III. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLVII. THE PRODUCTION OF POLYSACCHARIDES BY *PENICILLIUM LUTEUM* ZUKAL.

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It was shown by Raistrick and Rintoul [1931] that *Penicillium luteum* Zukal, when grown on Czapek-Dox medium containing glucose as the sole source of carbon, produced as the main metabolic product large yields of luteic acid, a malonyl-polyglucose, the products of acid hydrolysis of which consisted only of glucose and malonic acid. Alkaline hydrolysis of luteic acid gave rise to a neutral polysaccharide, luteose, by fission of the malonic acid groups. Later, Birkinshaw and Raistrick [1933] showed that luteic acid was elaborated by the same organism even if the glucose of the medium were replaced by any one of the following compounds as sole source of carbon: fructose, galactose, mannose, xylose, arabinose and glycerol.

During an investigation of the molecular constitution of luteose [Anderson *et al.*, unpublished] it was found that in the total crude material there was mixed with the luteose 8–10 % of another polysaccharide which yielded trimethyl-mannose after methylation and hydrolysis. The present work was undertaken in an attempt to trace the course of formation of this polymannose.

Flasks of Czapek-Dox medium containing 5 % of glucose as the only carbon source were sown with a spore suspension of *P. luteum* Zukal, incubated at 24° and examined for polysaccharide content at intervals. Half the flasks were worked up when approximately 3.5 % of glucose still remained (33 days) and the other half when the glucose had almost entirely disappeared (108 days). The product from each batch was separately submitted to an exhaustive fractionation from aqueous solution by alcohol. By this means the polysaccharide from the younger cultures yielded 8 fractions having different optical rotations and that from the older cultures 7 fractions.

The product from young cultures yielded large quantities of material having a specific rotation $[\alpha]_{5461} - 30^\circ$ to -40° (luteic acid has $[\alpha]_{5461}^{21^\circ} - 37.7^\circ$) together with smaller amounts of material having $[\alpha]_{5461} - 5^\circ$ to -15° , whilst that from old cultures yielded, in addition to the above products, considerable quantities of material having a high specific rotation, $[\alpha]_{5461} - 80^\circ$. Examination of each of these fractions for malonic acid, hexoses and pentoses showed that glucose, mannose and malonic acid were invariably present whatever the age of the culture; galactose was found in some of the fractions, particularly those from old cultures; fructose was found in small amount in some of the fractions, whilst pentoses were invariably absent. The results show that the main product is always a malonyl-polyglucose, that a mannose polysaccharide is found largely in the early stages of growth and that it tends to be replaced by a galactose polysaccharide in older cultures. The polysaccharides produced by young

cultures are hydrolysed less readily by acid than those found in old metabolism solutions.

It seems possible that the production by *P. luteum* of a series of polysaccharides containing different sugars may be closely linked with the formation of luteic acid from a variety of sugars reported by Birkinshaw and Raistrick [1933], both processes involving an intermediate product obtainable from all sugars and capable of being rebuilt into one or more carbohydrate units according to conditions. Support for this view is afforded by the observations of Coyne and Raistrick [1931] on the production of mannitol from hexoses and pentoses by a white species of *Aspergillus*, by the production of cellulose from glucose, sucrose, fructose, mannitol, glycerol and glyceraldehyde by *Bact. xylinum* [Hibbert and Barsha, 1931; Tarr and Hibbert, 1931] and confirmed by the work of Khouvine *et al.* [1932] and of Khouvine [1933], which showed that *Bact. xylinum* was capable of elaborating cellulose from glycerol, sorbitol, mannitol and α -glucoheptitol. A similar conversion of glucose into polysaccharides composed of other carbohydrate units was reported by Clutterbuck *et al.* [1934] who isolated a polymannose and a polygalactose as a result of the action of *P. Charlesii* G. Smith on glucose. Analogous also are the cases of the production of a polysaccharide containing galactose, glucose and idose or altrose found by Haworth *et al.* [1932; 1935] as a result of the cultivation of *P. varians* G. Smith on Czapek-Dox glucose medium. *P. rugulosum* Thom also produces a galactose polysaccharide when grown on Czapek-Dox medium containing glucose as sole source of carbon [Anderson and Raistrick, unpublished]. In this connection may also be cited the report by Dox and Neidig [1914] of the isolation of mycogalactan by extraction of mycelia of *Aspergillus niger* Van Tieghem grown on Raulin's medium.

EXPERIMENTAL.

50 one-litre conical flasks, each containing 350 ml. of Czapek-Dox medium of the following composition, were made up: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 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.; distilled water, 1 litre. After sterilisation each flask was sown with a spore suspension of *Penicillium luteum* Zukal (L.S.H.T.M. Catalogue No. Ad 30). This is the same strain as was used by Raistrick and Rintoul [1931], prepared from 17 Czapek-Dox glucose agar slopes 10 days' old. The flasks were incubated at 24° and single flasks were tested at intervals with the results given in Table I.

Table I.

Incubation period in days	α_{5780}	% Glucose by		Titration ml. 0.1 N NaOH/ 100 ml.	Yield of crude polysaccharide g. per flask
		Polarimeter	Shaffer- Hartmann		
25	+4.11°	3.78	3.76	12.0	1.28
31	+3.97°	3.64	3.60	12.0	0.94
46	+2.35°	2.15	2.51	10.0	1.40
59	+1.76°	1.60	1.86	7.0	1.42
108	-0.22°	—	0.04	2.5	1.75

(A) Examination of polysaccharides produced after short incubation period.

After 33 days' incubation the contents of 23 flasks were filtered through a "Metafilter" and concentrated *in vacuo* to 1 litre. 15 ml. of concentrated HCl were added and the polysaccharides fractionally precipitated with alcohol. The

precipitates obtained with different concentrations of alcohol were filtered, washed with alcohol and ether, dried and their optical rotations determined. The different fractions obtained were grouped according to rotations and were refractionated until a final series of 8 fractions was obtained (Fractions I to VIII—Table II). Each of these fractions was then examined as follows:

(i) Determination of equivalent before and after alkaline hydrolysis by titration with $N/10$ NaOH, treatment with excess of $N/10$ NaOH and back-titration.

(ii) Determination of specific rotation.

(iii) Hydrolysis by N , $2N$, or $3N$ H_2SO_4 at 85° followed polarimetrically.

(iv) Determination of the amount of reducing sugar produced on hydrolysis with H_2SO_4 (this determination was only carried out for fractions weighing 1 g. or more).

(v) Extraction of the hydrolysate from IV with ether and characterisation of the acid extracted by ether as malonic acid by sublimation, melting-point and acid equivalent.

(vi) Neutralisation of the ether-extracted hydrolysate from V with $BaCO_3$. The H_2SO_4 -free hydrolysate was concentrated *in vacuo* and tested for:

(a) Mannose by formation of mannosephenylhydrazone on treatment with phenylhydrazine in the cold.

(b) Glucose by formation of glucosazone after removal of mannose as the phenylhydrazone.

(c) Fructose by Bredereck's reaction [1931] (ammonium molybdate in HNO_3).

(d) Galactose by formation of mucic acid on oxidation with HNO_3 .

(e) Pentoses by Bial's reaction (orcinol + $FeCl_3$ in concentrated HCl).

In some cases it was possible to isolate glucose and galactose as such. The results obtained are summarised in Table II, Fractions I–VIII inclusive.

(B) *Examination of polysaccharides produced after long incubation period.*

The contents of the remaining 23 flasks were filtered after 108 days' incubation and concentrated to 350 ml. (this solution was considerably less viscous than that from the 33-day batch). 7.5 ml. of concentrated HCl were added and the

Table II.

Fraction	Weight g.	Equivalent		Rotation $[\alpha]_{5461}$		% reducing sugar by	
		Before hydrolysis	After hydrolysis	Initial	Final	Shaffer-Hartmann	Alkaline I
I insol. in cold water	1.06	996	413	—	+13°	—	—
II	7.29	864	335	-40.0°	+64.5°	82	93
III	8.35	1065	270	-30.0°	+73.5°	—	75
IV	4.59	2240	377	-15.0°	+60.4°	—	62
V	0.23	2500	300	-26.5°	+61.0°	—	—
VI	1.69	Neutral		-21.6°	+32.5°	—	—
VII	1.88	705	300	-32.7°	+54.5°	40	45
VIII	0.41	Neutral		-5.2°	+5.5°	—	—
IX insol. in cold water	11.95	8930	1880	-38.7°	+63.5°	84	93
X	0.24	6900	1530	-83.2°	+92.5°	—	—
XI	2.23	10000	1100	-80.5°	+86.0°	78	85
XII	3.11	15700	2245	-51.0°	+90.3°	70	86
XIII	4.03	13230	1235	-42.3°	+80.1°	75	90
XIV	0.55	6960	1245	-42.3°	+72.0°	—	—
XV	2.06	528	330	-11.6°	+52.4°	37	43

polysaccharides were fractionally precipitated with alcohol, refractionated and examined in the same way as is described under Section A. The results obtained are summarised in Table II, Fractions IX to XV inclusive.

Discussion of results.

In all the fractions, whether from the young or old cultures, glucose in large amounts and malonic acid in smaller amounts were present and pentoses absent. Mannose was present in appreciable amount in Fractions II, V, VI, VII, X, XIV and XV and in traces in all the remaining fractions. Galactose was actually isolated from Fractions XII and XIII, was detected in traces in Fractions I, II, V, VIII, XI and XIV, and was absent from the remaining fractions. Fructose was present in small amounts in Fractions II, III, XI and XIII, in traces in Fractions IV, IX, XII and XV, and was absent from the remaining fractions.

It is evident that young cultures up to 33 days produce a preponderance of material with a low laevorotation, that is $[\alpha]_{5461} - 40^\circ$ or below, and none above $[\alpha]_{5461} - 40^\circ$. On the other hand, the old cultures (108 days) produce a considerable amount of material having a much higher specific rotation $[\alpha]_{5461} - 80^\circ$, but little material having a small laevorotation. It is also seen that the older cultures produce considerably more polysaccharide material insoluble in cold water than do the younger cultures. Further, much of the polysaccharide produced by old cultures is much more readily hydrolysed than that from the young cultures. A notable difference between polysaccharides from old cultures and those from young cultures is the marked increase in the acid equivalent with age, both before and after alkaline hydrolysis.

It thus appears that whilst luteic acid, defined as a malonyl-polyglucose, is the principal metabolic product of *P. luteum* Zukal at all stages of growth, it is accompanied by a polymannose in the early stages of growth, and that this polymannose tends to be replaced by a polygalactose with increasing age of the culture.

SUMMARY.

Penicillium luteum Zukal, when grown on a Czapek-Dox medium containing glucose as the sole source of carbon, produces as the main metabolic product luteic acid, a malonyl-polyglucose. In addition however there are formed much smaller amounts of other laevorotatory polysaccharides, built up of mannose, galactose or fructose units in proportions varying with the age of the culture.

REFERENCES.

- Birkinshaw and Raistrick (1933). *Biochem. J.* **27**, 370.
Bredereck (1931). *Ber. deutsch. chem. Ges.* **64**, 1730.
Clutterbuck, Haworth, Raistrick, Smith and Stacey (1934). *Biochem. J.* **28**, 94.
Coyne and Raistrick (1931). *Biochem. J.* **25**, 1513.
Dox and Neidig (1914). *J. Biol. Chem.* **19**, 235.
Haworth, Raistrick and Stacey (1932). *Chem. Ind.* **51**, 980.
——— (1935). *Biochem. J.* **29**, 2668.
Hibbert and Barsha (1931). *J. Amer. Chem. Soc.* **53**, 3907.
Khouvine (1933). *Compt. Rend. Acad. Sci.* **196**, 1144.
——— Champetier and Sutra (1932). *Compt. Rend. Acad. Sci.* **194**, 208.
Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond. B* **220**, 255.
Tarr and Hibbert (1931). *Can. J. Res.* **4**, 373.