XVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVI. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH.

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DURING an examination of a sample of mouldy Italian maize our late colleague, Mr J. H. V. Charles, isolated a new species of *Penicillium* belonging to Thom's group "The Monoverticillata-Ramigena" [Thom, 1930]. A description of this mould, which has been named *P. Charlesii*, has been published elsewhere [Smith, 1933]. When grown on Czapek-Dox solution or on the medium which we have called Raulin-Thom solution [Clutterbuck *et al.* 1932] the culture medium becomes very strongly acid, reduces KMnO_4 instantly in the cold, gives an intense orange or scarlet colour with ferric chloride, and on addition of excess of alcohol gives a voluminous sticky precipitate. In addition, if the period of incubation is sufficiently prolonged, the metabolism solution becomes definitely laevorotatory.

Addition of acetone to the evaporated metabolism solution precipitated a mixture of two new polysaccharides, one of which was a polygalactose and the other a polymannose. The syrupy precipitate obtained with acetone both from Czapek-Dox solution and Raulin-Thom solution gave, on trituration with absolute alcohol and ether, an amorphous hygroscopic powder with a high content of ash consisting chiefly of phosphates. Most of this ash, however, could be removed by precipitation with alcohol.

The aqueous solution of the crude product from Raulin-Thom medium was acid to litmus, with an average rotation of $[\alpha]_{5790} = -40^\circ$, and had no action on Fehling's solution or on iodine. Its behaviour on hydrolysis suggested the presence of two polysaccharides. Treated at 100° with $0.4N$ sulphuric acid the rotation changed from -40° to $+61^\circ$, but on pouring the equilibrium solution into alcohol a considerable portion of a polysaccharide was precipitated. With $3N$ sulphuric acid, however, this precipitated portion hydrolysed readily, the rotation changing from $+50^\circ$ to $+14.3^\circ$, the latter value being identical with the equilibrium rotation of *d*-mannose. The crystalline hydrolysis product from the more labile polysaccharide was identified as *d*-galactose, and that from the more stable polysaccharide was identified as *d*-mannose. By a very tedious process of fractional precipitation with alcohol a sample of the polymannose $[\alpha]_{5790} + 63^\circ$ in water, and a sample of the polygalactose $[\alpha]_{5790} = -84^\circ$ in water were obtained, thus showing that the original product was a mixture. The crude polymannose was a white hygroscopic powder containing approximately 15 %

of moisture and 10 % of ash, most of the latter being removed by precipitation from hydrochloric acid solution with alcohol. It gave a colourless acid solution in water and did not react with iodine or Fehling's solution. Hydrolysis with 3*N* sulphuric acid gave only mannose. On acetylation a homogeneous triacetyl compound was obtained from which the original polymannose could be regenerated with sodium hydroxide. The polygalactose was identical in appearance with the polymannose. It contained only 1.8 % of ash when crude and approximately 10 % of moisture. It was readily hydrolysed by even dilute acid to give only galactose. An ash-free triacetyl derivative was prepared which could not, however, be satisfactorily deacetylated with sodium hydroxide. The aqueous solution of the polygalactose was colourless, acid to litmus and had no action on iodine or Fehling's solution.

From Czapek-Dox medium a mixture of the same two polysaccharides was obtained. This crude mixture had a very high ash content and contained a larger proportion of the polymannose.

After precipitation of the polysaccharides with acetone, the acetone mother-liquors were evaporated *in vacuo* to remove acetone, acidified with HCl and exhaustively extracted first with ether and then with chloroform. These solvents extract a mixture of at least six new optically active acids, which have been shown to have the following empirical formulae, and for which we propose the accompanying names: $C_9H_{10}O_4$, *carolic acid*; $C_9H_{12}O_7$, *carolinic acid*; $C_{10}H_{10}O_6$, *carlic acid*; $C_{10}H_{12}O_6$, *carlosic acid*; $C_{16}H_{20}O_6$, *ramigenic acid*; $C_{26}H_{32}O_{12}$, *verticillic acid*.

Carolic acid, $C_9H_{10}O_4$, is produced in the largest yields and occurs principally in the chloroform extracts and to a much smaller extent in the ether extracts. It is produced on both media, though in larger yield on the Czapek-Dox medium. It is a dextrorotatory monobasic acid and gives a characteristic orange-yellow coloration with $FeCl_3$.

Carolinic acid, $C_9H_{12}O_7$, is the acid produced in the next largest yield to carolic acid and occurs principally in the ether extracts. The largest yields of this acid were obtained from Raulin-Thom's medium. It is a dextrorotatory dibasic acid and gives a characteristic copper salt which crystallises in pure blue needles.

Carlic acid, $C_{10}H_{10}O_6$, is produced in small amounts on both media and is present almost entirely in the chloroform extracts. It is a laevorotatory dibasic acid.

Carlosic acid, $C_{10}H_{12}O_6$, a laevorotatory dibasic acid, was obtained only on Czapek-Dox medium, none being isolated from Raulin-Thom medium. It occurs entirely in the ether extracts and was obtained in moderate yields. Carlosic acid is differentiated from the other metabolic products of *P. Charlesii* by the heavy brownish-yellow precipitate with $FeCl_3$, insoluble in excess of this reagent, by the formation on standing of a crystalline yellow precipitate with uranium acetate and by the immediate heavy precipitate, soluble in excess, which is given on careful addition of normal lead acetate.

Ramigenic acid, $C_{16}H_{20}O_6$, a dextrorotatory acid, was obtained in small yields on both media, occurring entirely in the ether extracts.

Verticillic acid, $C_{26}H_{32}O_{12}$, a laevorotatory tetrabasic acid, was obtained on both media, the larger yields being given on Raulin-Thom medium. It occurs almost entirely in the ether extracts, only traces being isolated from the chloroform extracts. A neutral aqueous solution of its sodium salt reduces silver nitrate in the cold, gives a heavy precipitate with mercuric chloride from which it can be regenerated unchanged, a definite orange-yellow colour with uranium acetate and a very intense reddish brown colour with $FeCl_3$.

In addition to the products described above, a certain amount of non-crystalline syrup has been separated. Moreover, the aqueous phase after exhaustive solvent extraction is still laevorotatory. We propose to investigate both of these fractions further.

It is the purpose of this paper to describe the cultural methods employed for the production of the substances described above, the chemical methods used for their isolation and purification and their general properties. The question of their molecular constitution will be dealt with in a series of subsequent papers.

EXPERIMENTAL.

Organism and culture media.

The organism used during the whole of this work was the new species of *Penicillium*, *P. Charlesii* G. Smith (London School of Hygiene Catalogue Number P 146). (Cf. Smith, 1933.)

Preliminary experiments in test-tubes showed that the reactions characteristic of *P. Charlesii* are given when the mould is grown on either the well-known Czapek-Dox solution or on the modified Raulin's solution which we have called the Raulin-Thom medium. The compositions of the two media are the following:

Czapek-Dox solution. Water, to 1000 ml.; glucose, 50 g.; NaNO_3 , 2 g.; KH_2PO_4 , 1 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.

Raulin-Thom solution. Water, to 1500 ml.; glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; diammonium hydrogen phosphate, 0.6 g.; K_2CO_3 , 0.6 g.; MgCO_3 , 0.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.25 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.

The course of metabolism.

A number of one litre conical flasks, each containing 350 ml. of Czapek-Dox solution, and a similar number of flasks of Raulin-Thom solution were sterilised and sown with a spore-suspension of *P. Charlesii* and incubated at 24°. Single flasks were taken at intervals for examination of the metabolism solution.

On Czapek-Dox solution growth was rapid but the mycelial felt was thin, the surface being rich dark green in colour and with a white reverse. The solution became very tardily pink (21 days), then brownish yellow. The rate of metabolism, as shown by analysis of the contents of single flasks, was somewhat erratic. Representative analyses are given in Table I in which are shown the

Table I.

Days of incubation	p_{H}	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	FeCl_3 reaction
0	4.5	+6.25°	5.04	Nil
3	5.1	+6.18	4.98	Nil
6	4.8	+5.72	4.61	Pale yellow
9	4.7	+5.42	4.37	Yellow
17	4.6	+4.35	3.51	Very deep yellow
25	3.4	+3.05	2.46	Orange-yellow
32	3.1	+1.73	1.40	Deep orange
51	2.4	-0.39	—	Orange-red

apparent rate of disappearance of the sugar, as determined by polarimeter (these figures are obviously somewhat inaccurate owing to the presence of optically active metabolic products), the change of reaction of the medium and the colours

obtained on addition to 5 ml. of the solution of a few drops of ferric chloride solution. An attempt was made to estimate bromine absorption by Koppeschaar's method [1876], but although it was apparent in the case of the older solutions that very considerable absorption took place, back-titration was impossible owing to rapid decomposition of the bromine compounds.

On Raulin-Thom solution growth was more rapid and the course of metabolism less erratic than on Czapek-Dox solution. The mycelial felts were comparatively thick, being bright green on the surface with a reverse which became rapidly mauve and then brownish in colour, the solution being first bright rose-pink and then brownish yellow in colour. In view of the failure to obtain satisfactory results for the estimation of bromine absorption by the Koppeschaar method an attempt was made to obtain some measure of the reactive substances produced by the mould by estimating the amount of KMnO_4 reduced under carefully standardised conditions. The results obtained, however, were unsatisfactory. The results obtained on the Raulin-Thom medium are given in Table II.

Table II.

Days of incubation	p_{H}	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	FeCl_3 reaction
0	3.9	+6.61°	5.34	Bright yellow
3	3.6	+6.07	4.90	Bright yellow
6	2.5	+3.64	2.94	Deep golden yellow
9	2.3	+1.90	1.53	Bright orange
11	2.2	+1.22	0.98	Bright orange
14	2.0	+0.70	0.56	Bright orange
17	2.0	+0.30	0.24	Deep orange
20	2.0	+0.11	0.09	Reddish orange
23	2.2	0	0	Reddish orange

Cultivation of P. Charlesii in the routine metabolism experiments.

In each of twelve experiments 100 one litre conical flasks, each containing 350 ml. of the selected medium, were sterilised by steaming on three successive days and were then sown with an aqueous spore suspension made from four cultures, 2 to 3 weeks old, grown on beer wort-agar slopes. The flasks were incubated at 24° until the sugar had completely disappeared, as evidenced by the solution becoming optically inactive or slightly laevorotatory. The duration of incubation on the Raulin-Thom medium was 24–28 days and on the Czapek-Dox medium about 8 weeks.

Separation of metabolic products.

At the end of the incubation period the metabolism solution was filtered from the mycelium, and the latter was well pressed and washed with a small quantity of water, the washings being added to the main filtrate. The metabolic products were first separated from the metabolism solution in a series of fractions which were eventually examined individually. The general scheme of separation of these fractions was as shown on p. 98.

This scheme relates to the experiments with Raulin-Thom medium. With Czapek-Dox medium the method of separation employed was the same but the grouping of the extracts was slightly different. Thus for Czapek-Dox medium, fraction (IV) refers to ether extracts (2–3), fraction (V) to ether extracts (4–10) and fraction (VII) to chloroform extracts (2–5).

The combined filtered metabolism solution and washings from 100 flasks were evaporated *in vacuo* at 45–50°, using the apparatus described by Raistrick and

Smith [1933], to 800 ml. and allowed to stand for 1 day at 0°. An amorphous solid separated and was filtered off, washed and dried (fraction I). To the mother-liquor, now about 1000 ml. in volume, were added 4 litres of commercial acetone, the mixture being well stirred and allowed to stand until the sticky

precipitate produced had completely settled. The clear liquid was decanted off and the precipitate washed twice with small quantities of acetone, about 200 ml. in all. The residual sticky solid was dissolved in 200 ml. of water, the solution filtered and the crude carbohydrate reprecipitated in a less sticky form by the rapid addition of 9 volumes of ethyl alcohol. The solid was separated by decantation, then ground with absolute ethyl alcohol until the whole was in the form of a powder, which was filtered off, well drained at the pump and dried in air (fraction II).

The combined aqueous acetone mother-liquors and washings were evaporated *in vacuo* to about 600 ml. in order to remove all the acetone. To the concentrate, 100 ml. concentrated HCl were added. A small amount of oily material separated at this stage but was ignored as it was difficult to separate and was readily redissolved during the subsequent extractions. The acidified solution was extracted 12 times (Raulin-Thom) or 10 times (Czapek-Dox) with its own volume of ether. Using Raulin-Thom medium, the first extract (fraction III) was kept separate and extracts 2–5 were combined as fraction IV, as were also extracts 6–12 as fraction V. With Czapek-Dox medium extracts 2–3 were combined as fraction IV and extracts 4–10 were combined as fraction V. The aqueous mother-liquor was then extracted 6 times (Raulin-Thom) or 5 times (Czapek-Dox) with its own volume of chloroform, the first extract (fraction VI) being kept separate and the remaining extracts being combined as fraction VII. All the extracts were evaporated *in vacuo* to give orange syrups which on keeping for some time gradually solidified to sticky masses of small crystals.

The aqueous solution, after extraction with chloroform, still gave a faint orange colour with ferric chloride, readily reduced KMnO_4 and was definitely laevorotatory. Extraction with ether or with chloroform in a continuous extractor for several days removed only very small amounts of dark-coloured sticky syrup. It is possible that the residual unextracted acid is carlic acid $\text{C}_{10}\text{H}_{10}\text{O}_6$, since its relative solubility in water, ether and chloroform would render its extraction very slow (see p. 106).

The dry weights of all the above fractions for eight typical experiments (Nos. 5 to 12), four on each medium, are summarised in Table III. The fractions from Exp. 1-4 (twelve experiments in all, six on each medium, have been carried out) were used in the preliminary work of detecting and isolating the various products present, of obtaining their solubilities and investigating their general properties.

Table III.

Exp. No.	Medium	Fractions: weight in g.						
		I	II	III	IV	V	VI	VII
5	Czapek-Dox	10.9	53.6	30.0	35.4	40.2	51.8	25.6
6	"	25.4	48.7	30.3	18.1	30.2	50.0	15.7
7	"	5.9	51.6	30.0	25.6	36.0	44.0	20.5
10	"	11.5	45.6	22.0	14.2	20.8	40.2	20.8
8	Raulin-Thom	4.0	39.7	18.0	37.9	26.8	30.4	13.6
9	"	5.4	41.0	29.0	31.0	21.4	26.0	12.3
11	"	4.9	43.0	19.4	26.0	19.0	24.8	11.2
12	"	1.7	44.8	31.4	27.0	18.0	24.0	14.0

Since in each experiment 100 flasks were used containing in all 1750 g. glucose at the beginning of the experiment, and the flasks were taken off when the sugar had disappeared, the yields of these fractions in relation to the sugar utilised can readily be computed.

Isolation of products from fractions I-VII.

A. Fraction I.

This fraction consisted, using both media, of a yellowish-brown, amorphous powder which was insoluble in all the usual organic solvents, was non-acidic, contained no nitrogen and did not melt on heating but charred at about 250°. It was not further investigated.

B. Fraction II.

The following are the details of the preliminary examination of this fraction, which consisted essentially of a mixture of two polysaccharides together with mineral salts. A more detailed investigation of these polysaccharides is now in progress and will be reported on in a subsequent paper.

I. Investigation of the polysaccharides from Raulin-Thom's medium. The crude polysaccharide was precipitated as a red viscid mass by the addition of excess acetone to the concentrated metabolism solution. On trituration with absolute alcohol and washing with ether it was obtained as a yellowish hygroscopic powder which tended to form a glutinous mass on exposure to the atmosphere. The four batches of the material which were prepared had the following properties.

Table IV.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
8	39.7	9.5	-41.5° (c=2.31)
9	41.0	10.3	-39.5° (c=2.17)
11	43.0	4.0	-38.7° (c=2.07)
12	44.8	5.7	-40.1° (c=2.68)

The polysaccharide readily dissolved in water to give a milky yellow solution which rapidly cleared on centrifuging. It had no action on Fehling's solution or

on iodine and was acid to litmus. No pentose could be detected by the furfuraldehyde test but the solution after treatment with acid reduced Fehling's solution in the cold.

Hydrolysis of the crude polysaccharide with 0.4N H₂SO₄. A portion of the crude polysaccharide from experiment 8 containing 9.5 % ash was dissolved in 0.4N H₂SO₄, heated on the boiling water-bath and the rotation followed polarimetrically ($c=1.16$) with the following results.

0 minutes $[\alpha]_{5790} = -41.5^\circ$, 5 minutes $[\alpha]_{5790} = -20.1^\circ$, 10 minutes $[\alpha]_{5790} = 0^\circ$, 15 minutes $[\alpha]_{5790} = +27.2^\circ$, 45 minutes $[\alpha]_{5790} = +55.9^\circ$, 90 minutes $[\alpha]_{5790} = +60.5^\circ$ (equilibrium value). This equilibrium value, which on correction for ash = 66.2° , gave no definite indication of the sugar formed, and it was found that on pouring the rotation solution into absolute alcohol a polysaccharide was precipitated. This precipitate was removed by centrifuging, washed with absolute alcohol and ether and dried *in vacuo*. It was a white amorphous powder, soluble in water with an acid reaction to litmus and contained approximately 10 % of ash.

It was stable at 100° to 0.4N H₂SO₄ for 3 hours but was hydrolysed as follows by 3N H₂SO₄ at 100° ($c=0.97$ and rotation corrected for ash). 0 minutes $[\alpha]_{5790} = +48.7^\circ$, 10 minutes $[\alpha]_{5790} = +29.9^\circ$, 30 minutes $[\alpha]_{5790} = +15.5^\circ$, 60 minutes $[\alpha]_{5790} = +14.3^\circ$ (equilibrium value). This equilibrium value is very close to that of *D*-mannose $[\alpha]_{5790}^{20} = +15.2^\circ$.

The crude polysaccharide (3.5 g.) was dissolved in 0.4N H₂SO₄ (50 ml.) and heated for 90 minutes until the rotation was constant. Alcohol was added to the cold solution until there was no further precipitate. This precipitate was isolated by the method described above. Yield = 1.1 g. Ash content = 10.1 %.

Examination of the alcoholic mother-liquors. A little water was added and the sulphuric acid neutralised with barium carbonate. The barium sulphate was filtered off and the solution concentrated *in vacuo* at 30° . The syrupy product was extracted with hot 95 % ethyl alcohol until the residue showed no reducing properties. Concentration of the alcoholic extract gave a clear colourless syrup which crystallised on scratching. Yield, 1.9 g.; m.p. crude 160° . Recrystallised from aqueous alcohol the crystals had m.p. 165° alone or in admixture with *D*-galactose; $[\alpha]_{5790} = 148.7^\circ \rightarrow 83.8^\circ$ ($c=1.11$) after 2 days (equilibrium value for *D*-galactose $[\alpha]_{5790}^{20} = +83.6^\circ$). The identity of the galactose was confirmed by the formation of galactosazone (m.p. and mixed m.p. 196°) and by the preparation, on oxidation with nitric acid, of *D*-mucic acid (m.p. 214°); yield, 77 % theoretical. No other sugar could be detected.

Hydrolysis of the more stable polysaccharide. This polysaccharide (1.1 g.) was heated for 90 minutes at 100° with 50 ml. of 3N sulphuric acid. The acid was neutralised with barium carbonate, the barium sulphate filtered off and the solution concentrated *in vacuo* at 30° . The syrup was extracted with 95 % alcohol, the removal of which by evaporation gave a colourless product.

On treatment of a portion of the syrup (0.2 g.) with phenylhydrazine acetate (0.2 g.) in the cold for $\frac{1}{2}$ hour a sandy crystalline powder separated. After standing for an hour the crystals were filtered off and recrystallised from 50 % alcohol (0.28 g.), from which solvent they separated in the characteristic form of mannosephenylhydrazone and had m.p. and mixed m.p. with an authentic specimen 196° .

This phenylhydrazone, on heating with an excess of phenylhydrazine acetate at 100° for 5 minutes, yielded glucosazone identical with an authentic specimen.

The remainder of the syrup was scratched with a nucleus of *d*-mannose and crystallised completely in a few days, M.P. 135° (M.P. of mannose 132–133°).

Fractionation of the mixture of the two polysaccharides. It was found that the crude polysaccharide could be separated into various fractions by the graded addition of absolute alcohol to a concentrated aqueous solution. In a typical experiment 78 g. of crude polysaccharide $[\alpha]_{5790} = -41.0^\circ$ gave the following results.

Table V.

Fraction	$[\alpha]_{5790}$	% alcohol	% ash	Yield (g.)
1	0	50	40.3	12
2	-34.2°	66	3.5	40
3	-37.6°	80	5.8	9
4	-56.7°	Concentrated mother-liquor	3.7	14

Most of the ash was removed in the first fraction and it was found that the ash could be further separated as follows. 43 g. of crude polysaccharide with a high ash content were dissolved in 150 ml. of water. To this syrup, pyridine (200 ml.) was carefully added. A fraction (7 g.) was precipitated and removed by centrifuging. This fraction contained 90 % of ash. The remaining fractions could be precipitated by the addition of alcohol to the aqueous pyridine solution.

By an elaborate and tedious method of repeated precipitations it was possible to isolate specimens of a polymannose having $[\alpha]_{5790} = +63^\circ$ (15 g.) and a polygalactose $[\alpha]_{5790} = -84^\circ$ (12 g.). A series of intermediate fractions is in process of separation.

Properties of the purified polymannose. This compound was a pure white hygroscopic powder containing, before final purification, 15 % of moisture and 10 % of ash. The ash content was lowered to 2.5 % after one precipitation from weak hydrochloric acid solution by the addition of alcohol. Its aqueous solution was colourless, acid to litmus, non-reducing and without action on iodine. It was stable to *N* H₂SO₄ at 80° for 2 hours but was readily hydrolysed with 3*N* H₂SO₄ at 100°. A sample having an ash content of 0.7 % and dried to constant weight gave the following results on hydrolysis with 3*N* H₂SO₄ at 100°:

0 minutes $[\alpha]_{5790} = +63.0^\circ$, 10 minutes $[\alpha]_{5790} = +49.1^\circ$, 25 minutes $[\alpha]_{5790} = +31.4^\circ$, 45 minutes $[\alpha]_{5790} = +17.7^\circ$, 60 minutes $[\alpha]_{5790} = +15.7^\circ$, 90 minutes $[\alpha]_{5790} = +15.7^\circ$. Therefore the equilibrium rotation calculated as hexose = +14.1° (equilibrium rotation recorded for mannose = +15.2°). Mannose was the only hydrolysis product isolated.

An analysis was carried out on the incompletely dried polysaccharide and the moisture content estimated separately. The analysis was not entirely satisfactory owing to the hygroscopic nature of the ash.

(Found: Moisture, 15.0 %; ash, 9.6 %; C, 44.6; H, 7.5 %. (C₆ H₁₀ O₅)_n requires: C, 44.4; H, 6.2 %.)

Preparation of an acetate. The polysaccharide (1 g.) was dissolved in water (1 ml.) and pyridine (25 ml.) added slowly. A small amount of inorganic material separated and was removed by centrifuging. Acetic anhydride (25 ml.) was now added slowly and the solution maintained at 40° during the addition (10 minutes). It was then cooled to 20° and left overnight. Next morning the clear pale yellow solution was poured into 200 ml. of warm water, and the acetate was obtained as a flocculent precipitate. It was centrifuged and washed repeatedly with warm water until free from the acetylation mixture. It was dried *in vacuo*. Yield, 1.6 g. Ash content, 0.9 %; OAc, 46.1 %. $[\alpha]_{5790} = +52.6^\circ$ in acetone

($c=1.56$). Fractional precipitation of the acetate from acetone by means of ether showed that it was homogeneous.

Regeneration of the polysaccharide. The acetate (1 g.) was dissolved in acetone and a slight excess of N NaOH added. The product was precipitated twice from weak HCl solution, washed with absolute alcohol and dried *in vacuo*. Ash content, 0.7 %. $[\alpha]_{5790} = +62^\circ$ ($c=1.0$).

Properties of the purified polygalactose. This was a white hygroscopic powder containing 1.83 % of ash and 10 % of moisture; $[\alpha]_{5790} = -84.3^\circ$. It was analysed in the same manner as the polymannose and again the ash content prevented a satisfactory analysis.

(Found: C, 44.4; H, 7.0 %. $(C_6H_{10}O_5)_n$ requires: C, 44.4; H, 6.2 %.)

The solution in water was colourless, faintly acid to litmus and with no action on Fehling's solution or on iodine.

It readily hydrolysed as follows with 0.3 N sulphuric acid. ($c=0.711$.) 0 minutes $[\alpha]_{5790} = -84.3^\circ$, 10 minutes $[\alpha]_{5790} = -50.0^\circ$, 15 minutes $[\alpha]_{5790} = 0^\circ$, 20 minutes $[\alpha]_{5790} = +30.0^\circ$, 45 minutes $[\alpha]_{5790} = +72.0^\circ$, 60 minutes $[\alpha]_{5790} = +88.0^\circ$ (equilibrium value). Equilibrium value corrected for ash and as hexose = $+80.8^\circ$; $[\alpha]_{5790}^{20}$ for d -galactose = $+83.6^\circ$.

Acetylation. The polysaccharide (1 g.) was acetylated and the product isolated in the manner described for the polymannose. Yield, 1.5 g. of a white, ash-free powder. It had $[\alpha]_{5790}$ in acetone = -72.8° ($c=0.75$). Acetyl content, 44.4 %. (Theoretical for the triacetate of a polyhexose, $C_6H_7O_2(OCOCH_3)_3$, 44.8 %.)

Regeneration of the polysaccharide by deacetylation with sodium hydroxide was unsatisfactory, the product being extremely hygroscopic.

II. *Investigation of the polysaccharides from Czapek-Dox medium.* The products were obtained by precipitation by acetone from the concentrated metabolism solution and isolated as previously described for Raulin-Thom medium. The ash content was very high (approximately 30 %), and the product was more soluble in water giving a clear yellow solution. The yields and properties were as follows.

Table VI.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
5 + 6	102.3	30.0	0
7	51.6	31.2	-8.0°
10	45.6	28.5	-5.0°

Fractional precipitation from aqueous solution with alcohol removed most of the ash in the first fraction, and a series of fractions with different rotations was obtained. The crude material behaved on hydrolysis in a manner exactly similar to the product described from Raulin-Thom medium.

Hydrolysis of the crude polysaccharide with 0.5 N H_2SO_4 . A portion of the crude polysaccharide containing 9.5 % ash, and having an initial rotation of $[\alpha]_{5790} = -15.3^\circ$ dissolved ($c=0.96$) in 0.5 N H_2SO_4 , and heated at 100° reached an equilibrium value of $[\alpha]_{5790} = +48^\circ$ in 70 minutes.

Strong sulphuric acid was now added to make a 3 N solution and the solution heated again at 100° . The rotation fell to an equilibrium value of $[\alpha]_{5790} = +35.3^\circ$ in 60 minutes.

The crude polysaccharide (3 g.) was hydrolysed with 0.3 N H_2SO_4 as previously described. 2 g. of unhydrolysed polysaccharide were obtained on pouring the solution into absolute alcohol. This polysaccharide gave d -mannose on hydrolysis and after purification was identical with the polymannose previously described.

The mother-liquors on concentration and extraction with alcohol gave crystalline *d*-galactose, and it is probable that the crude polysaccharide is a mixture of the same polymannose and polygalactose as from the Raulin-Thom medium though this mixture has a higher polymannose content.

C. Fractions III, IV and V (Ether extracts).

Each of the above fractions, representing the material extracted by ether in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of chloroform (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with chloroform. In this way, each extract was separated into a quantity of white or light brown crystalline material and a chloroform mother-liquor which, after removal of chloroform, was dried down to a reddish brown syrup.

Using Czapek-Dox medium, the crystalline material obtained from fractions III and IV consisted of almost pure carlosic acid and from fraction V of almost pure carolinic acid, the total yields of which are given in Table VII. It appears therefore, that carlosic acid is completely extracted in the first three of the ten ether extracts, together with small amounts of carolinic acid, whilst the latter acid is extracted more slowly and is accumulated in the remaining ether extracts. This behaviour may be readily accounted for by considering the relative solubilities of these two acids in water and ether as shown later.

Using Raulin-Thom medium the crystalline material consisted in each of the fractions III, IV and V of a mixture of carolinic acid with varying but smaller amounts of verticillic acid, the total yields of which are given in Table VII. These two acids were then separated by fractionation with acetone in which verticillic acid is very much less soluble than carolinic acid.

Examination of the syrups obtained by drying down the chloroform mother-liquors from the above acids showed that in all cases appreciable amounts of carolic acid are present. In order to separate this acid, the combined syrups from experiments 5, 6, 7 and 10 on Czapek-Dox medium and, separately, the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium were thoroughly triturated with water. The combined syrups from the Czapek-Dox medium were extracted with a total volume of 450 ml. of water and the syrups from the Raulin-Thom medium with 300 ml. of water.

The aqueous extracts were then filtered and extracted once with an equal volume of chloroform. The chloroform extracts were evaporated and the resultant yellowish oil seeded with carolic acid and set aside to crystallise. The crystalline residue was finally rubbed up with a little absolute ethyl alcohol, filtered and washed with a small amount of alcohol, when almost pure carolic acid resulted. By this treatment the combined syrups from the experiments 5, 6, 7 and 10 on Czapek-Dox medium (total weight after drying at 50° *in vacuo* = 250 g.) yielded 53.5 g. of yellowish oil from which 30 g. of carolic acid were obtained, while the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium (total weight after drying at 50° *in vacuo* = 200 g.) yielded 35.4 g. of yellowish oil from which 18 g. of carolic acid were obtained.

Having removed the carolic acid from the combined syrups by water extraction as described above, the water-insoluble portion of the syrups was allowed to stand for four weeks when further crystallisation occurred, and small amounts of ramigenic acid separated in hard, well-formed prisms. These were filtered off, washed clean from syrup by rubbing up with chloroform and recrystallised

from acetone-light petroleum. The weights of pure ramigenic acid obtained from the syrups from the Raulin-Thom and the Czapek-Dox media were 3 g. and 1 g. respectively.

The syrups remaining after separation of ramigenic acid still contained small amounts of verticillic acid, amounting to about 7 % of the weight of the syrup from both media. In addition, in the case of the syrup from Czapek-Dox medium, but not from Raulin-Thom medium, a very small amount of carlosic acid was detected. These two acids were isolated by making use of specific precipitating reagents. Thus verticillic acid was separated by neutralising an aliquot portion of the syrup with sodium hydroxide to phenolphthalein and adding mercuric chloride which, as will be shown later, precipitates verticillic and ramigenic acids but does not precipitate the remaining four acids. The mercury precipitates from the two media were then suspended in water, the mercury removed with hydrogen sulphide, the excess of hydrogen sulphide removed in a stream of air and the solutions extracted with ether. After removal of the ether, the extracted material crystallised and was rubbed up with a small volume of acetone, filtered and washed with a little acetone. The residual crystalline material was shown by comparison with an authentic specimen to be verticillic acid. Finally, the presence of a very small amount of carlosic acid in the syrups from Czapek-Dox medium was detected by carefully adding normal lead acetate to the neutralised solution of an aliquot portion of the syrup. This reagent, as will be shown later, specifically precipitates carlosic acid, but its addition requires care as the precipitate redissolves in excess of the reagent. The lead precipitate was then rubbed up with 2N H_2SO_4 and the carlosic acid extracted with ether. After removal of the ether, the yellow crystalline mass was rubbed up with chloroform, filtered and washed with a little chloroform. The very small amount of crystalline material was shown by comparison with an authentic specimen to be carlosic acid $\text{C}_{10}\text{H}_{12}\text{O}_6$.

D. Fractions VI and VII (Chloroform extracts).

Each of the above fractions, representing the material extracted by chloroform in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of absolute ethyl alcohol (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with a little alcohol. In this way all the fractions VI gave a white crystalline powder consisting of almost pure carolic acid and amounting to 70 % of the total weight of the fractions, whilst all the fractions VII gave small yields of a white crystalline powder consisting of fairly pure carlic acid. The alcoholic mother-liquors from fractions VI and VII were evaporated *in vacuo* and extracted with water in which they were almost completely soluble. The aqueous solutions

Table VII. Yields in g. of isolated and almost pure crystalline acids.

Exp. No.	Medium	Carolic acid $\text{C}_9\text{H}_{10}\text{O}_4$	Carolinic acid $\text{C}_9\text{H}_{12}\text{O}_7$	Carlic acid $\text{C}_{10}\text{H}_{10}\text{O}_6$	Carlosic acid $\text{C}_{10}\text{H}_{12}\text{O}_6$	Verticillic acid $\text{C}_{26}\text{H}_{32}\text{O}_{12}$	Ramigenic acid $\text{C}_{16}\text{H}_{20}\text{O}_6$
5	Czapek-Dox	44.7	8.2	7.5	9.3	6.7	} 1 g.
6	"	43.7	10.2	5.4	9.0	4.4	
7	"	44.6	3.9	1.7	5.6	5.9	
10	"	42.4	7.8	5.3	9.6	3.3	
8	Raulin-Thom	29.7	16.3	4.1	—	13.6	} 3 g.
9	"	27.9	14.9	3.0	—	13.2	
11	"	29.6	14.5	1.3	—	11.5	
12	"	26.7	16.6	1.1	—	4.2	

were then extracted once with an equal volume of chloroform, the chloroform removed and the partially crystalline residue washed with alcohol, further small amounts of carolic acid being thus obtained. The total yield of carolic acid obtained from fraction VI was thus raised to 75 % of the weight of this fraction.

The final yields of the practically pure crystalline acids in the eight typical experiments are given in Table VII.

Properties and analysis of the crystalline acids.

We propose now to give details of the general properties and analysis of the different crystalline acids isolated, reserving for future communications the question of their molecular constitution. The equivalents were determined by dissolving a weighed amount in a suitable solvent and titrating to phenolphthalein with *N*/10 sodium hydroxide. Optical rotations were determined at room temperature on the free acids in a suitable solvent and also on neutral aqueous solutions of the sodium salts using the mercury green line λ_{5461} . Solubilities were determined roughly by shaking 0.1 g. of the finely powdered material at room temperature with a gradually increasing and measured amount of the solvent in question until dissolution was just complete. The reactions of aqueous solutions of the sodium salts of the different acids towards a series of inorganic reagents were determined by adding to 2 ml. of a 1 % aqueous solution of the acid, neutralised to phenolphthalein with sodium hydroxide, 1 ml., drop by drop, of a 10 % aqueous solution of the reagent in question. All micro-analyses were carried out by Schoeller (Berlin).

Carolic acid $C_9H_{10}O_4$. Carolic acid is best purified by recrystallising (2 g.) from boiling ethyl alcohol (20 ml.). Yield 1.4 g. of colourless radiating needles, m.p. 132° without obvious decomposition. On resetting and remelting the m.p. gradually falls to a constant value of 113°.

(Found: C, 59.45, 59.42; H, 5.61, 5.58 %; OCH_3 , nil; Mol. Wt. 217, 235. $C_9H_{10}O_4$ requires: C, 59.31; H, 5.54 %; Mol. Wt. 182.) The molecular weight found by the depression of the freezing-point of benzene was 196.

Titration. 0.2380 g. and 0.2318 g. required respectively 13.05 and 12.70 ml. *N*/10 NaOH, corresponding to equivalents of 182.4 and 182.5 respectively. ($C_9H_{10}O_4$ titrating as a monobasic acid requires 182.1.)

Rotations. (a) Free acid in water: ($c=0.50$) $[\alpha]_{5461} = +84^\circ$.

This value remained constant for 1 hour but had fallen after 24 hours to $+81^\circ$; the solution becoming faintly yellow in colour.

(b) Sodium salt: ($c=0.52$ calculated as monosodium salt) $[\alpha]_{5461} = +71.5^\circ$.

This value did not change overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 2 ml. chloroform, 12 ml. water or acetone, 28 ml. ethyl acetate, 36 ml. ethyl alcohol, 40 ml. benzene, 400 ml. ether, but was not completely dissolved in 500 ml. light petroleum, b.p. 40–50°.

Salts. Carolic acid gives no precipitate even on standing overnight with mercuric chloride, copper sulphate, uranium acetate, normal or basic lead acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate gives no precipitate even in concentrated solution and no reduction on standing overnight. Ferric chloride gives a characteristic bright orange colour but no precipitate (*cf.* carolinic acid).

An aqueous solution of the acid is strongly acid to Congo red, reduces acid or neutral permanganate immediately in the cold and decolorises bromine water instantly. With Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl), there is no immediate precipitate but on standing with excess of reagent a crystalline orange precipitate begins to separate after 48 hours and continues for 3 to 4 weeks. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, remaining colourless at 100° but at 140–150° becoming first rose-pink, then darkening to a characteristic intense bluish red colour.

Carolinic acid $C_9H_{12}O_7$. ($C_9H_{10}O_6$, H_2O .) Carolinic acid is best purified by recrystallisation from acetone (or acetone-light petroleum) from which solvent it separates in colourless prisms, m.p. 123° with evolution of gas.

(Found: C, 46.53, 46.43; H, 5.26, 5.21 %; OCH_3 , nil. $C_9H_{12}O_7$ requires: C, 46.53; H, 5.21%; Mol. Wt. 232.) The molecular weight of carolinic acid must be that corresponding to the formula $C_9H_{12}O_7$ since it readily loses $1H_2O$ giving a product having an observed molecular weight corresponding to $C_9H_{10}O_6$ (see below).

Titration. 0.1723 g. required 14.50 ml. $N/10$ NaOH corresponding to an equivalent of 117. ($C_9H_{12}O_7$ titrating as a dibasic acid requires 116.)

Rotation. (a) Free acid in water: ($c=0.34$) $[\alpha]_{5461} = +60^\circ$. The rotation did not change overnight but the solution became faintly yellow in colour.

(b) Disodium salt: ($c=0.41$; calculated as the disodium salt) $[\alpha]_{5461} = +54^\circ$. The rotation did not change overnight and the solution remained colourless.

Solubilities. 1 g. dissolves in 25 ml. ethyl alcohol, 30 ml. acetone, 150 ml. water, 170 ml. ethyl acetate, but was not completely dissolved in 500 ml. benzene, chloroform, ethyl ether or light petroleum, B.P. $40-50^\circ$.

Salts. Carolinic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brown precipitate, soluble in excess to a bright orange-coloured solution. Copper sulphate gives no immediate precipitate but on standing there is a copious separation of beautiful pure blue needles. Basic lead acetate gives a very heavy immediate precipitate completely soluble in excess. Barium or calcium chloride in presence of four volumes of ethyl alcohol gives a gelatinous precipitate tending to become crystalline on standing.

An aqueous solution of the acid is strongly acid to Congo red and only slowly reduces permanganate in the cold. With Brady's reagent it slowly gives a slight yellow precipitate. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes dark brownish red on heating to $140-150^\circ$.

Carolinic acid, crystallised from acetone, readily loses a molecule of water on drying at 80° . 0.2216 g. dried to constant weight at 80° lost 0.0170 g. $H_2O=7.67\%$ H_2O . (Theoretical for $C_9H_{12}O_7 \rightarrow C_9H_{10}O_6 + 7.76\%$.)

(Found for the dried substance: C, 50.81; H, 4.73 %; Mol. Wt. 223, 231; equivalent, 105. $C_9H_{10}O_6$ requires: C, 50.45; H, 4.71 %; Mol. Wt. 214; equivalent titrating as a dibasic acid, 107.) The dehydrated material on recrystallisation from acetone reverts to carolinic acid $C_9H_{12}O_7$.

Carlic acid $C_{10}H_{10}O_6$. Carlic acid is best purified by recrystallising from ethyl alcohol, from which it separates in colourless needles, m.p. 176° , giving a brown gummy melt which does not resolidify on cooling.

(Found: C, 53.23, 53.22; H, 4.53, 4.47 %; OCH_3 , nil. $C_{10}H_{10}O_6$ requires: C, 53.08; H, 4.46 %; Mol. Wt. 226.) The molecular weight found by the depression of the freezing-point of dioxan was 240. The empirical formula of carlic acid $C_{10}H_{10}O_6$ also follows from that of carolic acid $C_9H_{10}O_4$ since, from observations which will be published later, it is known that the molecule of carlic acid contains CO_2 more than that of carolic acid.

Titration. 0.1214 g. required 10.70 ml. $N/10$ NaOH corresponding to an equivalent of 113.4. ($C_{10}H_{10}O_6$ titrating as a dibasic acid requires 113.)

Rotation. (a) Free acid in water: ($c=0.28$) $[\alpha]_{5461} = -160^\circ$. This value was not changed overnight and the solution remained colourless.

(b) Disodium salt: ($c=0.29$; calculated as the disodium salt) $[\alpha]_{5461} = -147^\circ$. This value was not changed overnight and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. water, 45 ml. acetone, 75 ml. ethyl alcohol, 270 ml. ethyl acetate, 350 ml. chloroform, 500 ml. benzene, but was not completely dissolved in 500 ml. ethyl ether, light petroleum, B.P. $40-50^\circ$, or butyl alcohol.

Salts. Carlic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brownish-yellow precipitate soluble in excess to an orange-yellow solution. Copper

sulphate gives no immediate precipitate but after several days' standing blue-green nodular crystals separate, which are quite different in colour from those given by carolinic acid. Basic lead acetate gives a very heavy precipitate which is readily soluble in excess. Calcium chloride in presence of four volumes of alcohol does not give an immediate precipitate, but on standing for several days a moderate precipitate is formed. Barium chloride in presence of four volumes of alcohol gives an immediate heavy flocculent precipitate.

An aqueous solution is acid to Congo red, reduces acid permanganate fairly slowly and does not give a precipitate with Brady's reagent. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, which becomes only pale brown on heating for some time at $140\text{--}150^\circ$.

Carlosic acid $\text{C}_{10}\text{H}_{12}\text{O}_6$. Carlosic acid is best purified by crystallisation from a large volume of boiling benzene from which it separates in fine colourless needles, m.p. 181° .

(Found: C, 52.51, 52.62; H, 5.35, 5.30 %; OCH_3 , nil; Mol. Wt. 236, 242. $\text{C}_{10}\text{H}_{12}\text{O}_6$ requires: C, 52.61; H, 5.31 %; Mol. Wt. 228.)

Titration. 0.1040 g. required 9.20 ml. $N/10$ NaOH corresponding to an equivalent of 113.1. ($\text{C}_{10}\text{H}_{12}\text{O}_6$ titrating as a dibasic acid requires 114.)

Rotations. (a) Free acid in water: ($c=0.21$) $[\alpha]_{5461} = -160^\circ$. This value was unchanged overnight and the solution did not become coloured.

(b) Disodium salt: ($c=0.25$ calculated as the disodium salt) $[\alpha]_{5461} = -150^\circ$. This value was unchanged overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 14 ml. acetone, 30 ml. ethyl alcohol, 45 ml. ethyl acetate, 90 ml. water, 250 ml. of chloroform or ether, 500 ml. benzene, but was not completely dissolved in 500 ml. light petroleum, B.P. $40\text{--}50^\circ$.

Salts. Carlosic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, copper sulphate or calcium chloride in presence of four volumes of ethyl alcohol. Ferric chloride gives an immediate brownish-yellow precipitate insoluble in excess. Uranium acetate gives no immediate precipitate but on standing for 2–3 days a characteristic copious semi-crystalline yellow precipitate separates. Both normal and basic lead acetates give immediate very heavy precipitates soluble in large excess. Barium chloride in presence of four volumes of alcohol gives a flocculent white precipitate.

An aqueous solution is acid to Congo red, reduces acid KMnO_4 fairly slowly, and gives an immediate orange-yellow crystalline precipitate with Brady's reagent, m.p. 135° . The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes only pale brown on heating at $140\text{--}150^\circ$.

Ramigenic acid $\text{C}_{16}\text{H}_{20}\text{O}_6$. Ramigenic acid is precipitated on acidifying aqueous solutions of the sodium salt in large prisms containing $1\text{H}_2\text{O}$ (a), which is lost on standing *in vacuo* over P_2O_5 for several days. It crystallises in well-formed prisms from acetone-light petroleum (b). The acid $\text{C}_{16}\text{H}_{20}\text{O}_6 \cdot \text{H}_2\text{O}$ softens slightly at 125° owing to loss of water, but does not melt. The anhydrous acid melts at 171° .

(Found: (a) C, 59.24, 59.10; H, 6.62, 6.75 %; OCH_3 , nil. $\text{C}_{16}\text{H}_{20}\text{O}_6 \cdot \text{H}_2\text{O}$ requires: C, 58.86; H, 6.80 %. (b) C, 62.40, 62.30; H, 6.59, 6.51 %; OCH_3 , nil. $\text{C}_{16}\text{H}_{20}\text{O}_6$ requires: C, 62.30; H, 6.54 %.)

Titration. 0.2343 g. of the acid $\text{C}_{16}\text{H}_{20}\text{O}_6$ was dissolved in 20 ml. absolute alcohol and titrated to phenolphthalein. The first pink flush (rapidly disappearing) was obtained after adding 7.70 ml. $N/10$ NaOH corresponding to an equivalent of 304 ($\text{C}_{16}\text{H}_{20}\text{O}_6$ titrating as a monobasic acid requires 308). A further 12.30 ml. $N/10$ NaOH (total 20.0 ml.) were added and the solution heated on the water-bath for 2 hours. On cooling and titrating, the red colour first disappeared after adding 5.24 ml. $N/10$ H_2SO_4 , corresponding to an equivalent of 158.8 ($\text{C}_{16}\text{H}_{20}\text{O}_6$ titrating as a dibasic acid requires 154). On standing, the solution became slowly pink, was decolorised by addition of a few drops of $N/10$ H_2SO_4 and, on standing, again became pink. The process was repeatable, indicating that the more difficultly opened acidic group responsible for the dibasic character closes with great ease in more acid solution. The solution was then acidified and allowed to stand. Well-formed prisms having the characteristics of ramigenic acid separated, m.p. 171° (after loss of water at 125°) and not depressed on mixing with an authentic sample.

Rotations. (a) Free acid in alcohol: ($c=0.22$) $[\alpha]_{5461} = +28^\circ$. The rotation did not change overnight, and the solution did not become coloured.

(b) Sodium salt: ($c=0.27$; calculated as monosodium salt) $[\alpha]_{5461} = +85^\circ$. The rotation did not change overnight, and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. acetone, 16 ml. ethyl alcohol, 30 ml. chloroform, 90 ml. ethyl acetate, 180 ml. ether, but was not completely dissolved in 500 ml. water, benzene or light petroleum, B.P. 40–50°.

Salts. Ramigenic acid gives no precipitate with copper sulphate, uranium acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate is slowly reduced in the cold, giving a silver mirror on standing overnight. Ferric chloride gives a heavy pale brown crystalline precipitate, probably of ramigenic acid, the supernatant liquid being only light yellowish brown in colour. Mercuric chloride gives an immediate yellow colour with slow development of a heavy pale yellow precipitate. Normal and basic lead acetates give slight white precipitates.

The acid is insoluble in water and an aqueous suspension is not acid to Congo red and reduces KMnO_4 immediately in neutral and more slowly in acid suspension. No reaction occurs with Brady's reagent, but this may be due to the insolubility of the acid. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

Verticillic acid $\text{C}_{26}\text{H}_{32}\text{O}_{12}$. Verticillic acid is best purified by allowing the boiling saturated acetone solution to stand overnight, when the acid separates in fine needles, M.P. 171°, giving a brown viscous melt which does not reset on cooling.

(Found: C, 58.06, 58.24; H, 6.04, 6.06 %; OCH_3 , nil. $\text{C}_{26}\text{H}_{32}\text{O}_{12}$ requires: C, 58.17; H, 6.02 %.)

Titration. Verticillic acid on titrating in alcoholic solution behaves like an acid lactone; 0.1578 g. dissolved in 20 ml. alcohol gave the first pink flush after the addition of 11.09 ml. $N/10$ NaOH. The titration then became slow as for a lactone. After standing for three hours at room temperature with an excess of NaOH, the final titration was 11.74 ml. $N/10$ NaOH, corresponding to an equivalent of 134.5 ($\text{C}_{26}\text{H}_{32}\text{O}_{12}$ titrating as the lactone of a tetrabasic acid requires 134).

Rotations. (a) Free acid in alcohol: ($c=0.40$) $[\alpha]_{5461} = -53^\circ$. This value rapidly fell on standing and the solution became progressively more and more yellow in colour. The specific rotations after 10, 15 and 45 minutes, 2, 5, 24, 48 and 72 hours were respectively -47° , -44° , -36° , -35° , -34° , -28° , -20° , -14° .

(b) Sodium salt: ($c=0.49$, calculated as the tetrasodium salt) $[\alpha]_{5461}$ initially $= +46^\circ$ and only slowly decreases to $+41^\circ$ after 24 hours, the solution becoming faintly yellow in colour.

Solubilities. 1 g. dissolved in 45 ml. ethyl alcohol, 270 ml. acetone, but was not completely dissolved in 500 ml. water, benzene, chloroform, ethyl acetate, ether and light petroleum, B.P. 40–50°.

Salts. Silver nitrate gives no precipitate, but reduction commences after 2–3 hours in the cold with the formation of a silver mirror overnight. Mercuric chloride gives an immediate heavy white precipitate with a yellow supernatant fluid. Ferric chloride gives a characteristic very intense brownish-red colour. Uranium acetate gives no precipitate but a characteristic orange-yellow colour. Slight precipitates are given with copper sulphate, normal and basic lead acetates and calcium or barium chloride in presence of four volumes of alcohol.

An aqueous solution of verticillic acid is faintly acid to Congo red and reduces acid permanganate very readily in the cold. An alcoholic solution of the acid gives no immediate precipitate with Brady's reagent, but an orange precipitate begins to separate after 12 hours. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

The molecular weights by the Rast camphor method for both verticillic and ramigenic acids were unsatisfactory, owing to the fact that they both decompose on heating at a temperature below that of the melting-point of camphor. The molecular formulae $\text{C}_{26}\text{H}_{32}\text{O}_{12}$ and $\text{C}_{16}\text{H}_{20}\text{O}_6$ have been assigned to verticillic and ramigenic acids respectively for the following reasons:

(a) Verticillic acid (1 molecule) on mild hydrolysis gives rise to 1 molecule of ramigenic acid and a keto-acid which analyses for the empirical formula of $(\text{C}_5\text{H}_6\text{O}_3)_n$.

(b) Ramigenic acid titrates in the cold as a monobasic acid, equivalent 304 ($C_{16}H_{20}O_6$ requires 308).

(c) Ramigenic acid with diazomethane gives a crystalline monomethyl derivative containing 9.60 % OCH_3 and having an observed molecular weight by the Rast camphor method of 332 and 340 ($C_{16}H_{18}O_5OCH_3$ requires 9.63 % OCH_3 and molecular weight 322). It follows therefore that the empirical formula for ramigenic acid must be $C_{16}H_{20}O_6$ and hence that for verticillic acid is $C_{26}H_{32}O_{12}$.

SUMMARY AND DISCUSSION.

The metabolic products of a new mould species isolated from mouldy Italian maize, *Penicillium Charlesii* G. Smith, have been investigated. This mould was grown at 24° on Czapek-Dox medium in which glucose is the only source of carbon, and on Raulin-Thom medium in which glucose is the only carbohydrate present. The metabolic products isolated consist of two new polysaccharides and six hitherto undescribed optically active organic acids.

The polysaccharides are a polygalactose $[\alpha]_{5790} = -84^\circ$ and a polymannose $[\alpha]_{5790} = +63^\circ$ which give on hydrolysis *d*-galactose and *d*-mannose respectively as the only carbohydrates. Both of these polysaccharides were formed on both media and their production in considerable amounts affords an interesting example of the ready conversion by a mould of glucose into mannose and galactose.

The six organic acids isolated are as follows: (a) carolic acid $C_9H_{10}O_4$, (b) carolinic acid $C_9H_{12}O_7$, (c) carlic acid $C_{10}H_{10}O_6$, (d) carlosic acid $C_{10}H_{12}O_6$, (e) ramigenic acid $C_{16}H_{20}O_6$ and (f) verticillic acid $C_{26}H_{32}O_{12}$. Of these (a), (b) and (e) are dextrorotatory while (c), (d) and (f) are laevorotatory. The acids were isolated from the metabolism solution and separated from each other in a state of purity by taking advantage of their differing solubilities and of their varying precipitability by different metallic salts.

It is a point of considerable microbiological interest to compare the products of metabolism of three species of *Penicillium*, all of which were isolated from mouldy maize, *i.e.* of *P. brevi-compactum* Dierckx (syn. *P. stoloniferum* Thom), *P. Charlesii* G. Smith, each of which was isolated from mouldy Italian maize, and *P. puberulum* Bainier from mouldy American maize. *P. brevi-compactum* produces from glucose the following series of optically inactive phenolic acids, each containing a benzenoid nucleus: $C_{17}H_{20}O_6$, $C_{10}H_{10}O_7$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_5$ and $C_8H_6O_6$ [Clutterbuck *et al.*, 1932; Oxford and Raistrick, 1932; 1933, 1, 2; Clutterbuck and Raistrick, 1933]. *P. Charlesii* on the other hand produces from glucose the following series of optically active non-benzenoid acids; $C_{26}H_{32}O_{12}$, $C_{16}H_{20}O_6$, $C_{10}H_{12}O_6$, $C_{10}H_{10}O_6$, $C_9H_{12}O_7$ and $C_9H_{10}O_4$. It will be noticed that two acids having the empirical formula $C_{10}H_{10}O_6$, but differing widely in chemical constitution, are produced by *P. brevi-compactum* and *P. Charlesii*, which curiously enough were isolated from the same sample of mouldy Italian maize. *P. puberulum* produces from glucose three organic acids, penicillic acid $C_8H_{10}O_4$ [Alsberg and Black, 1913], puberulic acid $C_8H_6O_6$, and an acid $C_8H_4O_6$ [Birkinshaw and Raistrick, 1932]. Each of these three acids is optically inactive and non-benzenoid in nature.

In view of the amazing variety of the metabolic products of different species of *Penicillia* occurring in mouldy maize, some of which are known to be definitely toxic, it is tempting to enquire whether the possibility has been entirely ruled out that mould infection of maize has a connection with human pellagra. Most workers on human pellagra appear to agree that this disease is intimately connected in some way with the consumption of maize. Aykroyd [1933] divides the views which have been expressed as to the origin of pellagra under three headings:

- (a) theory of a maize toxin,
- (b) theory of amino-acid deficiency due to the low biological value of maize proteins,
- (c) theory of vitamin deficiency.

It would be outside the scope of this paper to discuss (b) and (c), but we venture to raise the question as to whether the presence of toxic materials elaborated by organisms infecting maize may not form a contributory factor. In this connection it is of interest to note, to quote Aykroyd (page 343), that "Italian antipellagra policy is enshrined as a law passed in 1902 which was based on the theory of causation generally accepted at that date. The sale of 'spoiled' (*i.e.* infected) maize as human food was strictly prohibited, and local authorities were forced to acquire public desiccators and municipal reservoirs in which maize could be kept in good condition." This is particularly interesting in view of the fact that the death rate per 100,000 of population from pellagra reported officially in Italy was 10.7 in the period 1896-1900, a figure which steadily decreased to 0.2 in 1928. On the other hand, in the United States of America where, so far as we are aware, no such law is in force as exists in Italy, the death rate per 100,000 in 1924 was 2.5, increasing steadily to 6.4 in 1928 and being 5.9 in 1930.

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