PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

On the Production and Chemical Constitution of a New Yellow Colouring Matter, Citrinin, Produced from Glucose by Penicillium citrinum Thom

Arthur Clement Hetherington and Harold Raistrick

Phil. Trans. R. Soc. Lond. B 1931 **220**, 269-295
doi: 10.1098/rstb.1931.0025

Studies in the Biochemistry of Micro-organisms.

PART XIV.—*On the production and chemical constitution of a new yellow colouring matter, citrinin, produced from glucose by* Penicillium citrinum THOM.

By ARTHUR CLEMENT HETHERINGTON *and* HAROLD RAISTRICK.

It has been known for some time that *Penicillium citrinum* THOM when grown on suitable media produces a characteristic lemon yellow colour. Thus THOM in his monograph on "Cultural Studies of species of *Penicillium*" (1910), says, "*P. citrinum* produces a lemon yellow colour, soluble in alcohol, in media containing sugars, milk, gelatin, bread and potato." This colouring matter was not isolated, however, nor was its nature further investigated.

In the course of an investigation into the colour reactions given with ferric chloride by metabolism solutions produced by various species of *Penicillium*, it was found that a very characteristic reaction was given by each of the strains of *Penicillium citrinum* THOM in our possession, but was not given by any other species in pure culture whether of *Penicillium* or of any other genus tested.

The three strains of *P. citrinum* THOM used in this work were sent to Dr. CHARLES THOM for his opinion. Their history is as follows :—

1. *P. citrinum* THOM. Catalogue Number Ad. 23, received from Mr. F. T. BROOKS, of Cambridge, in 1925. Dr. THOM says of this culture, "Ad. 23 varies somewhat in structure from the usual forms of *P. citrinum* while harmonising in general reactions with that species. In microscopic examination and cultural reactions, however, it is closest to a strain received from Baarn and carried as 4876·24 but labelled by Dr. WESTERDIJK as *P. fellutanum* BIOURGE. As this organism was placed in my original draft of the manuscript for my book, it would have been very near to *P. citrinum*, but because the more frequent type of conidial mass was monoverticillate, I transferred it in the final write-up to that section, cross-referencing it to indicate its possible relationship to the *P. citrinum* series."
2. *P. citrinum* THOM. Catalogue No. Ad. 95. This strain is Dr. THOM'S own culture No. 4733·14, and was purchased from the American Type Culture

Collection, Catalogue No. 1109. Dr. THOM says of this culture, "No. 95 is the organism Miss CHURCH sent to you and to Mr. BROOKS as *P. citrinum*."

3. *P. citrinum* THOM. Catalogue No. Ad. 114. This strain was received from Mr. L. D. GALLOWAY, of the Shirley Institute of the Cotton Research Association, under the label *P. aurifluum* (No. 94), which, according to BIOURGE, is almost synonymous with *P. citrinum*. Dr. THOM says of this organism, "No. 114 is another of the variant forms but is near enough to this group to be left in it."

When each of these three strains is grown on a modified CZAPEK-DOX glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7, in vessels having an ample supply of air, *e.g.* in flasks loosely plugged with cotton wool, the metabolism solution gradually takes on a yellow colour which deepens in tone until it becomes orange yellow. The filtered metabolism solution which is orange yellow in colour gives the following reactions :—

(1) Addition of ferric chloride solution gives first a heavy buff coloured precipitate which dissolves in excess of the reagent to give a very intense iodine-brown coloured solution.

(2) Potassium permanganate solution is immediately decolorised giving rise to a solution which no longer gives a colour with ferric chloride.

(3) Acidification by means of HCl or H_2SO_4 causes immediate discharge of the orange yellow colour of the medium and the deposition on standing of a yellow microcrystalline powder having a characteristic appearance under the microscope.

These crystals consist of yellow, flat, narrow, elliptical plates having pointed ends and serrated edges and being usually combined in groups of two or three by their extremities. This substance to which we propose to give the name "citrinin" appears to be characteristic of *P. citrinum* THOM, since, apart from the above three strains of *Penicillium citrinum*, no other species of *Penicillium* nor indeed of any other genus of fungi amongst the 400 to 500 species tested gives the typical ferric chloride reaction or the equally typical micro-crystalline deposit on acidification. Of the above three strains of *P. citrinum* the best yields of citrinin are given by the strain Catalogue No. Ad. 23, though considerable quantities have been prepared from each of the other two strains Catalogue Nos. Ad. 95 and Ad. 114.

The preparation in fair quantity of this substance and the investigation of its chemical properties and constitution are given in the following paper.

Preparation of citrinin.

Thirty litres of CZAPEK-DOX glucose solution were made and distributed in 350 c.c. quantities in 85 1-litre conical flasks plugged with cotton wool. After sterilizing by steaming for half an hour on each of three consecutive days, the flasks were sown with a suspension of spores of *P. citrinum* Ad. 23 in distilled water, prepared from ROUX bottle cultures on beer wort agar. The flasks were then incubated at 28° C. until the production of citrinin had reached a maximum.

A rough measure of the amount of citrinin present is given by the figure obtained on subtracting the percentage of glucose present as estimated by the polarimeter, from the percentage of glucose present as estimated by alkaline iodine. This figure, however, is, at the best, only an approximation to the true amount of citrinin present, since citrinin itself is lævorotatory. It will be shown on p. 276 that citrinin is readily acted on by alkaline iodine. The point of maximum production of citrinin is generally reached when the glucose has decreased to about 1 per cent., and it has been found that if incubation is continued after this point there is a gradual decomposition and loss of citrinin.

The metabolism solution is now filtered and the filtrate acidified by the addition of 100 c.c. of concentrated HCl. There is an immediate production of a yellow turbidity which on standing gradually resolves itself into a yellow micro-crystalline deposit. This is filtered off, washed with water and dried, and consists almost entirely of practically pure citrinin. Citrinin is so very slightly soluble in water that only a very small amount is left in the acidified solution after standing overnight, and attempts to recover this are not worth while. The yields of citrinin obtained from 30 litres of metabolism solution varied between 45 and 60 gm. As thus obtained citrinin is a very light yellow powder which on inhalation gives rise to violent fits of sneezing.

A table, Table I, is attached giving particulars of the various batches of citrinin produced, the various data included being the time of incubation, residual glucose, production of material as estimated by alkaline iodine (see p. 276) and yield of crude product actually recovered from 30 litres of medium in each case.

It will be noted on examination of Table I that with batches 8 and 10 the cultures used were spp. Ad. 95 and Ad. 114 respectively, as mentioned on pp. 269 and 270. These batches were grown with a view to ascertaining definitely that these two strains would produce citrinin when grown under identical conditions with Ad. 23.

Effect of temperature on the yield of citrinin.

While engaged on the collection of sufficient material for the purposes of investigation, it was considered advisable to study the temperature conditions for the optimum yield of citrinin.

An experiment was carried out on test tube scale to ascertain the effect of various temperatures on the production of citrinin from glucose by Ad. 23. The following temperatures were employed :—Room temperature, 24°, 28°, 30°, 32°, 34° and 37° C. After 19 days' incubation, tubes were taken off for analysis and the results are shown in Table II.

From these figures it appears that, in general, there is an optimum temperature range of approximately 28° to 32° C.

TABLE I.—Details of experiments for the preparation of citrinin from glucose.

Expt.	Period of Incubation.			Containing Vessel.	Residual Glucose by Polari-meter.	Residual Glucose by Alkaline Iodine.	Difference	Yield per 30 litres.	Organism used.	Remarks.
	Start.	Finish.	Time.							
			Days.		Per cent.	Per cent.	Per cent.	Gm.		
1	30.6.27	14.7.27	14	Tank, 60 litres	0·712	0·799	0·087	4·0	Ad. 23	Quality of product poor. Grown at room temperature.
2	19.7.27	10.8.27	22	Tank, 60 litres	0·497	0·545	0·048	9·5	Ad. 23	Grown at room temperature. Owing to poor results with tank, future experiments conducted in flasks plugged with cotton wool.
3	3.8.27	19.8.27	16	Flasks, 30 litres	0·492	0·966	0·474	45·3	Ad. 23	Grown at 28–30° C.
4	8.8.27	22.8.27	14	Flasks, 30 litres	0·773	1·343	0·570	46·0	Ad. 23	Grown at 28° C.
5	27.8.27	12.9.27	16	Flasks, 30 litres	0·790	0·928	0·138	12·7	Ad. 23	Grown at 28° C. Product dark in colour, amorphous.
6	2.9.27	15.9.27	13	Flasks, 30 litres	1·172	1·781	0·609	41·2	Ad. 23	Grown at 28° C. Product lighter and more crystalline.
7	15.9.27	27.9.27	12	Flasks, 30 litres	1·045	1·756	0·711	40·0	Ad. 23	Grown at 28° C.
8	19.9.27	4.10.27	15	Flasks, 30 litres	0·591	0·911	0·320	15·3	Ad. 95	Product dark and somewhat tarry.
9	1.10.27	13.10.27	12	Flasks, 30 litres	0·954	1·589	0·635	58·0	Ad. 23	The best yield so far obtained.
10	11.6.29	29.6.29	18	Flasks, 21·75 litres	1·04	1·38	0·34	19·3	Ad. 114	Productdark and somewhat tarry, *cf.* experiment 8.
11	14.11.27	26.11.27	12	Flasks, 30 litres	0·995	1·647	0·652	41·9	Ad. 23	

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE II.

Temperature of Incubation.	Total Glucose by Alkaline Iodine.	Glucose by Polarimeter.	Net Iodine Figure.	Reaction with HCl.	Reaction with excess $FeCl_3$.
					Comparative Dilution.
	per cent.	per cent.	per cent.		
Room	4·118	4·016	0·102	Slight precipitate after standing	0 (standard).
24° C.	2·425	2·041	0·384	Medium precipitate ...	0·7 c.c.
28° C.	2·161	1·656	0·505	Medium precipitate ...	6·0 c.c.
30° C.	1·990	1·574	0·416	Medium precipitate ...	6·5 c.c.
32° C.	1·634	0·516	1·118	Medium precipitate ...	7·0 c.c.
34° C.	0·878	0·775	0·103	Slight opalescence on standing	3·7 c.c.
37° C.	1·765	1·734	0·031	Slight opalescence on standing	1·5 c.c.

Note.—The figures in the last column represent the degrees of dilution to correspond with the depth of colour in an undiluted tube grown at room temperature.

Production of citrinin by P. citrinum *Ad.* 95.

A number of flasks were prepared containing a medium exactly as used for Ad. 23. These were filled and sterilized in the manner described on p. 270 and after sterilization were sown with an emulsion of spores of *P. citrinum* Ad. 95. The flasks were then incubated in the usual fashion at 28° C., and after the production of citrinin had reached a maximum (*i.e.*, after 15 days) as shown by the difference between the glucose estimated by polarimeter and the glucose estimated by alkaline iodine, the metabolism solution was filtered, acidified as before, and the precipitated product filtered off for purification.

The product in this case, being somewhat dark in colour and apparently not very pure, was placed in a Soxhlet thimble and extracted for some hours with ether. This gave rise to a crop of yellow crystals which, after recrystallising from alcohol, melted with decomposition at 170°–172° C., *cf.* p. 274. On titration 0·1668 gm. of this product required 6·78 c.c. of N/10 NaOH corresponding to a combining weight of 249·6, thus showing that the crystalline substance isolated is the same as that produced by Ad. 23, *cf.* p. 275.

Production of citrinin by P. citrinum *Ad.* 114.

A similar experiment was carried out using cultures of *P. citrinum* Ad. 114. In this case the production of citrinin had reached a maximum after 18 days. The product, which was yellow brown in colour, was isolated by precipitation with acid as before. A portion of this dried product was extracted with ether in a Soxhlet apparatus and the dried extract was recrystallised from alcohol. Typical yellow prisms were obtained which gave the characteristic colour reaction with $FeCl_3$ and melted with decomposition at 170° C. The yield of crude citrinin obtained from 21·75 litres of modified Czapek-Dox solution was 14 gm.

Purification of citrinin.

The samples of citrinin obtained as described on p. 271 by simple acidification of the metabolism solution are sufficiently pure for most purposes, but in order to obtain an absolutely pure sample for analysis the crude citrinin was recrystallised several times from boiling absolute alcohol in which it is readily soluble, and from which it separates in golden yellow prismatic needles, which if left undisturbed during crystallisation may attain a length of 1 cm. These crystals are highly refractive.

General properties of citrinin.

Citrinin crystallises from alcohol in golden yellow prismatic needles which are practically insoluble in cold water but dissolve to a very slight extent on boiling and separate on cooling in the form of a fine suspension.

It is readily soluble in aqueous NaOH, or aqueous Na_2CO_3 (with evolution of CO_2), giving rise to an orange yellow solution which on standing changes colour to orange red. It is also soluble in aqueous solutions of sodium acetate giving rise to a pale yellow solution, thus indicating the presence in the molecule of a carboxyl group. It is immediately precipitated on acidification of its aqueous solution in NaOH, Na_2CO_3 or sodium acetate.

It is slightly soluble in hot ether from which solvent it crystallises in needles on evaporation. It is fairly readily soluble in both chloroform and acetone. Alcohol is the most suitable solvent for recrystallising citrinin since it is not very soluble in cold alcohol but is readily soluble on heating.

A neutral aqueous solution of citrinin in sodium hydroxide gives a heavy buff coloured precipitate with ferric chloride which is soluble in excess of ferric chloride, giving rise to a very intense iodine-brown coloured solution. A similar colour reaction is given by ferric chloride with an alcoholic solution of citrinin. It immediately decolorises acid potassium permanganate solution in the cold and on treatment with alkaline iodine is oxidized with production of iodoform.

On heating with soda lime pungent vapours are given off with an odour strongly reminiscent of phenol.

On heating, citrinin melts at 166°–170° C. with obvious decomposition.

Analysis of citrinin.

A sample of citrinin recrystallised from absolute alcohol, and air dried, does not lose weight on drying at 100° C., thus indicating the absence of solvent of crystallisation.

A sample of citrinin recrystallised from absolute alcohol and dried at 100° C. gave the following results on combustion :—

TABLE III.

Weight of Citrinin Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Gm.	Gm.	Gm.		
0·1434	0·3271	0·0721	62·20	5·63
0·1278	0·2921	0·0638	62·32	5·58
Theoretical for $C_{13}H_{14}O_5$	—	—	62·37	5·64

Determination of combining weight of citrinin by titration.

0·3941 gm. of dry citrinin was suspended in water and N/10 NaOH added to the first appearance of a permanent pink with phenolphthalein. 15·77 c.c. of N/10 NaOH were required, corresponding to a combining weight of 249·9.

Assuming that citrinin titrates as a monobasic acid, its molecular weight is thus 249·9, or if it is a dibasic acid, 499·8. The molecular weight of $C_{13}H_{14}O_5$ is 250 so that it appears probable that citrinin has the formula $C_{13}H_{14}O_5$ with a molecular weight of 250 and titrates to phenolphthalein as a monobasic acid.

Molecular weight of citrinin.

In a determination of the molecular weight by RAST'S method, 0·00354 gm. citrinin caused a depression of 9·89° C. in the melting point of 0·03994 gm. camphor. This corresponds to a molecular weight of 356. It was noted, however, that distinct signs of some decomposition or interaction between the citrinin and camphor were visible during the experiment, a fact which would render the above estimation invalid.

Optical activity of citrinin.

Citrinin is lævorotatory in alcoholic solution. 0·4996 gm. of citrinin was dissolved in 100 c.c. of absolute alcohol and polarised in the mercury green light in a 20 cm. tube. The mean of a number of rotations was — 0·4167° corresponding to $[\alpha]_{\text{Hg. green}} = -41 \cdot 7°$.

0·2543 gm. dissolved in 25·08 c.c. absolute alcohol gave an average rotation of —0·891° in a 20 cm. tube with the mercury green light. This corresponds to a specific rotation of $[\alpha]_{\text{Hg. green}} = -43 \cdot 9°$.

Determination of methoxyl groups in citrinin.

An ordinary Zeisel estimation was carried out in the usual Perkin form of apparatus. 0·1968 gm. of dry citrinin gave 0·0059 gm. silver iodide thus indicating the absence of any methoxyl groups in the molecule of citrinin.

Action of alkaline iodine on citrinin.

0·2559 gm. of citrinin was dissolved in the theoretical amount of sodium hydroxide and made up to 100 c.c. with water, this representing an approximately M/100 solution of citrinin. Varying amounts of this standard solution were then treated with 50 c.c. of N/10 iodine and to the mixture were added during the space of three minutes 75 c.c. of N/10 NaOH. This mixture was then allowed to stand for one hour, acidified with 10 c.c. of N/1 HCl and the iodine liberated was titrated with N/10 sodium thiosulphate. The results are given in Table IV.

Table IV.

c.c. of Solution of Citrinin used.	c.c. of N/10 Iodine absorbed.	c.c. N/10 Iodine absorbed by Indicated Volume of exactly M/100 citrinin.	c.c. N/10 Iodine absorbed per 10 c.c. M/100 Citrinin.
1·0	1·15	1·13	11·27
2·0	1·99	1·94	9·70
5·0	4·56	4·45	8·90
10·0	8·45	8·25	8·25
15·0	11·37	11·11	7·41
20·0	14·00	13·68	6·84

The figures in the last column thus represent the number of atoms of iodine taken up by one molecule of citrinin. Under the particular conditions of the experiment this figure seems to vary between 7 and 11.

The solution containing the higher concentrations of citrinin contained a yellow precipitate which smelt of iodoform and those with 10 c.c. or more were pink in colour, the intensity of colour being proportional to the concentration of citrinin.

It was definitely established that iodoform is formed by the action of alkaline iodine on citrinin by the following procedure: 1·25 gm. of citrinin were dissolved in 50 c.c. of N/10 NaOH to which were added 3,825 c.c. of water and 2,500 c.c. of N/10 iodine. To this mixture 375 c.c. of N/1 NaOH were added slowly and with constant shaking. The mixture was allowed to stand for about an hour by which time a yellow precipitate had formed and the solution was coloured pale pink. The precipitate was filtered off and recrystallised from acetone. It crystallised in typical yellow hexagonal plates having a melting point of 118°–122° C. and the characteristic odour of iodoform.

Derivatives of citrinin.

Various attempts were made to obtain crystalline derivatives of citrinin and the particulars of the methods adopted are as follows :—

(1) *Acetylation of citrinin.*—An attempt to acetylate citrinin by treatment with acetic anhydride and anhydrous sodium acetate resulted in an oil which could not be crystallised, but an estimation of the hydroxyl content of citrinin was carried out, according to the method of PETERSON and WEST (1927).

0·7795 gm. of citrinin gave an acetyl value corresponding to 3·16 c.c. of N/1 NaOH which corresponds to the acetylation of one hydroxyl group in citrinin under the conditions employed, assuming that acetylcitrinin titrates as a monobasic acid.

(2) *Ethylation of citrinin.*—2 gm. of citrinin were suspended in a little dry ether and were ethylated by the addition of an excess of an ethereal solution of diazoethane. A vigorous reaction occurred immediately, slowing down quickly until in about 10 minutes no further evolution of gas was evident. The mixture, still containing a large excess of diazoethane, was allowed to stand overnight. In the morning the ether and residual diazoethane were evaporated off and the ethylated citrinin dried at 60° C. *in vacuo.*

As thus obtained, ethylated citrinin consisted of a pale yellow syrup insoluble in water or sodium hydroxide and resisted all attempts to crystallise it. It distilled at a pressure of about 1 mm. with a bath temperature of 170°–210° C. The distillate consisted of a pale yellow oil which did not crystallise. As there were obvious signs of decomposition the undistilled material was used for analysis.

A methyl compound, similar in appearance, physical and chemical properties, to the ethyl compound, is produced when citrinin is methylated by means of dimethyl sulphate and sodium hydroxide.

The ethoxyl content of the undistilled ethylated citrinin dried to constant weight at 60° C. *in vacuo*, was determined by a ZEISEL estimation with the results given in Table V.

TABLE V.

Weight of Substance Analysed.	Weight of Silver Iodide.	Percentage OC_2H_5.
Gm.	Gm.	
0·2460	0·3846	29·96
0·1792	0·2822	30·17
0·2265	0·3528	29·85
Theoretical for $C_{13}H_{12}O_3(OC_2H_5)_2$	—	29·41

The product is thus a diethyl compound, and since it is insoluble in sodium hydroxide it appears that the carboxyl group has been esterified and that the product is therefore an ethyl ester of *O*-mono-ethylcitrinin, $C_{12}H_{12}O_2.(OC_2H_5).(COOC_2H_5)$.

3. *Reduction of citrinin.*—When citrinin is reduced by means of nascent hydrogen it loses its orange yellow colour and gives rise to a colourless product.

5 gm. of citrinin were dissolved in methyl alcohol, 1–2 c.c. of glacial acetic acid added and then an excess of zinc dust was added in small amounts, the mixture being kept cold. The yellow colour of the citrinin quickly disappeared and when the liquid was quite colourless it was diluted with water, acidified with a little dilute sulphuric acid and shaken out with ether. Although the reduction product is colourless it absorbs oxygen so quickly that the ethereal solution is distinctly yellow in colour in a few minutes. Owing to the instability of the reduction compound an analysis was not carried out on it but it showed the following properties: Reduced citrinin when freshly prepared is a white crystalline product which melts at about 240° C. Its alcoholic solution gives a bright blue colour with a trace of ferric chloride, but with excess of ferric chloride an iodine brown colour is obtained exactly like that given by citrinin itself, reduced citrinin being apparently readily oxidized by ferric chloride to citrinin.

The formula for reduced citrinin can be inferred from :—

4. *Diacetyl compound of reduced citrinin.*—5 gm. of crude citrinin were dissolved by warming in 30 c.c. of methyl alcohol and 2 c.c. of glacial acetic acid were added. To this, zinc dust was added in small portions with shaking until the mixture was nearly colourless and hydrogen began to be evolved vigorously. The temperature was maintained at about 40°–50° C. during the reduction which took about 10 minutes. The mixture was transferred to a separating funnel, acidified with H_2SO_4 and extracted with ether. The white precipitate which forms on acidifying is immediately soluble in the ether. The ether was filtered and quickly evaporated and the residue in the flask dried overnight *in vacuo* over H_2SO_4 and KOH.

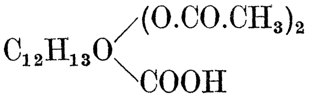
Next day 15 c.c. of acetic anhydride and 7·5 c.c. of pyridine were added, and the acetylation effected by leaving for 24 hours at 37° C. The mixture was diluted with water, filtered from a small amount of insoluble material, and the acetyl derivative precipitated from the clear filtrate by acidification with dilute H_2SO_4. The acetyl derivative was purified by redissolving in aqueous sodium acetate solution, decolorising with blood charcoal and acidifying the clear colourless filtrate. It was finally washed thoroughly and dried for several days *in vacuo* over P_2O_5.

This material was analysed as follows :—

A weighed quantity was dissolved in alcohol and titrated with N/5 sodium hydroxide to phenolphthalein (column 2, Table VI). An excess of 100 c.c. of N/5 sodium hydroxide was now added and the mixture hydrolysed by boiling for three hours in an atmosphere of nitrogen. After cooling, the residual excess of N/5 sodium hydroxide was titrated with N/5 H_2SO_4, the difference representing the amount of acid formed on hydrolysis being given in column 4, Table VI. Finally the acetic acid formed during hydrolysis was estimated by exhaustive distillation *in vacuo.* The combined distillates were titrated with N/10 sodium hydroxide and this figure was corrected for a small amount of formic acid formed during hydrolysis, the amount of which was estimated by a modification of FINCKE's method (see Part VIII). The corrected figures are given in Table VI, column 6.

TABLE VI.

Weight of Material Analysed.	Initial Titration.		Acidity formed on Hydrolysis.		Acetic Acid found on Hydrolysis.	
	Found.	Calculated.	Found.	Calculated.	Found.	Calculated.
Gm.	c.c. N/5.	c.c. N/5.	c.c. N/5.	c.c. N/5.	c.c. N/10.	c.c. N/10.
0·6084	9·14	9·06	17·84	18·11	36·04	36·22
0·6110	9·06	9·09	17·31	18·19	36·05	36·37

Assuming that the acetyl compound has the formula  $C_{12}H_{13}O\begin{cases}(O.CO.CH_3)_2\\COOH\end{cases}$ the following calculations have been made :—

(*a*) Initial titration value ... ... Table VI, column 3.

(*b*) Acidity formed on hydrolysis... ... ... Table VI, column 5.

(*c*) Acetic acid formed on hydrolysis ... ... Table VI, column 7.

The agreement between the calculated values and those found by analysis is very good.

The diacetyl derivative of reduced citrinin is not easy to recrystallise from any organic solvent. Three different samples were prepared for combustion :—

(*a*) Ex acetone ; (*b*) ex acetone and ether ; (*c*) ex methyl alcohol.

The first two samples (*a* and *b*) contained solvent of crystallisation which is lost on drying at 100° C. The product recrystallised from acetone separated in colourless prisms which give no colour with ferric chloride, and are readily soluble in aqueous solutions of sodium hydroxide, sodium carbonate, or sodium acetate. On heating, this product begins to darken in colour about 200° C., finally becoming very dark and melting with decomposition at 322°–323° C.

Combustion results obtained on each of the above three samples after drying to constant weight *in vacuo* over P_2O_5 at 50° C. are given in Table VII (SCHOELLER, Berlin).

The results given in Tables VI and VII indicate that while citrinin ($C_{13}H_{14}O_5$) only contains one hydroxyl group, since it gives a mono-acetyl derivative (p. 277) and a mono-ethyl ether of the ethyl ester (p. 277), reduced citrinin contains two hydroxyl groups since it forms a diacetyl derivative. The formation of an additional hydroxyl group on

TABLE VII.

Sample Analysed.	Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Per cent. C.	Per cent. H.
	Mgm.	Mgm.	Mgm.		
(*a*) Ex acetone	4·773	10·485	2·62	59·90	6·14
(*b*) Ex ether-acetone	4·815	10·610	2·65	60·10	6·16
(*c*) Ex methyl alcohol	4·829	10·650	2·57	60·16	5·96
Theoretical for diacetyl dihydrocitrinin $C_{12}H_{13}O_3$ $(COCH_3)_2$ COOH ...				60·69	6·00
Theoretical for diacetyl citrinin $C_{12}H_{11}O_3$ $(COCH_3)_2$ COOH				61·05	5·43

reduction is further indicated by the fact that while citrinin gives an iodine brown colour with $FeCl_3$, reduced citrinin gives an initial blue colour becoming iodine brown on addition of excess of $FeCl_3$.

(5) *Bromine compound of citrinin.*—A bromine compound was obtained by treating citrinin with bromine in glacial acetic acid. This gave rise to a yellow crystalline compound, M.Pt. 121° C., which decomposed readily on treatment with water, with the formation of hydrobromic acid.

(6) *Distillation of citrinin with zinc dust.*—An attempt was made to obtain the basal aromatic nucleus contained in the molecule of citrinin by distillation with zinc dust, but no product could be isolated except a small quantity of tarry distillate with a strong phenolic odour.

This phenolic odour is also noticed when citrinin is heated with soda lime.

(7) *Phenylhydrazide of citrinin.*—It was found possible to prepare a crystalline derivative of citrinin by acting on it with phenylhydrazine in acetic acid solution.

5 gm. of citrinin were dissolved in a mixture of 5 c.c. of phenylhydrazine and 10 c.c. of glacial acetic acid. The whole formed an oily mixture on warming slightly. 100 c.c. of water were then added and the mixture heated on a boiling water bath for 15 minutes with constant stirring. On cooling the product separated as a sticky lump which was washed several times by boiling with water and finally purified by crystallising from absolute alcohol. The substance crystallises in pale yellow, flat prisms, not quite plates, and melts at 207° C. with decomposition. One striking property of this substance is the great rapidity with which its solutions in alkali darken on exposure to air, the colour being brownish yellow at the start and rapidly changing through green and dark blue until practically black.

This compound is insoluble in water, in aqueous potassium acetate or bicarbonate solution but dissolves in dilute sodium hydroxide solution. It gives a cherry red colour with $FeCl_3$ solution, contains nitrogen, and with $FeCl_3$ and concentrated H_2SO_4 gives a deep blue colour. The last-mentioned colour reaction is one which is commonly shown by phenylhydrazides.

Nitrogen analyses by DUMAS' method gave 8·44 per cent. and 8·33 per cent. N_2.

A phenylhydrazide or a phenylhydrazone of citrinin would require a nitrogen content of 8·24 per cent. corresponding to $C_{19}H_{20}O_4N_2$ while a phenylhydrazine salt of citrinin would require a nitrogen content of 7·82 per cent. corresponding to $C_{19}H_{22}O_5N_2$.

Taking the above figures for the nitrogen content of this compound, and also considering the fact that it is insoluble in potassium acetate solution and that citrinin cannot be shown to exhibit ketonic properties with other ketonic reagents, it appears probable that the derivative obtained is a phenylhydrazide of citrinin.

As little success had been obtained in attempts to make derivatives of citrinin the breakdown products of citrinin were now investigated.

DECOMPOSITION PRODUCTS OF CITRININ.

A. BY ACID HYDROLYSIS.

It was observed on a small scale that when citrinin is boiled with dilute sulphuric acid it loses its colour and obvious decomposition takes place.

An acid hydrolysis on quantitative lines was therefore carried out as follows :—

1·1036 gm. of citrinin were boiled with 50 c.c. of $2N.H_2SO_4$ for four hours by which time the citrinin had gone completely into solution. The hydrolysis was carried out in an atmosphere of nitrogen, freed from oxygen and carbon dioxide, and the gaseous products were passed up a reflux condenser and bubbled through a measured quantity of barium hydroxide. CO_2 was obviously evolved and was estimated by titration of the barium hydroxide.

CO_2 equivalent to 16·97 c.c. of N/2 HCl was evolved, corresponding to 16·9 per cent. of CO_2. Assuming that one molecule of citrinin, $C_{13}H_{14}O_5$, loses one molecule of CO_2, 17·6 per cent. of CO_2 would be theoretically evolved.

The residual hydrolysis solution was now diluted and distilled exhaustively *in vacuo* until the volatile acids present had been completely removed. The volatile acid distilled was titrated with N/10 NaOH, 43·40 c.c. being required for neutralisation. Assuming that one molecule of citrinin, $C_{13}H_{14}O_5$, gives rise on acid hydrolysis to one molecule of volatile acid, 1·1036 gm. of citrinin would require 44·14 c.c. of N/10 NaOH to neutralise the volatile acid produced.

Hence the inference from the above figures is that on hydrolysing with $2N.H_2SO_4$ one molecule of citrinin gives rise to one molecule of CO_2 and one molecule of a volatile acid.

Identification of the volatile acid formed by acid hydrolysis of citrinin.

5 gm. of citrinin were hydrolysed by boiling with 250 c.c. of $2N.H_2SO_4$ for four hours, the hydrolysis mixture was evaporated *in vacuo* to complete removal of the volatile

acid, and the distillate neutralised with sodium hydroxide. This solution of the sodium salt of the volatile acid was evaporated to dryness and qualitative tests on a portion of this showed the following reactions :—

(1) Decolorisation of acid permanganate solution.
(2) Reduction of aqueous silver nitrate.
(3) Reduction of mercuric chloride solution on boiling, with the formation of mercurous chloride.

This indicated the presence of formic acid.

The presence of formic acid was confirmed by the preparation of lead formate. A portion of the sodium salt was dissolved in water, acidified with phosphoric acid, and distilled *in vacuo*. The acid distillate was boiled with lead carbonate, filtered while still hot, and the clear filtrate evaporated. On cooling, lead formate crystallised in typical long needles which were recrystallised for analysis.

0·2651 gm. gave 0·2851 gm. of lead chromate corresponding to a percentage of lead in the above lead salt of 68·9 per cent. (Theoretical percentage of lead in lead formate = 69·7 per cent.)

The question now arose as to whether formic acid is the only volatile acid formed by the acid hydrolysis of citrinin. It was definitely proved that it is really so by the following means: The acids from a portion of the sodium salts were recovered by dissolving the latter in water, acidifying with phosphoric acid and distilling *in vacuo*. The aqueous distillate was made up to 500 c.c. and the following estimations carried out on this solution :—

(1) The formic acid content was estimated specifically by a modification of FINCKE's method (see Part VIII). The amount of formic acid present by this method in 500 c.c. was 0·360 gm.
(2) The *total* acid content was estimated by titration with sodium hydroxide to phenolphthalein and gave a total acidity calculated as formic acid of 0·374 gm.

It is thus evident that formic acid is the only volatile acid product of the acid hydrolysis of citrinin.

Investigation of the products of acid hydrolysis of citrinin other than CO_2 and formic acid.

5 gm. of citrinin were hydrolysed by boiling for four hours with 100 c.c. of $2N.H_2SO_4$. The solution was cooled, filtered from a little tar that was formed and polarised. The rotation in a 10 cm. tube was − 1·35°. The rotation for a 5 per cent. solution of citrinin under these conditions is approximately − 2·2°.

The hydrolysis solution was now exhaustively extracted with ether. After extraction the hydrolysis solution had no longer any optical activity. The ether solution on evaporation smelt strongly of a volatile acid (presumably formic acid) and was dried overnight *in vacuo* over potassium hydroxide. An orange mass was left containing crystals and weighing 4·16 gm.

This orange mass and a further quantity of material obtained from a second hydrolysis of 5 gm. of citrinin were fractionally crystallised from chloroform giving rise to two different crystalline products.

Product A soluble in hot chloroform and crystallised from this solvent.

Product B separated from Product A by its slight solubility in chloroform and crystallised from ethyl acetate.

Product A.

This is a white crystalline solid crystallising in plates which melt at 128°–130° C. after drying to constant weight at 100° C. As originally prepared this compound contains about 30 per cent. by weight of $CHCl_3$ as solvent of crystallisation which is lost on drying. It is soluble in water and the aqueous solution gives a blue colour with ferric chloride. The substance is lævorotatory. 0·2541 gm. dissolved in 25·08 c.c. of absolute alcohol gave a negative rotation of 0·885° in a 20 cm. tube with mercury green light corresponding to a rotation of $[\alpha]_{\text{Hg. green}} = -43 \cdot 7°$. Its solution in alcohol is practically neutral, 0·2037 gm. requiring 0·87 c.c. of N/10 NaOH, using phenolphthalein as indicator.

On drying the original material, 0·3405 gm. lost 0·1132 gm. in weight corresponding to a loss in weight of 33·25 per cent. In a repeat experiment on a different sample 1·1249 gm. lost 0·3126 gm. corresponding to a loss of 27·79 per cent. (Theory for $C_{11}H_{16}O_3$ + 1 mol. $CHCl_3$ = 37·78 per cent.)

TABLE VIII.—Analysis of Product A dried to constant weight at 100° C.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm.	Gm.	Gm.		
0·1236	0·3024	0·0912	66·72	8·27
0·1368	0·3352	0·1004	66·82	8·21
Theory for $C_{11}H_{16}O_3$	—	—	67·31	8·22

In a ZEISEL estimation, 0·1479 gm. gave 0·0041 gm. of silver iodide indicating that the substance contains no methoxyl groups. In a molecular weight estimation by the improved RAST method 0·00433 gm. depressed the freezing point of 0·03648 gm. of camphor by 23·35° C. corresponding to a molecular weight of 202. (Theory for $C_{11}H_{16}O_3$ = 196.) In a quantitative acetylation 0·2614 gm. gave an acetyl value equivalent to 2·37 c.c. of N/1 NaOH corresponding to an acetyl value of 9·07 c.c. of N/1 NaOH per gm.

1 gm. of $C_{11}H_{16}O_3$ containing two hydroxyl groups would have an acetyl value of 10·20 c.c of N/1 NaOH.

This product therefore appears to have a molecular formula of $C_{11}H_{16}O_3$, contains no methoxyl groups, and has two of the oxygen atoms present as hydroxyl groups which are acetylated by acetic anhydride and pyridine.

Product B.

This product is a white crystalline solid crystallising in prisms from ethyl acetate with a melting point of 169°–170° C. It is not readily soluble in cold water but is soluble in dilute NaOH. The aqueous solution gives a blue colour with $FeCl_3$ identical with that obtained with Product A. On drying at 100° C. to constant weight 2·0475 gm. lost 0·0021 gm. showing this material contains no solvent of crystallisation. It is optically *inactive.* 0·2545 gm. dissolved in 25·07 c.c. of absolute alcohol gave no rotation in a 20 cm. tube with mercury green light. On titration 0·2041 gm. dissolved in alcohol required 0·96 c.c. of N/10 NaOH, using phenolphthalein as indicator, showing that the substance is practically neutral in reaction.

TABLE IX.—Analysis of Product B dried to constant weight at 100° C.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm.	Gm.	Gm.		
0·1433	0·3519	0·1067	66·98	8·33
0·1151	0·2826	0·0848	66·95	8·24
Theory for $C_{11}H_{16}O_3$	—	—	67·31	8·22

In a molecular weight estimation 0·00283 gm. depressed the freezing point of 0·03140 gm. of camphor by 19·07° C., corresponding to a molecular weight of 188. (Theory for $C_{11}H_{16}O_3$ = 196.)

This compound appears to be the optically inactive stereoisomer of product A.

The following table shows the yields of these two materials which are obtained in a typical acid hydrolysis of a batch of crude citrinin as obtained from the metabolism solution by simple acidification.

From the Table X it can be seen that the yield of Product A is eight to nine times that of product B and the course which this reaction takes would appear to be as follows :—

$$\underset{\text{Citrinin}}{C_{13}H_{14}O_5} + 2H_2O \longrightarrow \underset{\substack{\text{Products}\\ \text{A and B}}}{C_{11}H_{16}O_3} + CO_2 + \underset{\text{Formic acid.}}{HCOOH}$$

TABLE X.—Distribution of products from a typical acid hydrolysis of citrinin.

—	Weight of Material.	Percentage Yield on original citrinin.
	Gm.	
Citrinin	53	—
Tar	4·16	7·9
Total ether extract	43	81·1
Ether extract fractionated as follows :—		
Product A	26·03	49·1
Product B	3·04	5·7
Residual syrup (dark red)	13·84	25·9

As the hydrolysis product A, melting point 128°–130° C., was produced in preponderating amounts it was decided to confine investigations into the nature of the $C_{11}H_{16}O_3$ compounds (*i.e.*, products A and B) to this compound, and the following derivatives were made to obtain what information was possible as to its structure.

Derivatives of acid hydrolysis product A.

(1) *Acetyl compound of product A.*—4 gm. of product A, 4 gm. of sodium acetate and 12 c.c. of acetic anhydride were refluxed for one hour on an oil bath, the mixture diluted withw ater and the product separated as a colourless sticky oil. This was extracted with ether, the ether solution dried and evaporated. The product separated in small white crystalline plates. Yield 6·4 gm. The acetyl compound crystallises from 40 per cent. alcohol with a melting point of 90°–91° C., is insoluble in water, and gives no colour with $FeCl_3$ in alcoholic solution.

In an estimation of the acetic acid produced by hydrolysis from this compound 0·4011 gm. gave acetic acid equivalent to 37·29 c.c. of N/10 acid. This weight of diacetyl compound, *i.e.*, $C_{11}H_{14}O\ (OCOCH_3)_2$ would yield 28·64 c.c. of N/10 acid and of a triacetyl compound, *i.e.*, $C_{11}H_{13}\ (OCOCH_3)_3$ would yield 37·36 c.c. of N/10 acid. Product A therefore appears to contain three hydroxyl groups which can be acetylated with acetic anhydride and sodium acetate, though only two are acetylated by acetic anhydride and pyridine (see p. 283).

Note on the hydrolysis of the acetyl compound of A.—The non-volatile product from the hydrolysis of the acetyl product of A consists of a whitish solid residue with a melting point of 165°–167° C. A mixed melting point of this and product B gave 168°–170° C. Apparently the process of acetylation and deacetylation of Product A causes racemisation of the optically active compound, a fact which confirms the view expressed on p. 284 that Products A and B are optical isomerides.

TABLE XI.—Analysis of acetyl compound of product A.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm.	Gm.	Gm.		
0·1323	0·3078	0·0776	63·43	6·56
0·1064	0·2459	0·0665	63·03	6·99
0·1163	0·2700	0·0705	63·31	6·78
Theory for $C_{17}H_{22}O_6$, *i.e.*, $C_{11}H_{13}$ $(OCOCH_3)_3$ … …			63·30	6·89
Theory for $C_{15}H_{20}O_5$, *i.e.*, $C_{11}H_{14}O$ $(OCOCH_3)_2$ … …			64·25	7·20

In a molecular weight estimation 0·0090 gm. of this acetyl compound depressed the melting point of 0·03171 gm. of camphor by 13·07° C. corresponding to a molecular weight of 278 (Theoretical for $C_{17}H_{22}O_6 = 322$). This low figure may be due to the product being slightly unstable in molten camphor.

(2) *Dimethyl derivative of product A.*—The hydrolysis product A was methylated as follows: 4 gm. of material were dissolved in 100 c.c. of water and 10 c.c. of dimethyl sulphate added. 10 per cent. NaOH was now slowly added with vigorous shaking to keep the mixture just alkaline, 60 c.c. of 10 per cent. NaOH being used in all. The solution was extracted with ether while still alkaline and the extract obtained was purified by distillation. The weight of extract obtained was 4·10 gm. and after being twice distilled in a high vacuum it gave a boiling point of 136°–138° C. at 1 mm. The product obtained is a colourless, very viscous oil, insoluble in water and dilute NaOH, and gives no ferric chloride reaction in alcoholic solution.

In a ZEISEL estimation 0·2773 gm. and 0·1592 gm. gave 0·5496 gm. and 0·3047 gm. of silver iodide, corresponding respectively to 26·18 per cent. and 25·29 per cent. of methoxyl.

Theory for $C_{11}H_{14}O$ $(OCH_3)_2$ is 27·68 per cent. methoxyl and for $C_{11}H_{13}$ $(OCH_3)_3$ is 39·06 per cent. methoxyl.

TABLE XII.—Analysis of dimethyl derivative of product A.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm.	Gm.	Gm.		
0·2209	0·5629	0·1775	69·50	8·99
0·1791	0·4563	0·1428	69·48	8·92
Theory for $C_{11}H_{14}O$ $(OCH_3)_2$ … … … … …			69·58	8·99

As the methyl derivative produced by this method is only a dimethyl derivative, whereas a triacetyl derivative was obtained by acetylation with acetic anhydride and sodium acetate, a second methylation was carried out on a 1 gm. scale using an ample excess of dimethyl sulphate and the reaction mixture was heated to boiling during the process. The product obtained was an oil boiling at 143° C. at 1·5 mm., which in a methoxyl estimation on 0·1592 gm. gave 0·3179 gm. of silver iodide, corresponding to 26·37 per cent. OCH_3. This is thus the same product as was obtained by the cold methylation process.

An attempt to recover a product from the hydriodic acid used in the various methoxyl estimations was not successful.

(3) *Monomethyl derivative of product A.*—During one methylation of product A where a large excess of methyl sulphate was not used and the alkalinity was never allowed to become pronounced, a different methylated product was obtained. This was a crystalline solid crystallising from benzene with a melting point of 144°–147° C. 0·1567 gm. of a purified specimen of this material gave 0·1702 gm. of silver iodide in a Zeisel estimation, corresponding to a methoxyl content of 14·35 per cent. (Theory for $C_{11}H_{15}O_2$ (OCH_3) is 14·76 per cent.)

In a molecular weight estimation 0·0034 gm. of this compound caused a depression of 16·64° C. in the melting point of 0·04324 gm. of camphor corresponding to a molecular weight of 201. The theoretical figure for $C_{11}H_{15}O_2.OCH_3$ is 210.

Table XIII.—Analysis of monomethyl derivative of Product A.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm. 0·1213	Gm. 0·3058	Gm. 0·0955	68·74	8·81
Theory for $C_{11}H_{15}O_2$ (OCH_3) …	…	… … …	68·56	8·63

Acetylated dimethyl compound of product A.—As product A forms a triacetyl, but only a dimethyl, compound an attempt was made to introduce an acetyl group into the dimethyl compound. 1 gm. of dimethyl A, 1·5 gm. of sodium acetate and 5 c.c. of acetic anhydride were refluxed for one hour, cooled, the mixture diluted and the product extracted with ether. The ether extract after drying over H_2SO_4 and KOH weighed 1·13 gm. This product was purified by distillation *in vacuo* giving a viscid colourless liquid boiling at 136°–138° C. at 1 mm.

In an estimation of the acetic acid formed by hydrolysis of this product, 0·4366 gm. gave a volatile acid equivalent to 15·19 c.c. of N/10 acid. 0·4366 gm. of $C_{11}H_{13}$ $(OCH_3)_2$ $OCOCH_3$ would require 16·40 c.c. of N/10 acid, on a theoretical basis, for one acetyl group. As a check on this experiment a similar amount of dimethyl A was subjected to an identical estimation, but only a negligible amount of volatile acid was produced.

This shows definitely that the product A contains three hydroxyl groups, two of which can be methylated or acetylated while the third can be acetylated but not methylated under the conditions employed. As a result of the information obtained from the various derivatives of product A it appears that this compound has the empirical formula $C_{11}H_{16}O_3$, contains an asymmetric carbon atom, and three hydroxyl groups, one of which appears to be alcoholic in nature, while the other two are phenolic.

(B) Degradation Products of Product A.

As the most favourable method of obtaining information as to the structure of citrinin appeared to be a study of the breakdown products of product A, a considerable quantity of this material was prepared.

The methods of dealing with this substance can be divided into three groups :—

(1) Direct oxidation, reduction, etc., of product A.
(2) Oxidation products obtained from dimethyl A.
(3) Products obtained by fusion of product A with potassium hydroxide.

(1) Attempts were made to obtain oxidation products of Product A by various methods including acid potassium permanganate, alkaline perhydrol, etc., but none of these methods gave rise to a product which could be isolated in a pure state.

Zinc and sulphuric acid at 100° C. have apparently no action on product A, which can be recovered unchanged from the reaction mixture.

An attempt was also made to break down product A by boiling for three hours with 30 per cent. sodium hydroxide, but this process also has no action on the material.

(2) A quantity of dimethyl A was prepared and oxidized by the method recommended by Luff, Perkin and Robinson (1910) for the production of asaronic acid.

7·5 gm. of purified dimethyl A were placed in a flask (1,500 c.c.) with about 200 c.c. of warm water and arranged on a boiling water bath under a reflux condenser. A 5 per cent. solution of alkaline permanganate was added slowly to the point where the disappearance of the pink colour took more than three minutes. This required about 800 c.c. of permanganate solution. The whole was allowed to cool slightly, filtered from precipitated MnO_2 and the filtrate acidified with hydrochloric acid until just acid to congo red paper. The acidified liquor was now evaporated to dryness, the residue mixed with kieselguhr and the whole extracted in a Soxhlet apparatus with ether. This gave rise to about 5·5 gm. of crude product. The extract was now treated with 50–60 c.c. of water and carefully washed to remove all oxalic acid. The water insoluble portion was then dried (weight = 2·20 gm.), and purified by distillation in a high vacuum. An oily material was obtained which distils at 180°–190° C. at 0·5 mm. and on cooling sets to a hard transparent glassy material which on treatment with ether separates as a white prismatic crystalline substance with melting point 122°–124° C.

These crystals are insoluble in *cold* dilute NaOH but are very soluble in chloroform. Quantity obtained, 0·39 gm.

On analysis this product gave the following results:—

TABLE XIV.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm. 0·1194	Gm. 0·2894	Gm. 0·0728	66·10	6·82
Theory for $C_{13}H_{16}O_4$	...	... ...	66·07	6·83

In a ZEISEL estimation 0·1075 gm. gave 0·2098 gm. of silver iodide corresponding to a methoxyl content of 25·77 per cent. Two methoxyl groups in $C_{13}H_{16}O_4$ would require 26·28 per cent. methoxyl.

In a molecular weight estimation by the RAST method, 0·00284 gm. caused a depression of 13·88° C. in the melting point of 0·03432 gm. of camphor. This figure corresponds to a molecular weight of 237 while the molecular weight of $C_{13}H_{16}O_4$ is 236.

To test the possibility of this material possessing a lactone or anhydride structure, a small quantity of it was boiled with very dilute NaOH for one hour, cooled, and the mixture titrated to neutrality using phenolphthalein as indicator. The amount of NaOH used in this hydrolysis by 0·0719 gm. of material was 2·89 c.c. of N/10 NaOH. This amount of oxidation product, $C_{13}H_{16}O_4$, would require 3·05 c.c. of N/10 NaOH to neutralise an opened lactone structure and 6·10 c.c. to neutralise an anhydride structure. The product is therefore probably of the lactone type.

As far as can be assumed from the foregoing facts it appears that the oxidation product obtained by the action of alkaline permanganate on dimethyl A has the empirical formula $C_{13}H_{16}O_4$, contains two methoxyl groups, and has probably a lactone structure.

(3) *Products obtained by fusing product A with potassium hydroxide.*—As already noted, p. 288, product A is resistant to the action of boiling aqueous 30 per cent. alkali. Accordingly 4 gm. of A were fused in a nickel crucible with 15 gm. of potassium hydroxide and 3 c.c. of water. The crucible was heated in a bath of fusible metal maintained at a temperature of 300° C. for 1½ hours. The mass became semi-fluid, darkened in colour and swelled, with apparent evolution of gas. At the close of the heating period the mixture was allowed to cool, dissolved in about 100 c.c. of cold water, and this solution saturated with CO_2 gas. This caused the precipitation of a crystalline phenolic

body, which was filtered off and washed, first with saturated potassium bicarbonate solution and then with water. Finally the filtrate was extracted with ether to remove the last traces of this product. The yield obtained was 2·9 gm. and on purification by sublimation the product melted at 97°–99° C.

Investigation of phenolic body produced by potash fusion of product A.—This compound is a white crystalline solid which crystallises from water in prisms, from benzene and petrol ether in plates, and sublimes as small polyhedric crystals. It is not readily soluble in cold water nor in an aqueous solution of potassium bicarbonate but dissolves readily in sodium hydroxide solution. Dilute aqueous solutions give a blue colour with a trace of ferric chloride. In dilute alkaline solution it absorbs oxygen from the air and develops a reddish brown colour on standing.

An aqueous solution of this material when titrated with alkali to phenolphthalein has no appreciable titratable value, 0·1045 gm. requiring 0·12 c.c. of N/10 NaOH. When tested for optical activity 0·2514 gm. dissolved in 25 c.c. of water gave a rotation of + 0·003° in a 20 cm. tube using mercury green light, the substance therefore being optically inactive.

In a molecular weight estimation 0·00283 gm. of this product caused a depression in the freezing point of 0·03255 gm. of camphor of 24·19° C. corresponding to a molecular weight of 143. A compound of the formula $C_9H_{12}O_2$ would have a molecular weight of 152.

On analysis the substance gave the following figures :—

TABLE XV.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Gm.	Gm.	Gm.		
0·1440	0·3759	0·1048	71·17	8·14
0·1284	0·3344	0·0920	71·00	8·02
Theory for $C_9H_{12}O_2$	… …	… … …	71·00	7·96

Derivatives of the phenol obtained by potash fusion of product A.

(1) *Acetyl derivative.*—0·5 gm. of the phenol was refluxed for 30 minutes with 0·75 gm. of anhydrous sodium acetate and 2 c.c. of acetic anhydride. On allowing the mixture to cool, diluting with water, and extracting the solution with ether, a syrupy product was obtained which could not be readily purified.

(2) *Dimethyl derivative.*—4 gm. of the phenol were treated with 15 c.c. of dimethyl sulphate and 10 per cent. NaOH added to keep the reaction just alkaline. As the reaction proceeded the rate of methylation was maintained by warming the reaction

mixture to 50° C. After adding 60 c.c. of 10 per cent. NaOH the reaction appeared to be almost complete, a slight excess of NaOH was added and the cooled mixture extracted with ether. The ether extract was washed with a little NaOH solution and then with water to ensure the removal of any unchanged or partially methylated material.

The yield of product obtained by this method is about 4 gm., and it is in the form of a colourless oil which gives no ferric chloride reaction, is insoluble in NaOH, and distils at 89°–91° C. at 1 mm. In a Zeisel estimation 0·1567 gm. gave 0·3531 gm. of silver iodide, corresponding to a methoxyl content of 29·76 per cent. A monomethyl derivative of the phenol, $C_9H_{11}O\,.\,(OCH_3)$ would contain 18·68 per cent. methoxyl and a dimethyl derivative, $C_9H_{10}\,(OCH_3)_2$, 34·42 per cent. methoxyl. The product in this case is apparently a dimethyl derivative.

(3) *Monomethyl derivative.*—If a sufficient excess of methyl sulphate and sodium hydroxide is not employed a monomethyl derivative of the phenol can be obtained which boils at 100°–120° C. at 1 mm. and crystallises from petrol ether with a melting point of 63°–65° C. This product is soluble in NaOH and is reprecipitated by CO_2.

In a Zeisel estimation, 0·1875 gm. gave 0·2619 gm. of silver iodide, corresponding to a methoxyl content of 18·45 per cent. Theory for $C_9H_{11}O.OCH_3$ is 18·68 per cent.

From the foregoing results it appears that the product obtained by the potash fusion of product A is a phenolic body with the empirical formula of $C_9H_{12}O_2$ and contains two hydroxyl groups.

This formula would be satisfied by either a propyldihydroxybenzene, a methylethyldihydroxybenzene, or a trimethylhydroxybenzene. The properties of this compound, however, do not appear to agree with any such compounds which are described in the literature.

(C) Oxidation Products of the Dimethylphenol, $C_9H_{10}\,(OCH_3)_2$.

As the products obtained by the oxidation of dimethyl A and by the potash fusion of product A are compounds which do not appear to have been described in the literature, it was decided to attempt the preparation of some oxidation product of the methylated phenol from the potash fusion, with a view to obtaining something of the nature of a substituted dimethoxybenzoic acid. With this purpose in view a quantity of the dimethylphenol was prepared as already described on p. 289.

The method which was adopted for this oxidation was the same one as that described on p. 288 for the oxidation of dimethyl A. 11·3 gm. of the dimethylphenol, $C_9H_{10}(OCH_3)_2$ were suspended in 200 c.c. of water, heated to 100° C. and 1,000 c.c. of 5 per cent. alkaline permanganate added gradually in 25 c.c. portions. The solution was then allowed to cool, filtered from MnO_2 and acidified to congo red. The acidified solution was now evaporated *in vacuo* to a small bulk, the concentrated liquor made alkaline and extracted with ether to remove any unchanged material. The ether extract containing the unchanged material was combined with an ether extract of the precipitated MnO_2, and an ether extract of the condensed distillate from the evaporation, thus ensuring the complete recovery of all unchanged material, which in this case amounted to 5·05 gm. This unchanged material was now subjected to an identical oxidation process on a suitable scale and this repetition was carried on until all the available material had been converted into an alkali soluble oxidation product.

The combined oxidation solutions were acidified with HCl, evaporated to dryness, and the dry residue extracted with ether in a Soxhlet apparatus. The ether extract was evaporated and the residue fractionally crystallised from water. By this means four fractions were finally obtained :—

(1) Oxalic acid.
(2) A product melting at 97°–99° C.
(3) A product melting at 142°–146° C.
(4) An oily residue.

Oxidation product melting at 97°–99° *C.*

This product crystallises out of water in white prisms melting at 97°–99° C. without decomposition. It gives no colour with ferric chloride and is strongly acid in reaction. Combustion and ZEISEL results (SCHOELLER, Berlin) correspond to the empirical formula $C_{11}H_{14}O_4$, *i.e.*, C_8H_7 $(OCH_3)_2$ COOH. These results are given in Tables XVI and XVII.

TABLE XVI.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Mgm.	Mgm.	Mgm.		
4·980	11·455	2·95	62·75	6·63
4·730	10·880	2·83	62·76	6·70
Theoretical for $C_{11}H_{14}O_4$ … … … … …			62·82	6·72

TABLE XVII.

Weight of Substance Analysed.	Weight of Silver Iodide.	Percentage OCH_3.
Mgm.	Mgm.	
3·314	7·330	29·24
3·321	7·370	29·33
Theoretical for $C_9H_8O_2$ $(OCH_3)_2$ … … …		29·53

Oxidation product melting at 142°–146° *C.*

This product crystallises from water in very fine white needles. It gives no colour with ferric chloride and is strongly acid in reaction. Combustion results (SCHOELLER, Berlin) given in Table XVIII, show that it has the empirical formula $C_{10}H_{12}O_4$.

TABLE XVIII.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage C.	Percentage H.
Mgm.	Mgm.	Mgm.		
5·029	11·185	2·66	60·67	5·92
4·960	11·040	2·65	60·67	5·98
Theoretical for $C_{10}H_{12}O_4$			61·19	6·17

ZEISEL estimations (SCHOELLER, Berlin), given in Table XIX, indicate the presence of two methoxyl groups.

TABLE XIX.

Weight of Substance.	Weight of Silver Iodide.	Percentage OCH_3.
Mgm.	Mgm.	
3·460	8·240	31·46
3·420	8·080	31·21
Theory for $C_8H_6O_2(OCH_3)_2$		31·64

0·0254 gm. titrated to phenolphthalein in alcoholic solution required 1·31 c.c. of N/10 NaOH corresponding to a combining weight of 194 (theoretical for $C_{10}H_{12}O_4 = 196$).

This oxidation product is therefore a dimethoxy carboxylic acid of the formula $C_7H_5(OCH_3)_2 . COOH$.

The question of the constitution of citrinin is discussed in Part XV of this series, but the following is a summary of the salient points established in the work already described and having a bearing on this question.

1. Citrinin is a yellow product having the empirical formula $C_{13}H_{14}O_5$.
2. It contains one hydroxyl group and one carboxyl group, since it forms a mono-acetyl compound (p. 277) and an *O*-ethyl ethyl ester (p. 277).
3. It does not contain any methoxyl groups (p. 276) or ketonic groups (p. 280).
4. Since it is optically active it contains an asymmetric carbon atom.

5. The yellow citrinin $C_{13}H_{14}O_5$ containing one hydroxyl group is readily reduced by nascent hydrogen to a colourless dihydroxy compound of the formula $C_{13}H_{16}O_5$ which in its turn is readily re-oxidized to citrinin even by atmospheric oxygen (p. 278).

6. Citrinin is readily hydrolysed by boiling with dilute sulphuric acid, when one molecule of citrinin, $C_{13}H_{14}O_5$, gives rise to one molecule of CO_2, one molecule of formic acid, and the product $C_{11}H_{16}O_3$, Product A, according to the equation $C_{13}H_{14}O_5 + 2H_2O = CO_2 + HCOOH + C_{11}H_{16}O_3$ (p. 281).

7. Product A ($C_{11}H_{16}O_3$) contains an asymmetric carbon atom and three hydroxyl groups, two of which are phenolic in nature while the third one is alcoholic (p. 283).

8. The dimethyl derivative of product A, $C_{11}H_{14}O\ (OCH_3)_2$, on oxidation with permanganate gives rise to the product $C_{13}H_{16}O_4$ which is a dimethoxy derivative of an acid lactone (p. 289).

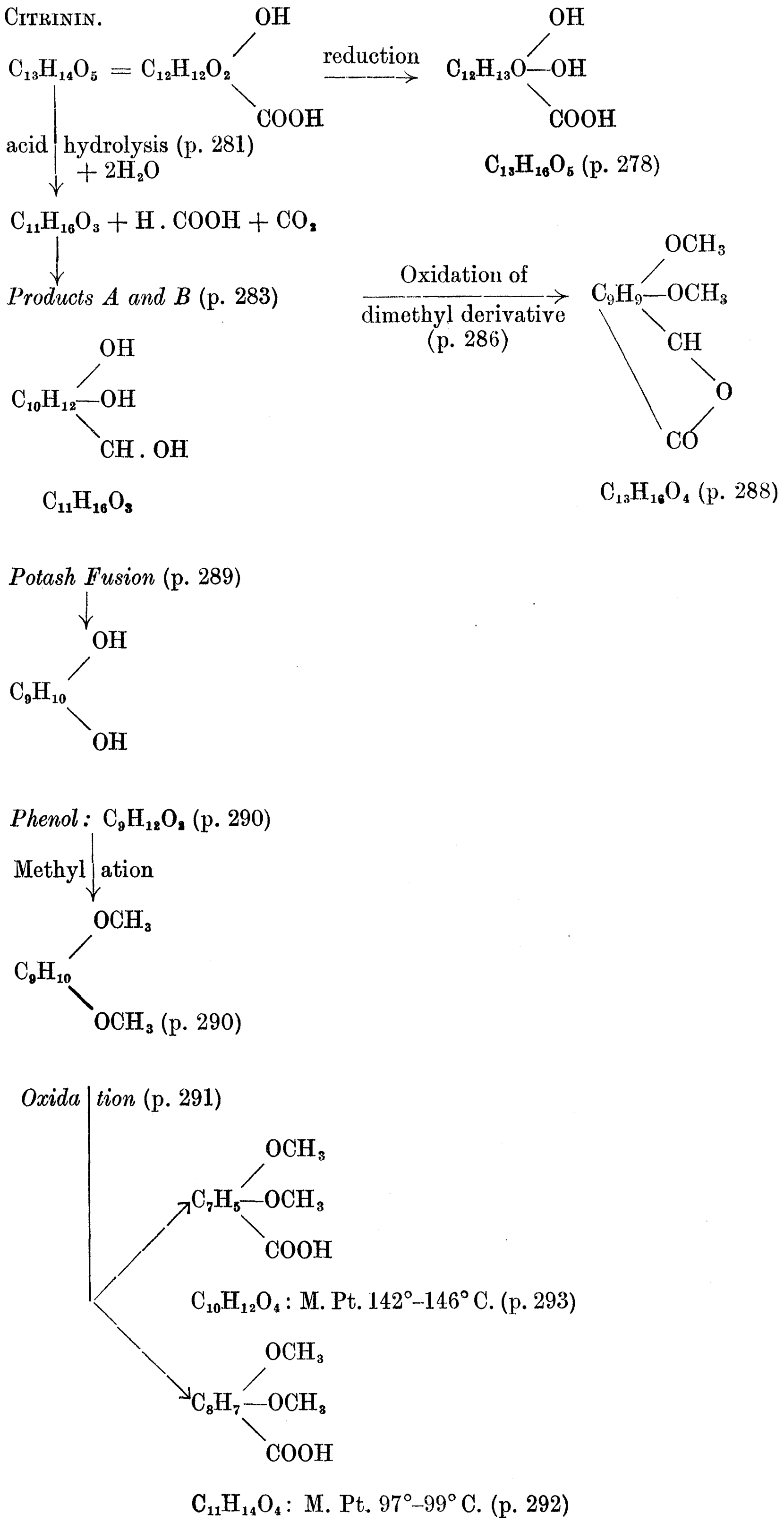
9. Product A ($C_{11}H_{16}O_3$) on fusion with potash gives the phenol $C_9H_{12}O_2$ containing two hydroxyl groups (p. 289).

10. This phenol $C_9H_{12}O_2$ was methylated to the dimethyl compound $C_9H_{10}\ (OCH_3)_2$ and oxidized with permanganate. Two acids were produced having the empirical formulæ $C_{10}H_{12}O_4$ and $C_{11}H_{14}O_4$. Both are dimethoxy-carboxylic acids, *i.e.*, $C_7H_5\ (OCH_3)_2\ COOH$ and $C_8H_7\ (OCH_3)_2\ COOH$ (pp. 291–293).

The relationship to citrinin of the various decomposition products is given graphically on p. 295.

Summary.

P. citrinum THOM, when grown on modified CZAPEK-DOX glucose solution, gives rise to a new metabolic product. This is a rich yellow crystalline colouring matter of the empirical formula $C_{13}H_{14}O_5$, to which the name citrinin has been given. A description is given of its preparation, properties, derivatives and breakdown products. It is apparently produced only by *P. citrinum* THOM and seems to be specific for that species.


CITRININ.
$C_{13}H_{14}O_5 = C_{12}H_{12}O_2$ OH COOH
reduction
$C_{12}H_{13}O$—OH OH COOH
$C_{13}H_{16}O_5$ (p. 278)
acid hydrolysis (p. 281) + $2H_2O$
$C_{11}H_{16}O_3 + H.COOH + CO_2$
Products A and B (p. 283)
$C_{10}H_{12}$—OH OH CH.OH
$C_{11}H_{16}O_3$
Oxidation of dimethyl derivative (p. 286)
C_9H_9—OCH_3 OCH_3 CH O CO
$C_{13}H_{16}O_4$ (p. 288)
Potash Fusion (p. 289)
C_9H_{10} OH OH
Phenol: $C_9H_{12}O_2$ (p. 290)
Methylation
C_9H_{10} OCH_3 OCH_3 (p. 290)
Oxidation (p. 291)
C_7H_5—OCH_3 OCH_3 COOH
$C_{10}H_{12}O_4$: M. Pt. 142°–146° C. (p. 293)
C_8H_7—OCH_3 OCH_3 COOH
$C_{11}H_{14}O_4$: M. Pt. 97°–99° C. (p. 292)