{13}H_8O_3 \cdot (OCH_3)_2$  = 22.62 per cent.)

(13) *O-Monomethylcitromycin*.  $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3)$ .—The monomethyl compound was prepared by methylating citromycin with methyl iodide and alcoholic potassium hydroxide. 1 gm. of citromycin was heated with 2 gm. of methyl iodide (which is rather more than 3 molecules of  $CH_3I$  to 1 molecule of citromycin) and a solution of 0.79 gm. of potassium hydroxide in 6.6 c.c. of methyl alcohol, this being the amount of KOH necessary to decompose 2 gm. of  $CH_3I$ . The mixture was placed in a small Carius tube previously filled with nitrogen, and then sealed and heated in a boiling water bath for one hour. The mixture was diluted with water and the precipitated methyl derivative recrystallised from 50 per cent. aqueous alcohol. The *O*-monomethylcitromycin so prepared crystallises in small yellow needles having a melting point of 183–185° C. It is readily soluble in sodium hydroxide solution but insoluble in carbonates, and its alcoholic solution gives a yellow colour with ferric chloride, *i.e.*, a practically negative reaction.

When recrystallised from aqueous alcohol it appears to contain one molecule of water of crystallisation. 0.2020 gm., which had been previously dried *in vacuo* over sulphuric acid, was dried to constant weight in nitrogen at 140° C. Loss in weight = 0.0103 gm. corresponding to 5.10 per cent. of water. (Theoretical for  $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3) \cdot H_2O$  = 6.48 per cent.)

0.1917 gm. of this dried material gave in a ZEISEL estimation 0.1877 gm. of AgI = 12.93 per cent.  $OCH_3$ . (Theoretical for  $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3)$  = 11.93 per cent.)

#### *Decomposition products of citromycetin.*

##### *Section 1.—By acid hydrolysis.*

As previously stated (p. 227) citromycetin on boiling with dilute sulphuric acid gives rise to one molecule of carbon dioxide and one molecule of citromycin. No other hydrolytic products have been found. 1 gm. of citromycetin was boiled with 150 c.c. of 2N sulphuric acid in an atmosphere of nitrogen with a reflux condenser for nine hours

until no further carbon dioxide was evolved. At the end of this time the hydrolysis liquid was distilled and the distillate tested :—

(a) For acetone, with sodium nitroprusside and ammonia (ROTHERA's test), and with 2 : 4-dinitrophenylhydrazine. Both gave negative results.

(b) For acetic acid. This also gave a negative result.

*Section 2.—With alkaline iodine.*

When citromycetin is treated with alkaline iodine obvious decomposition takes place and iodoform is formed. Since oxidation with alkaline iodine has been applied successfully to the estimation of kojic acid (see Part VIII), the method was tested for its possible application to the estimation of citromycetin.

0.2900 gm. (M/100) of citromycetin, dried in nitrogen at 150° C. to constant weight, was dissolved in the minimum amount of N/10 sodium hydroxide and made up to 100 c.c. Portions of this were then treated with a known volume of N/10 iodine solution, and a definite volume of N/10 sodium hydroxide was added, drop by drop, during the space of three minutes. The mixed solutions were allowed to stand for two hours, and were then acidified with 20 c.c. of N/1 sulphuric acid and the excess of iodine titrated with N/10 sodium thiosulphate solution. The results are summarized in Table VII.

TABLE VII.—Reaction of citromycetin with alkaline iodine.

c.c. of Citromycetin Solution used.	c.c. of Water added.	c.c. N/10 Iodine added.	c.c. N/10 NaOH added.	c.c. N/10 Thio. used.	c.c. N/10 Iodine absorbed per 10 c.c. of Citromycetin Solution.
2	98	40	50	38.44	6.55
5	95	40	50	35.88	7.74
10	90	40	50	32.09	7.66
15	85	40	50	27.89	7.91
20	80	40	50	24.02	7.87
15	35	20	25	8.17	7.81
20	30	20	25	5.49	7.20
Blank experiment	100	40	50	39.75	—

The end-point is fairly sharp but the blue colour of the starch iodine quickly reappears. A further experiment was carried out to determine how long it is necessary to allow the mixture of citromycetin and alkaline iodine to react to give consistent results. A solution of citromycetin was made having the same composition as that described above. 10 c.c. of this were measured into each of nine flasks, together with a blank containing 10 c.c. of distilled water. Into each of these flasks were then measured in turn 100 c.c. of water, 40 c.c. of N/10 iodine, and 50 c.c. of N/10 sodium hydroxide

added drop by drop during the space of three minutes. The flasks were now kept at 24° C. for varying lengths of time as indicated in the following table :—

TABLE VIII.—Effect of time on the reaction between citromycetin and alkaline iodine.

Flask No.	Duration of Oxidation with Alkaline Iodine.	Thio. Reading c.c. N/10.	c.c. N/10 Iodine absorbed per 10 c.c. Citromycetin Solution.
	Min.		
1	30	33·14	6·68
2	86	32·61	7·21
3	120	32·70	7·12
4	150	32·58	7·24
5	180	32·54	7·28
6	255	32·30	7·52
	Hrs.		
7	21	31·90	7·92
8	23	31·90	7·92
9	69½	31·41	8·41
Blank	21½	39·82	—

It appears from these results that while there is a slight increase in the amount of iodine absorbed if the oxidation is continued for a very long time a fairly steady figure is reached after 1½ to 2 hours, and in subsequent work a 2-hours' oxidation was used in all estimations.

The results are not so good as those obtained with kojic acid, but seemed to support the opinion that one molecule of citromycetin (molecular weight 290) absorbs eight atoms of iodine, and that this may be used as a roughly quantitative method for the estimation of citromycetin in solution.

The following routine method was adopted in estimating citromycetin in metabolism solutions. The sum of the glucose and citromycetin present is found by estimating the total iodine absorbed on treating a portion of the solution with alkaline iodine. Glucose alone is estimated by polarising a portion of the decolorised metabolism solution. The difference between these two figures gives a roughly quantitative idea of the amount of citromycetin present. The best method of decolorising the highly coloured metabolism solutions for polarising was found to be treatment with colloidal iron.

25 c.c. of the metabolism solution are neutralised to phenolphthalein with sodium hydroxide in a 50 c.c. measuring flask. 10 c.c. of colloidal iron are added drop by drop, followed by a little magnesium sulphate solution, and the whole made up to the mark. The mixture is filtered through a dry paper and, if it is still coloured, this residual colour may be discharged by adding one drop of 50 per cent. sulphuric acid. The solution is then polarised as usual. Comparative experiments carried out with colloidal iron,

Merck's blood charcoal, Norite and basic lead acetate showed that while all give satisfactory decolorisation, the use of any of them except colloidal iron is rendered inadvisable because of the large loss of glucose by adsorption.

The effect of alkaline iodine solution on the following derivatives of citromycetin has also been investigated :—

- (a) Citromycin.
- (b) *O*-Dimethylcitromycetin.
- (c) *O*-Dimethylcitromycetin methyl ester.

The results showed that while citromycin is readily oxidized by alkaline iodine, absorbing approximately eight atoms of iodine per molecule of citromycin, in a similar manner to citromycetin itself, neither *O*-dimethylcitromycetin nor its methyl ester are appreciably attacked by alkaline iodine. The details of the experiment with citromycin follow :—

0.2460 gm. (M/100) of citromycin was dissolved in the minimum amount of N/10 sodium hydroxide and made up to 100 c.c. Different amounts of this solution were treated with alkaline iodine in a similar manner to that described for citromycetin. The oxidation was continued for two hours before acidifying and titrating the excess of iodine. The results are given in Table IX.

TABLE IX.—Reaction between citromycin and alkaline iodine.

Cubic centimetres Citromycin Solution used.	Cubic centimetres Water added.	Cubic centimetres N/10 Iodine.	Cubic centimetres N/10 NaOH.	Cubic centimetres N/10 Thiosulphate.	Cubic centimetres N/10 Iodine absorbed per 10 c.c. Citromycin Solution.
2	98	40	50	37.75	9.30
5	95	40	50	35.22	8.78
10	90	40	50	31.28	8.33
15	85	40	50	27.05	8.37
20	80	40	50	22.99	8.31
15	35	20	25	7.84	7.98
20	30	20	25	4.64	7.58
Blank	100	40	50	39.61	—

An attempt was made to isolate the breakdown products resulting from the oxidation of citromycetin by alkaline iodine but did not meet with great success. 3.9 gm. of citromycetin were dissolved in the theoretical amount of barium hydroxide solution and diluted to 7,400 c.c., iodine equivalent to eight atoms was added and the whole shaken until the iodine had dissolved. At the end of three hours the solution smelt strongly of iodoform and contained a yellowish precipitate. This was filtered off and recrystallised from acetone in shining yellow hexagonal plates having a melting point of 122° C.

It was evidently iodoform. In spite of repeated attempts the only other oxidation products identified with certainty were oxalic and acetic acids and carbon dioxide. For this reason the attempt to obtain information as to the constitution of citromycetin by a study of its oxidation products with alkaline iodine was abandoned.

*Section 3.—By hydrolysis with alkali.*

When citromycetin was subjected to the action of alkali in the presence of air it immediately became dark brown to black in colour, and all attempts to isolate end products by potash fusion of citromycetin in air failed to give any product. For this reason the alkaline hydrolysis of citromycetin was studied in the following manner: 30 gm. of citromycetin were introduced into a hydrolysis flask fitted with a reflux condenser. The whole apparatus was then filled with nitrogen. A solution of 100 gm. of potassium hydroxide in 200 c.c. of water was now added, and the mixture was boiled in an atmosphere of nitrogen for three hours. Air was rigorously excluded during the whole hydrolysis. Even so the hydrolysis solution became very dark brown in colour. The water in the reflux condenser was kept warm and the constant stream of nitrogen bubbled through the apparatus was subsequently scrubbed in ice-cold water. At the end of the hydrolysis this aqueous solution gave strong positive tests for acetone. The solution smelt strongly of acetone, gave a very intense sodium nitroprusside test, and gave a copious precipitate with 2:4-dinitrophenylhydrazine, which on recrystallising from alcohol had a melting point of 126°–127° C. (The melting point of acetone 2:4-dinitrophenylhydrazone is 128° C.)

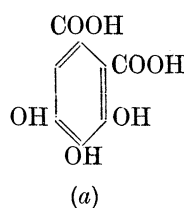
Carbon dioxide was now passed through the hydrolysis mixture to saturation point, contact with air still being avoided. Nothing separated and the liquid retained its dark colour. It was extracted exhaustively with ether, and the ether extract on evaporation gave rise to a very small amount of dark brown material from which nothing crystalline could be isolated. The ether-extracted solution was then acidified with sulphuric acid and the acidified solution extracted exhaustively with ether. The ether solution, on evaporation, yielded large quantities of acetic acid, and, since pure ether was used for extraction, acetic acid must be a product of hydrolysis. The ether extract was dried *in vacuo* over potassium hydroxide and sulphuric acid, giving rise to a brownish black residue containing an obvious white crystalline material. The residue, freed from acetic acid by drying, was then treated with water and warmed for some time on the water bath. This treatment dissolved the dark brown material and left a small amount of insoluble residue in the form of minute rosettes. These were filtered off while still warm, and recrystallised by dissolving in hot alcohol, decolorising with a little charcoal and adding boiling water to the alcoholic solution until crystallisation started. By this means a small quantity of white needles was obtained having the following characteristics:—

They were almost insoluble in water, but readily soluble in hot alcohol. On heating, no change was obvious until 260°–270° C. at which temperature the material became

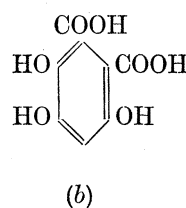
brown in colour. The colour slowly darkened as the temperature rose until the material finally melted at  $290^{\circ}$ – $291^{\circ}$  C. and decomposed sharply at  $292\cdot5^{\circ}$  C., with gas evolution, leaving a brownish black residue. An aqueous solution of this material gave with a very small amount of ferric chloride a pure blue colour changing to green on the addition of further amounts of ferric chloride. An alcoholic solution gave a very dark ivy green colour with ferric chloride.

The yield of this material was so very small, however, that there was not sufficient for analysis, but, in view of later results and the characteristics described above, it seems probable that it was a trihydroxyphthalic acid.

Only one trihydroxyphthalic acid has been described, although two are possible, having the two following formulæ :—

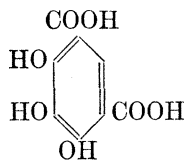


3 : 4 : 5-trihydroxyphthalic acid.



3 : 5 : 6-trihydroxyphthalic acid.

3 : 4 : 5-trihydroxyphthalic acid was prepared by PRATT and PERKINS (1918). It is described by them as being insoluble in boiling xylene, and on crystallising from water it gave slightly brownish crystals, which melted slowly, with decomposition, beginning at about  $280^{\circ}$  C. (corr.). It gave a greenish blue colour with a little ferric chloride and a yellow green with excess. PRATT and PERKINS considered their 3 : 4 : 5-trihydroxyphthalic acid to be identical with the so-called gallocarbonic acid of SENHOFER and BRUNNER (1880). Gallocarbonic acid has a melting point of over  $270^{\circ}$  C. with evolution of carbon dioxide. With very dilute ferric chloride it gives a violet colour, with more concentrated, a greenish brown colour. As the result of more recent work, however, by VOSWINCKEL and DE WEERTH (1912), and FEIST and SANDSTEDT (1918) it seems certain that gallocarbonic acid is not a phthalic acid derivative but an *iso*-phthalic acid derivative of the following formula :—



In view of these facts, together with considerations arising from the results obtained by the hydrolysis of *O*-dimethylcitromycetin methyl ester (see p. 237), it is probable that the trihydroxyphthalic acid isolated from the hydrolytic products of citromycetin is represented by formula (b) and is indeed 3 : 5 : 6-trihydroxyphthalic acid.

A further experiment was carried out with a view to estimating the volatile materials produced by alkaline hydrolysis of citromycetin. 3·38 gm. of pure citromycetin were

introduced into a 100 c.c. Claisen distilling flask connected to a condenser and an adapter, the end of which passed under a little water contained in a 200 c.c. measuring flask immersed in ice. Nitrogen was bubbled through the whole apparatus until the latter was free from air, and then a solution of 10 gm. of potassium hydroxide in 20 c.c. of water was added through a tap funnel and hydrolysis carried out for two hours. The liquid which slowly distilled over from the Claisen flask was replaced by adding water from time to time through the tap funnel. The distillate A was made up to 200 c.c. and the residue B treated as described later.

*Treatment of Distillate A.*—An estimation by MESSINGER'S method of the acetone present in a portion of the distillate gave 0.223 gm. corresponding to 0.37 gm. molecules per gm. molecule of citromycetin. The 2:4-dinitrophenylhydrazone was prepared and recrystallised. It melted at 127°–128° C., and its melting point was unchanged on admixture with acetone 2:4-dinitrophenylhydrazone prepared from a sample of pure acetone. On the other hand, a sample of the 2:4-dinitrophenylhydrazone of methyl ethyl ketone, with which acetone easily might be confused, was found to melt at 114°–115° C. while the mixed melting point of this with the hydrazone isolated from the hydrolysis mixture proved to be 94°–99° C. On combustion 2.969 mgm. of the hydrazone of the hydrolysis product gave 0.597 c.c. nitrogen at 23.5° C. and 762 mm. pressure (SCHOELLER, Berlin), corresponding to 23.2 per cent. of nitrogen (theoretical for acetone 2:4-dinitrophenylhydrazone = 23.5 per cent., and for methyl ethyl ketone 2:4-dinitrophenylhydrazone = 22.2 per cent.).

*Treatment of Residue B.*—The condenser and adapter were now detached from the Claisen distilling flask which was then connected to a large bubbler containing standard barium hydroxide. A slight excess of acid was run through the tap funnel, and the CO<sub>2</sub> liberated was passed over in a current of nitrogen, absorbed and estimated. 0.54 gm. CO<sub>2</sub> was produced corresponding to 1.09 gm. mol. CO<sub>2</sub> per gm. mol. of citromycetin.

The acidified hydrolysis mixture was now exhaustively evaporated *in vacuo* to remove all volatile acids and the combined distillates titrated with N/10 sodium hydroxide, of which 75.1 c.c. were needed to neutralise, corresponding to 0.72 gm. mol. volatile acid per gm. mol. citromycetin. The volatile acid was shown to be acetic acid by conversion into the silver salt. 0.0788 gm. of the silver salt gave 0.0506 gm. of silver, corresponding to 64.23 per cent. Ag (theoretical for silver acetate = 64.64 per cent.).

The volatile hydrolysis products per gm. mol. of citromycetin are thus:—0.37 gm. mol. acetone, 1.09 gm. mol. carbon dioxide and 0.72 gm. mol. acetic acid.

In a repeat experiment the volatile hydrolysis products obtained per gm. mol. of citromycetin were 0.36 gm. mol. acetone, 1.14 gm. mol. carbon dioxide, and 0.92 gm. mol. acetic acid. The volatile acid was examined specially for formic acid but none was found.

SECTION 4.—*Hydrolysis of the methyl ester of O-dimethylcitromycetin with alcoholic potassium hydroxide.*

In view of the failures so far experienced in attempting to isolate decomposition products from the hydrolytic products from citromycetin itself, all subsequent work was devoted to the examination of the products of hydrolysis with alcoholic potassium hydroxide of the methyl ester of *O*-dimethylcitromycetin. In this way, by replacing OH groups with methoxyl groups before hydrolysis, most of the trouble due to the formation of black decomposition products was overcome and it was found possible to isolate various decomposition products. 30 gm. of the methyl ester of *O*-dimethylcitromycetin were boiled with alcoholic potassium hydroxide made from 60 gm. of potassium hydroxide sticks + 60 c.c. of water + 240 c.c. of absolute alcohol. The methylated citromycetin was placed in a hydrolysis flask fitted with reflux condenser. A stream of nitrogen free from oxygen was bubbled through this, and when all the air had been driven out, the alcoholic potassium hydroxide was run in through a tap funnel placed at the top of the condenser. The contents were boiled for 3½ hours, and at the end of this period the alcohol was completely removed *in vacuo*, the residue taken up in 800–900 c.c. of distilled water and carbon dioxide passed in to saturation. The hydrolysis mixture which, previous to passing in carbon dioxide, was dark brown in colour was now much lighter and was thoroughly extracted with ether. The ether extract on evaporation gave Fraction A, treatment of which is described below. The ether-extracted solution was acidified with sulphuric acid, giving rise to a precipitate which was filtered off, and the filtered acid solution extracted with ether. This ether extract gave Fraction B.

*Treatment of Fraction A.*—Fraction A consisted of a yellow crystalline solid (Product A). It was purified by crystallising from boiling absolute alcohol from which it separated in light yellow, very fine, feathery, needles having a melting point of 177°–180° C. and showing signs of decomposition on melting. An alcoholic solution of this substance gave an olive brown colour with ferric chloride.

On the addition of alcoholic potassium acetate solution to a solution of Product A in absolute alcohol a potassium salt was formed. This separated at first in a gelatinous condition, but on standing gradually crystallised in thick rosettes of orange prisms. Product A also gave a hydrobromide, crystallising in bunches of orange needles, on the addition of concentrated hydrobromic acid to its solution in glacial acetic acid. In both cases there was immediate darkening in colour from yellow to orange, on the addition of potassium acetate in the one case, and of hydrobromic acid in the other. A darker colour was obtained with hydrobromic acid than with potassium acetate. These properties indicate that the pyrone ring originally present in citromycetin still remains unbroken in Product A.

It did not contain water of crystallisation and could be dried unchanged at 110° C. in nitrogen, although it showed some signs of decomposition at 150° C. in nitrogen. It

had a molecular weight, determined by BARGER's method, of between 231 and 250 (molecular weight of  $C_{12}H_{12}O_5 = 236$ ). ZEISEL estimations on material dried at  $110^\circ C$ . in nitrogen gave the following results: 0.1664 gm. of substance gave 0.3412 gm. of AgI and 0.1904 gm. substance gave 0.3872 gm. AgI corresponding to 27.06 per cent.  $OCH_3$  and 26.86 per cent.  $OCH_3$  respectively. (Theoretical for  $C_{10}H_6O_3 \cdot (OCH_3)_2$ , i.e.,  $C_{12}H_{12}O_5 = 26.28$  per cent.) The following combustion results were obtained on different samples dried in nitrogen at  $110^\circ C$  :—

Weight of Substance Analysed.	Weight of $CO_2$ .	Weight of $H_2O$ .	Percentage Carbon.	Percentage Hydrogen.
0.1212 gm.	0.2717	0.0589	61.14	5.44
0.1194 gm.	0.2664	0.0569	60.87	5.33
Theoretical for $C_{12}H_{12}O_5$	—	—	61.02	5.10

Product A appears to be a dimethoxy-hydroxy-methylbenzopyrone.

*Treatment of Fraction B.*—This fraction was the main hydrolytic product of the methyl ester, 8.2 gm. being obtained from 30 gm. of ester. It consisted of a light brown syrup smelling strongly of acetic acid, which is thus one of the hydrolytic products of the methyl ester. It gave a brownish purple colour with ferric chloride. On standing it gradually set to a thick sticky mass of needles which could not be purified by draining on a porous tile. It was finally fractionated in the following manner: it was dried *in vacuo* over potassium hydroxide to remove acetic acid, and the residue dissolved in 200 c.c. of hot absolute alcohol, filtered and heated to boiling. To the boiling solution were added 80 c.c. of 5 per cent. alcoholic solution of potassium acetate. On cooling there was a copious separation of a potassium salt which, however, did not crystallise but appeared under the microscope as small spherical globules. These were filtered off and washed and constituted Fraction B1. (Weight = 5.9 gm.)

The filtrate constituting Fraction B2 will be dealt with later.

The potassium salt (Fraction B1) was dissolved in water and acidified with a little 50 per cent. sulphuric acid. This produced an immediate milkiness which, on shaking vigorously, coalesced into a single piece of brown, tarry material. The clear aqueous solution, which was only pale yellowish brown in colour, was extracted with ether, and the ether solution evaporated to dryness, leaving a transparent non-crystalline residue which was now fractionated from a considerable volume of dry chloroform. The main product separating from a hot chloroform solution on cooling consisted of a substance crystallising in white needles, an aqueous solution of which gave a pure purple colour with ferric chloride (Product B). It was readily soluble in water and alcohol and could be crystallised by dissolving in a large volume of chloroform and evaporating the solution quickly until crystals appeared. On heating it melted with decomposition at  $181^\circ$ – $182^\circ C$ , but the melting point varied very considerably with the rate of heating.

The crystals clung tenaciously to chloroform which could only be driven off by heating to constant weight at 110° C. in nitrogen, when they lost about 14 per cent. of their weight as chloroform.

This substance on analysis gave the following results :—

0·2389 gm. dried to constant weight at 110° C. in nitrogen was titrated with N/10 sodium hydroxide to phenolphthalein : A very sharp end-point was obtained, and 19·45 c.c of N/10 sodium hydroxide solution were required, corresponding to a combining weight of 122·8 and a molecular weight of 122·8 or 245·6. (Theoretical for  $C_{10}H_{10}O_7 = 242$ .)

ZEISEL estimations gave the following results : 0·1210 gm. of dried material gave 0·2308 gm. of AgI and 0·1248 gm. gave 0·2376 gm. of AgI, corresponding to 25·19 per cent. and 25·14 per cent.  $OCH_3$  respectively. (Theoretical for  $C_{10}H_{10}O_7 = C_8H_4O_5 \cdot (OCH_3)_2 = 25·63$  per cent.  $OCH_3$ .)

It gave the following results on combustion :—

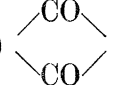
Weight of Substance.	Weight of $CO_2$ .	Weight of $H_2O$ .	Percentage Carbon.	Percentage Hydrogen.
0·1122 gm.	0·2068	0·0405	50·27	4·04
0·1357 gm.	0·2482	0·0496	49·89	4·09
Theoretical for $C_{10}H_{10}O_7$	—	—	49·58	4·17

The substance appears to be a dimethoxy-hydroxy-phthalic acid.

That it is a true phthalic acid derivative and not an *iso*- or *tere*-phthalic acid derivative seems to be indicated by the following fact : A small amount of the dimethoxy-hydroxy-phthalic acid was heated in an open test tube in a castor oil bath, the temperature of which was maintained at 180°–200° C. Water was given off, as evidenced by the condensation of moisture on the cool parts of the tube. A substance sublimed (Product C), with apparent gaseous decomposition, in the form of beautiful long white needles some of which were half a centimetre long. These melted at 203°–204° C. without decomposition but with partial sublimation during heating. It was readily soluble in water but gave no colour with aqueous ferric chloride.

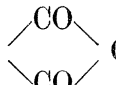
Product B was definitely proved to be a true phthalic acid derivative as follows : A quantity of the dry material was dissolved in dry ether and ethylated by treatment with an excess of an ethereal solution of diazoethane. On removal of the excess diazoethane and ether an oil remained which no longer gave a colour with ferric chloride. This oil was hydrolysed by heating on the boiling water bath with aqueous potassium hydroxide solution and the hydrolysis solution was acidified and extracted with ether. On evaporating the ethereal solution a crystalline residue remained which was repeatedly sublimed in an oil bath at 150°–200° C. at a pressure of 14 mm. until a constant melting point was obtained. It was finally sublimed at ordinary pressure and analysed.

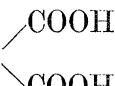
The final product (Product D) consisted of beautiful slender prisms of a very pale lemon-yellow colour, which melted sharply at 195°–196° C. It is insoluble in water, cold alcohol, ligroin and petroleum ether. It is soluble in acetone, chloroform, benzene and is readily crystallised from hot alcohol or aqueous alcohol.

The sublimed product (Product D) was proved by analysis (SCHOELLER, Berlin) to be dimethoxy-ethoxy-phthalicanhydride,  $C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5)$    $O = C_{12}H_{12}O_6$  and not dimethoxy-ethoxyphthalic acid,  $C_{12}H_{14}O_7$ .

4.289 mgm. gave 8.990 mgm.  $CO_2$  and 1.90 mgm.  $H_2O$  corresponding to 57.18 per cent. carbon and 4.92 per cent. hydrogen. (Theoretical for  $C_{12}H_{12}O_6 = 57.15$  per cent. carbon and 4.76 per cent. hydrogen; for  $C_{12}H_{14}O_7 = 53.31$  per cent. carbon and 5.22 per cent. hydrogen.)

2.758 mgm. gave 7.640 mgm. AgI.

(Theoretical for  $C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5)$    $O = 7.680$  mgm. AgI; for

$C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5)$    $= 7.170$  mgm. AgI.)

Product D is thus the anhydride and not the free acid and hence it follows that the substance from which it arose, *i.e.*, Product B, is a true phthalic acid derivative and not an *iso*- or a *tere*-phthalic acid derivative.

In view of the lack of published information on the dimethoxy-hydroxy-phthalic and benzoic acids an attempt was made to prepare the parent phenolic acid from the residual products of the treatment of the dimethoxy-hydroxy-phthalic acid with hydriodic acid as carried out in the ZEISEL estimation. 1.87 gm. of the dimethoxy-hydroxy-phthalic acid, isolated as described on p. 238, were boiled with 15 c.c. of freshly distilled, constant boiling point, hydriodic acid in an atmosphere of carbon dioxide. The hydriodic acid solution was cooled, diluted with four times its volume of water and extracted with ether. The ether extract was washed with water to remove hydriodic acid and with sodium thiosulphate solution to remove iodine. The purified ether solution on evaporation to dryness left a crystalline residue (Product E) which was crystallised from a large volume of chloroform, from which it separated in white prisms having a melting point which varied considerably with the rate of heating. The substance melted in any case with decomposition, but if heated slowly had a melting point of 208°–209° C., while if heated quickly it melted at 214°–215° C. An aqueous solution of the material gave a deep blue colour with ferric chloride, fading to brown with excess of reagent. An aqueous solution immediately reduced ammoniacal silver solution in the cold, and aqueous silver nitrate solution, also in the cold, after standing for a short time.

A portion of this material was heated in a castor oil bath at a temperature somewhat above its melting point. The tube containing the material was fitted with a short inner tube kept cold by a current of cold water. After some time there appeared on the inner tube a sublimate consisting of a very light white deposit of fine needles (Product F). These were readily soluble in water, giving a colourless solution which quickly turned pink and later brown in colour. On heating, the crystals shrank at  $139^{\circ}\text{C}$ . and melted sharply at  $140\cdot5^{\circ}\text{C}$ ., without decomposition.

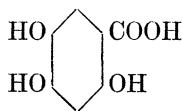
An aqueous solution of the material gave a very transient blue colour with ferric chloride.

*Treatment of Fraction B2.*—The alcoholic filtrate constituting Fraction B2 was evaporated to dryness *in vacuo*, the residue dissolved in water and acidified with sulphuric acid. A resinous precipitate which formed was filtered off and the sulphuric acid solution was extracted with ether. The ether residue was taken up with chloroform, which dissolved the greater part, and from the chloroform solution was obtained a further amount of dimethoxy-hydroxy-phthalic acid as described on p. 238, with a melting point of  $181^{\circ}$ – $182^{\circ}\text{C}$ . The insoluble residue, consisting of fine needles, was crystallised from 50 per cent. aqueous alcohol, from which it separated in small white needles which were apparently only very slightly soluble either in water or 50 per cent. alcohol. These had a melting point of  $242^{\circ}$ – $243^{\circ}\text{C}$ ., with decomposition, and gave no colour with ferric chloride either in alcoholic or aqueous solution. This fraction, which was small in amount, has not been further investigated. It appeared to be much less readily extracted from the acidified hydrolysis mixture than was dimethoxy-hydroxy-phthalic acid since, while the latter was almost completely extracted by two extractions with ether, the former was still being extracted in small amounts after six or seven extractions.

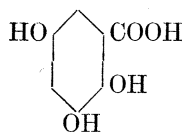
*Discussion of results obtained and their bearing on the constitution of citromycetin.*

Product F is almost certainly hydroxyquinol. A sample of hydroxyquinol made by THIELE's method (1898) from benzoquinone shrinks at  $139^{\circ}\text{C}$ . and melts at  $140\cdot5^{\circ}$ – $141^{\circ}\text{C}$ . There is no obvious change in melting point of a mixture of the synthetic material with Product F.

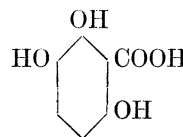
Product E is therefore a hydroxyquinolcarboxylic acid. There are three possible isomers of this compound.



(a)



(b)

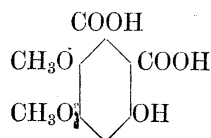


(c)

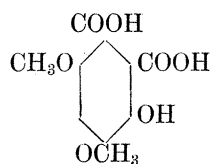
Of these only (*a*) has been described and this is apparently identical with Product E. The hydroxyquinolcarboxylic acid of the formula (*a*) was first prepared by THIELE and JAEGER (1901), by heating together hydroxyquinol and sodium bicarbonate in water. Its constitution was considered by VON HEMMELMAYR (1911) to be represented by formula (*c*) but it was finally settled by BERGELLINI and MARTEGANI (1912), who showed that, since on methylation with methyl sulphate it gives asaronic acid, it must have the constitution given in formula (*a*). A specimen of synthetic 1:3:4-trihydroxybenzoic acid (*a*) was prepared by THIELE and JAEGER's method given above and was shown to behave similarly to the product isolated from citromycetin. The variation in melting point with the rate of heating makes a mixed melting point of less diagnostic value than usual, but the melting point of a mixture of synthetic 1:3:4-trihydroxybenzoic acid and Product E was the same as Product E itself when the two melting points were determined side by side in the same apparatus.

Accepting that Product E is 1:3:4-trihydroxybenzoic acid then Product B, dimethoxy-hydroxy-phthalic acid, must of necessity have one of the two following formulæ since:—

- (*a*) It is a true phthalic acid and not an *iso*- or a *tere*-phthalic acid, and hence has its carboxyl groups vicinal to each other and
- (*b*) the free hydroxyl group must be in the *ortho* position to one of the carboxyl groups since the material arises from fully methylated citromycetin, which is a pyrone derivative.

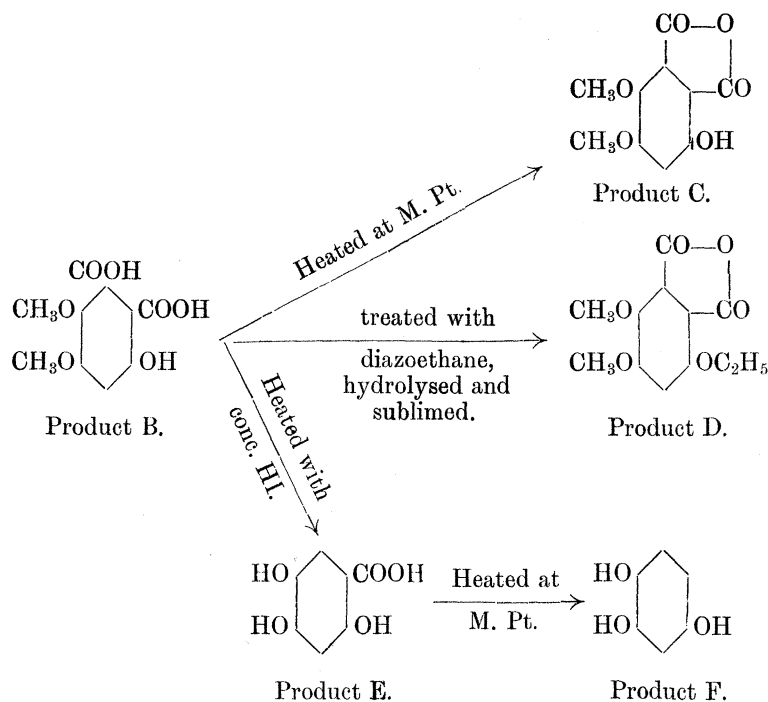


(1)

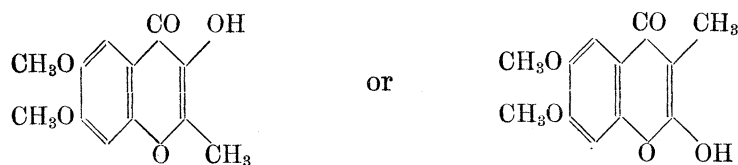


(2)

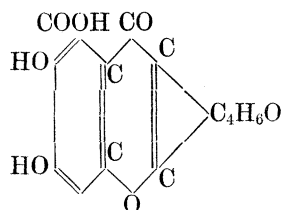
It has not yet been definitely established which of these formulæ is correct but formula (1) seems the more probable because of the strong dyeing properties of citromycetin (p. 244). It has been shown by LIEBERMANN and V. KOSTANECKI that marked dyeing properties in the natural colouring matters are almost invariably associated with the presence in the molecule of two hydroxyl groups in the *ortho* position to one another. Hence formula (1) may be taken as representing the dimethoxy-hydroxy-phthalic acid, and accepting this, the relationship of the various breakdown products arising from methylated citromycetin is shown in the following scheme:—



The formula for Product A then becomes one of the two following :—



and the formula for citromycetin becomes



The question of the seventh oxygen atom present in the group  $\text{C}_4\text{H}_6\text{O}$  has not been satisfactorily settled, and work is at present in progress to settle the constitution of this group. It is of interest in this connection, however, to note that alkaline hydrolysis of citromycetin or of its fully methylated derivative gives rise to considerable quantities of acetone, together with acetic acid and carbon dioxide (p. 236).

*Dyeing properties of citromycetin.*

A sample of citromycetin was submitted to Prof. A. G. PERKIN of the Department of Colour Chemistry and Dyeing, Leeds University, with the request that Prof. PERKIN should give an opinion as to whether citromycetin has any marked dyeing properties. The following reply was received from him, for which we desire to offer him our thanks.

“ 29th October, 1926.

“ I have made a cursory examination of your sample of citromycetin and find it to be quite a strong dyestuff. The shades given closely resemble those of luteolin, the colouring matter of WELD, though your product is not the same as this.

“ The reactions of the compound correspond to some extent with dyes of the xanthone or flavone group, more nearly perhaps to the former, in that it yields a green fluorescent solution with sulphuric acid.”

*Summary.*

The production of a new biochemical product—citromycetin—( $C_{14}H_{10}O_7 \cdot 2H_2O$ ) by various species of *Citromyces* is described. The influence of different factors on the yield of this material is given on pp. 212–215. Details of the method of preparation of citromycetin on a large laboratory scale are given on pp. 215–217. The general properties of the substance are described on pp. 218–221. The preparation and properties of a number of derivatives of citromycetin and of its decarboxylated product citromycin are given on pp. 221–230. The decomposition products of citromycetin are described (*a*) by acid hydrolysis (p. 230), (*b*) with alkaline iodine (p. 231), (*c*) by alkaline hydrolysis (p. 234), (*d*) by hydrolysis of methylated citromycetin with alcoholic potassium hydroxide (p. 237). The question of the constitution of citromycetin is discussed on pp. 241–243. A report on the dyeing properties of citromycetin, submitted by Prof. A. G. PERKIN, is given on p. 244.

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TABLE V.—Details of “Tank” experiments for preparation of Citromycetin. Organism used—*Citromyces* sp. Ad. 7.

Tank.	Carbon Compound Fermented.	Period of Incubation.			Location of Tank.	Type of Tray used.	Residual Glucose by Polarimeter.	Estimated Citro-mycetin as c.c. N/10 Iodine per 100 c.c. Medium.	Yield of Crude Citro-mycetin.	Remarks.
		Start.	Finish.	Duration in Days.						
1	Glucose	21.12.25	Tray 6 taken off 11.1.26 Remainder 18.1.26	21 28	Tank incubated in sterilizing room of old Biochemical Lab.	Enamelled iron	Per cent. 0.13	—	3.8 gm.	Tray 6 was the only tray of the whole 12 giving a reasonable ferric chloride reaction, and it was from the contents of this tray that the 3.8 gm. were obtained. None of the other trays gave a good reaction with ferric chloride and no citromycetin could be isolated from any of them. The temperature of the sterilizing room in which Tanks 1–10 were incubated fluctuated very widely ranging between 10°–26° C. in the earlier tanks and 20°–26° C. in the later tanks where an attempt was made to lessen the fluctuations.
2	Glucose in 9 trays. Maltose in 3 trays.	29.1.26	3 trays on 9.2.26 5 trays on 10.2.26 4 trays on 11.2.26	11 12 13	Ditto	Ditto	0.2–0.6	—	125.7 gm. from 9 glucose trays. 50.5 gm. from 3 maltose trays.	The yield of citromycetin judged from the ferric chloride reaction varied considerably from tray to tray and although a considerable amount of crude material was isolated subsequent investigations showed that the content of citromycetin was not very large. No advantage was evident in using maltose as compared with glucose.
3	Glucose	22.4.26	4 trays on 3.5.26 5 trays on 4.5.26 3 trays on 5.5.26	11 12 13	Ditto	Ditto	0.14–0.58	—	116.4 gm.	In this tank 0.02 per cent. of ferrous sulphate was used in the medium for the first time, Tanks 1 and 2 having only contained 0.001 per cent. It was noticed that the mycelium in all trays was covered with spore heads and was of a fairly uniform dark greenish colour. The results in different trays were still erratic and investigation showed that aeration had not been uniform. The quality of the crude citromycetin was very much superior to that obtained in Tank 2.
4	Glucose	8.5.26	3 trays on 18.5.26 4 trays on 19.5.26 5 trays on 20.5.26	10 11 12	Ditto	Ditto	0.11–0.35	—	131.3 gm.	The yield of citromycetin in the different trays still varied very considerably although in this tank an attempt was made to secure uniform and ample aeration. The quality of crude citromycetin was quite good.
5	Glucose	22.5.26	6 trays on 1.6.26 6 trays on 2.6.26	10 11	Ditto	Ditto	0.15–0.4	223.3	142.2 gm.	In this tank each tray was sown with the spores from two Roux bottles instead of the customary one. Even as early as the fourth day the mycelium of all trays visible was this time seen to be green. The trays were more uniform than previously. It was definitely shown by analysis that very little citromycetin is left in the acidified and evaporated solution after standing for some time.
6	Glucose	24.12.26	2 trays on 6.1.27 3 trays on 8.1.27 1 tray on 10.1.27 3 trays on 12.1.27 3 trays on 14.1.27	13 15 17 19 21	Ditto	Ditto	nil–0.180 (average 0.095)	107–236 (average 164.3)	84.6 gm.	Sowing consisted of one Roux bottle per tray. In this tank for the first and only time aluminium grids were placed in each of the trays to support the mycelial felt. The poor results and small yields of citromycetin appear to be due in part at any rate to the presence of aluminium grids, since considerable quantities of potash alum crystals separated from the concentrated mother liquors on standing indicating that considerable erosion of the aluminium grids must have taken place.
7	Glucose	12.2.27	6 trays on 22.2.27 5 trays on 23.2.27 1 tray on 24.2.27	10 11 12	Ditto	Ditto	0.11 (average of 12 trays)	247.1 (average of 12 trays)	125.7 gm.	The different trays gave more uniform results than previously with the exception of one tray in which growth was not quite so good as in the remainder. The quality of citromycetin obtained was quite good.
	Glucose	19.3.27	1 tray on 31.3.27 11 trays on 2.4.27	12 14	Ditto	Ditto	0.17 (average sample of all 12 trays)	120.0 (average of 12 trays)	70 gm.	This tank gave entirely unsatisfactory results for which no explanation has been forthcoming, but the unsatisfactory nature of it is evidenced not only by the low yield of crude citromycetin isolated but also by the estimated figure with alkaline iodine.
	Glucose	2.4.27	12 trays on 18.4.27	16	Ditto	Ditto	See remarks	See remarks	25.5 gm. See remarks.	In experiments 9–12 the new aluminium tank was used whereas in experiments 1–8 the old iron tank constructed in the factory had been used throughout. On opening the tank on 18.4.27 it was seen that only one tray had given a really typical growth with a characteristic colour reaction. Three others were fairly satisfactory but by no means good. These four trays were worked up in the usual way and gave a yield of 25.5 gm. of crude citromycetin. All the other trays gave little or no ferric chloride reaction and microscopic examination revealed the fact that they were all infected by extraneous organisms. These eight trays were therefore rejected and were not worked up. The cause of infection seems to have been splashing of the wash water from the air wash bottle into the trays.
	Glucose	29.4.27	12 trays on 10.5.27	11	Ditto (average temperatures 24°–26° C.)	Ditto	0.018 (average sample)	289.8 (average sample)	187 gm.	This tank was uniformly good throughout and gave the best yields and best quality of material so far obtained. The method of aeration adopted was to leave the tank for three days after inoculation with cotton wool plugs in the sampling holes. Aeration was carried out simultaneously through the main perforated pipe and through the twelve side tubes. At no time were any of the trays aerated separately and aeration of all trays was continuous from 2.5.27 to 10.5.27. The rate of aeration was about 50 cubic feet per hour. The sowing consisted of a suspension of spores from six Roux bottle cultures on beer wort agar. These were half of twelve sown on 25.3.27, the other six having been used for Tank 9. The six cultures used for Tank 10 had been stored from 2.4.27 till the date of using in an unused incubator at room temperature with the necks of the Roux bottles plugged but uncovered.
	Glycerol (Price's pure)	21.11.27	6 trays on 15.12.27  6 trays on 17.12.27	24  26	New Biochemical Lab. (Average temperature about 18° C.) Ditto	6 silica trays.  6 enamelled iron trays	—  —	400.2  186.9	117.0 gm.  58 gm.	In this tank six fused quartz trays referred to on p. 213 were used for the first time and in the other half of the tank six of the old enamelled iron trays were used. The results obtained with the silica trays were very much better than those obtained with the enamelled iron trays. The yield of citromycetin was almost twice as large, amounting for a full tank to the equivalent of 234 gm. The quality of the crude citromycetin was very much superior and the colour of the metabolism solution in the silica trays was a dark orange compared with a brownish black in the iron trays. This indicates that the mould is attacking and dissolving the enamel and the metal of this type of tray with deleterious results to the yield and quality of citromycetin. The appearance of the mycelium at the end of the experiment is quite different from that usually obtained on a glucose medium. On glucose this mould spores freely giving a dark green felt, whereas in a glycerol medium the mycelium is practically white at the end of the experiment.
2	Glycerol (Price's pure)	23.12.27	6 trays on 17.1.28 6 trays on 18.1.28	26 27	Ditto Ditto	6 silica trays 6 enamelled iron trays	— —	326.3 251.2	89.4 gm. 70.9 gm.	The results obtained with Tank 12 were very similar to those from Tank 11 except that the difference in yield between the silica and enamelled iron trays was not quite so marked.