LIII. STUDIES IN THE BIOCHEMISTRY
OF MICRO-ORGANISMS.
XXVII. THE PRODUCTION OF LUTEIC ACID
FROM VARIOUS SOURCES OF CARBON BY
PENICILLIUM LUTEUM ZUKAL.

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RAISTRICK AND RINTOUL [1931] isolated from the metabolic products of *Penicillium luteum* Zukal, when grown on an aqueous medium consisting only of glucose and inorganic salts, a mucilaginous, laevorotatory material which they termed luteic acid. This substance, the salts of which form very viscous solutions, is a colloidal material of high molecular weight, built up of polysaccharide units, which in the typical molecule arise from the condensation, with the loss of 2 molecules of water, of 2 molecules of glucose with 1 molecule of malonic acid in such a way as to leave only one carboxyl group of the malonic acid free. On mild alkaline hydrolysis luteic acid gives rise to a neutral laevorotatory polysaccharide which was named luteose, and which, on acid hydrolysis, gives glucose as the sole product of hydrolysis.

Luteic acid has up to the present been obtained solely from glucose as substrate, but in view of the unexpected results recorded by Coyne and Raistrick [1931] in obtaining mannitol from various sugars by the agency of the unnamed white species of *Aspergillus*, Catalogue No. Ac 55 (Thom and Church, No. 4640-489), it became of interest to determine whether the mould *P. luteum* Zukal would convert other sugars than glucose into the polyglucose complex, luteic acid, or whether each sugar employed as substrate would afford a different polysaccharide. It was hoped that the results obtained would throw some light on the initial stages of carbohydrate metabolism by moulds, and in particular by the species studied.

The organism used was a non-ascosporic form of *P. luteum* Zukal, Catalogue No. Ad 30, which was originally obtained from the Centraalbureau voor Schimmelcultures at Baarn in May 1925. It is the same strain as that described by Thom [1930, p. 448] as *P. luteum* Zukal, No. 11.

The medium employed in this work consisted of the usual Czapek-Dox salts, together with one of the following sources of carbon, in amount equivalent in carbon to 5 % glucose: fructose, galactose, mannose, xylose, arabinose, glycerol, citric and succinic acids. Acetic and pyruvic acids were also tried, but the mould did not grow on these substrates. The medium had the following composition: 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.; water to 1000 cc., with the addition of one of the sources of carbon mentioned, in amount equivalent in carbon to 50 g. of glucose. In the

case of the media containing the different carbohydrates or glycerol, the p_H was 4.2. In the case of citric acid and succinic acid sufficient 2*N* NaOH was added before final dilution with water to bring the p_H of the medium approximately to 4.2.

The medium was distributed in quantities of 350 cc. in 1 litre flasks, which were sterilised by steaming on each of 3 successive days and then inoculated with a spore suspension of *P. luteum* Zukal which had been grown on Czapek-glucose agar. The flasks were incubated for about 30 days at 24°. Very good characteristic growth was obtained with all the carbohydrates tried and also with glycerol. The growth obtained with succinic and citric acids was moderately good, being better with the former than with the latter. The metabolism solution was filtered from the mycelium, which was well washed with hot water, the washings being added to the main filtrate. The filtrate was then evaporated *in vacuo* to about 1/20 of the original volume and transferred as completely as possible to a beaker. After stirring in 1 cc. of concentrated HCl per flask of culture medium, four volumes of absolute alcohol were added, whereupon the polysaccharide was precipitated as a sticky mass.

The crude material at this stage contains a considerable amount of ash owing to its tendency to salt formation. Attempts were made to purify it by dissolving it in water and subjecting the solution to electro-dialysis. These attempts, however, were unsuccessful since the product appeared to be decomposed under the action of the electric current, breaking up into smaller units which passed through the membrane.

It was therefore decided to adhere closely to the original method of purification [Raistrick and Rintoul, 1931], which consisted in the precipitation of the aqueous solution acidified with HCl by means of absolute alcohol. After five such precipitations the product usually contained only a small amount of ash. The last precipitation was undertaken from neutral solution. The product was ground up in a mortar under alcohol to reduce it to a powdery consistency, filtered, washed with alcohol and finally with a little dry ether. After drying in a vacuum desiccator the product, which now consisted of a white friable powder, was subjected to analysis according to the methods described below.

The following determinations were made.

1. *Rotation of luteic acid.* (Table I, column 2.)

The specific rotation of the metabolism product was determined at 20° in 1 % solution using the mercury green line λ 5461. In order to obtain this value, it was necessary to neutralise the product with NaOH as described by Raistrick and Rintoul [1931]. In any case, owing to experimental difficulties, this figure can only be regarded as an approximation.

2. *Titration.* (Column 3.)

0.25–0.5 g. was titrated in the cold with *N*/10 NaOH, from which the equivalent was calculated.

3. *Ash.* (Column 4.)

The ash was determined on 0.1–0.15 g.

4. *Rotation of luteose.* (Column 5.)

The residual solution from the titration of luteic acid (item 2) was treated with 20 cc. of *N* NaOH and left overnight. 20 cc. of *N* HCl were added, and the neutral polysaccharide was precipitated with 4 volumes of absolute alcohol. The precipitate was allowed to settle, the supernatant liquid was poured off, and the precipitate was dissolved in water. After two further precipitations the luteose was separated and dried. Its specific rotation was determined in 1 % solution in a jacketed tube at 80° using the mercury green line λ 5461.

5. *Sugar determination.* (Columns 6 and 7.)

Acid hydrolysis. 1.0 g. of the metabolism product was treated with 50 cc. N H_2SO_4 and heated in a boiling water-bath under reflux for 9 hours. The volume was then made up to 100 cc. The sugar in the hydrolysate was determined by the polarimeter and by the Wood-Ost method. For the Wood-Ost determination 20 cc. of the hydrolysate were placed in a 25 cc. flask, and the volume was made up to the mark with $2N$ $NaOH$. In this way a practically neutral solution was obtained. 10 cc. were used for each Wood-Ost determination.

6. *Titration.* (Column 10.)

10 cc. of the hydrolysate from item 5 were treated with 5 cc. of N $NaOH$ to neutralise exactly the mineral acid used in hydrolysis. The subsequent titration to phenolphthalein with $N/10$ $NaOH$ then gave the organic acid produced during hydrolysis. This was expressed in terms of the weight of substance taken for hydrolysis as the "apparent equivalent" after hydrolysis. As it represents the difference between two large titrations it is subject to a large experimental error.

7. *Isolation of the acid produced on hydrolysis.* (Column 11.)

The remainder of the hydrolysate from item 5, about 70 cc. in volume, was extracted with ether for about 6 hours in a continuous extraction apparatus. The ether solution was poured into a sublimation tube, the ether evaporated and the residue sublimed in a high vacuum. The sublimed product was tested for malonic acid by determination of the M.P. and mixed M.P. with an authentic sample and by determination of the equivalent.

8. *Osazone from sugar.* (Columns 8 and 9.)

The extracted liquid was neutralised with $2N$ $NaOH$ to phenolphthalein and filtered, and 2 cc. of glacial acetic acid and 3 cc. of phenylhydrazine were added. The mixture was heated in boiling water for about 30 minutes, allowed to cool, filtered, and the osazone separating washed with water, then with a little alcohol and finally with dry ether. When dry, 0.1 g. of the product was dissolved in 3 cc. of alcohol and 2 cc. of pyridine, and the optical rotation was determined immediately and after standing for 24 hours, using the mercury green line.

In some cases the solution did not transmit enough light for accurate determination of its optical rotation without recrystallisation. This was accomplished by cautious addition of water to the alcohol-pyridine solution.

The results obtained for the metabolic products isolated from the different sources of carbon are given in Table I. The figures given for luteic acid produced from glucose are extracted from Raistrick and Rintoul's paper [1931].

Considering now the results obtained for the hexoses, pentoses and glycerol, it is obvious that the figures obtained agree well with the values for luteic acid obtained from glucose as determined by Raistrick and Rintoul [1931], and it may be safely asserted that all these substances give rise to a malonylpolyglucose. Presumptive evidence for the presence of glucose as the only sugar is obtained from the close agreement of the glucose determinations by polarimeter and by the Wood-Ost method. The preparation of the phenylosazone and the determination of its characteristic initial and final rotations afford further evidence. In the case of the product from fructose, 5 g. were hydrolysed by heating with 250 cc. of N H_2SO_4 for 9 hours, and after extraction of the malonic acid the residual solution was neutralised with barium hydroxide. The barium sulphate was removed by filtration, and the filtrate was evaporated *in vacuo* almost to dryness. It was taken up in methyl alcohol, filtered and again evaporated. After redissolving in methyl alcohol, glucose crystallised on standing. It was recrystallised from methyl alcohol, weighed 1.85 g., now melted at

Table I. *Identification of luteic acid from various substrates.*

	Products of acid hydrolysis									
	Luteic acid			Luteose Sp. rotation [α] ₅₄₆₁ (at 90°)	Glucose				Appar- ent equi- valent by titra- tion	Equiv- alent of malonic acid isolated
	Sp. rotation [α] ₅₄₆₁ 20°	Titra- tion equi- valent	Ash %		By polari- meter g./100 cc.	By Wood- Ost g./100 cc.	Osazone			
							Initial rotation	Final rotation		
Glucose	-47°	435	0.79	-46.4°	0.840	0.846	-0.87° (calc.)	—	222	51.6
Fructose	-41°	768	0.87	-46°	0.841	0.843	-0.90°	-0.43°	407	51.7
Galactose	-38°	601	3.62	-46°	0.782	0.795	-0.86°	-0.42°	307	52.0
Mannose	-37°	817	3.46	-45°	0.756	0.769	-0.85°	-0.42°	322	51.8
Xylose	-37°	839	2.33	-43°	0.700	0.727	-0.72°	-0.44°	382	51.9
Arabinose	-37°	772	3.85	-35°	0.833	0.795	-0.80°	-0.42°	361	52.5
Glycerol	-30°	489	1.03	-46°	0.817	0.820	-0.79°	-0.42°	265	52.2
Succinic acid	-41°	8370	0.70	—	0.908	0.902	-0.72°	-0.39°	2630	—
Citric acid	-27°	2000	3.26	-32°	0.760	0.778	—	—	662	—
1	2	3	4	5	6	7	8	9	10	11

144–148° and gave no depression when mixed with authentic glucose. The osazone was prepared and the rotation determined as usual. The actual isolation of glucose from this particular product affords final proof of the formation of glucose from sugars other than glucose.

The production of malonic acid on hydrolysis of the metabolic product is equally definite, since in each case it was actually isolated by ether extraction, purified by sublimation, and its mixed m.p. and equivalent determined. There was in no case any depression of m.p. when the product was mixed with authentic malonic acid, and the equivalent as determined corresponds in each case very closely with the theoretical figure of 52.

The proof that the malonylpolyglucose obtained is identical with luteic acid rests upon the comparison of the figures obtained for the metabolic products from the various sugars with those given by the product from glucose. Though there are certain divergences here, they can be satisfactorily explained. The difficulties in obtaining the specific rotation on the original material, owing to its difficult solubility and the great viscosity of the neutralised solution, make this figure only an approximation. In Table I the values given, though somewhat variable, are of the same order and sign and agree sufficiently well in view of the above explanation.

The titration figures vary widely, and it seems quite certain that in the prolonged purification to which the product is subjected it is to some extent hydrolysed, the extent of hydrolysis depending on the duration of the purification process. We desire to state here that we do not regard luteic acid as a chemical entity with a constant chemical composition and a constant molecular weight. Rather are we of the opinion that luteic acid is analogous with acetylcellulose and nitrocellulose, in that although the ultimate products of hydrolysis are invariably solely malonic acid and glucose, these compounds are not necessarily produced in fixed and unvarying proportions. On the other hand, we regard luteose as a chemical entity in exactly the same sense as cellulose may be so regarded.

In spite of variations referred to above, it will be observed that the equivalent of the product is approximately twice the "apparent equivalent" of the hydrolysed material, findings which agree with the presence in the metabolic products

of malonic acid having one free and one bound carboxyl group, the latter becoming titratable after hydrolysis.

The values for the specific rotation of the luteose, obtained on alkaline hydrolysis agree sufficiently well with the figures obtained from authentic luteose, and in view of these facts it may be definitely stated that luteic acid is produced by *P. luteum* Zukal from glucose, fructose, galactose, mannose, xylose, arabinose and glycerol.

Coming now to the consideration of the products from succinic and citric acids, it will be observed that the equivalent is, in each case, very high, indicating that if luteic acid, having a relatively small equivalent, is produced from these substrates, it must have undergone considerable hydrolysis. Though it is possible that this may have occurred during purification as already explained, it appears much more probable that the hydrolysis is here due to an increase in the p_H of the medium during the course of the experiment owing to the consumption of the organic acids used as substrates. The final p_H values of the media for succinic and citric acids were 8.8 and 6.8. Since only mild alkaline conditions are necessary to eliminate completely the malonic acid from luteic acid, the product obtained from succinic acid would be expected to contain practically no malonic acid, as is seen to be the case. Citric acid occupies an intermediate position since, in this case, a very small amount of acid was extracted by ether after acid hydrolysis which after sublimation melted slightly below the m.p. of pure malonic acid, but gave no depression on mixing. The amount was too small for an equivalent determination, but it seems reasonably certain that the metabolic product in this case did contain malonic acid.

The production of glucose in each case is indicated by the agreement between the Wood-Ost and polarimeter figures, and is proved in the case of the succinic acid product by the production of the osazone. On the other hand, no osazone could be obtained from the citric acid product, as the acid hydrolysate, which was rather dark in colour, only gave rise to a small amount of a tarry product on heating with phenylhydrazine. Further, the specific rotations of the metabolic product and the neutral carbohydrate obtained from it by alkaline hydrolysis are abnormal, and hence the production of luteic acid from citric acid must be regarded as unproven, although it is fairly certain that a malonylpolyglucose is produced.

The most interesting feature of these observations is the demonstration of the power of the organism employed to convert all these varied materials into a polyglucose. Whether we assume that glucose is first formed and then condensed into a polysaccharide, or whether we suppose that the materials offered are first built up into polysaccharides containing their own type of unit, the complex then being in some way directly converted into the polyglucose, it seems remarkable that the interconversion of sugars, apparently so difficult of accomplishment by purely chemical synthesis, is performed with such ease by the mould. That such behaviour may not be by any means rare, however, in the metabolism of micro-organisms is indicated by the observation of Coyne and Raistrick [1931], who obtained mannitol from a whole series of sugars, *viz.* glucose, mannose, galactose, xylose and arabinose, by the agency of a white species of *Aspergillus*. Further, Haworth, Raistrick and Stacey [1932] isolated from the metabolic products of *Penicillium varians* G. Smith [1933], when grown on a medium containing glucose as the sole source of carbon, a polysaccharide the molecule of which consists of a chain of about eight hexose units. The chain is built up from one unit of glucose at the one end of the chain, one unit of either altrose or idose at the other end, and about six units of galactose placed intermediately.

The elucidation of the mechanism by which these changes are brought about must await further work. At present the most attractive hypothesis would appear to be the degradation of the source of carbon to relatively simple molecules containing only a few carbon atoms, which are then built up to form carbohydrates of the peculiar pattern required by the organism, acting as a template, much in the manner that ingested proteins are broken down in the animal body to the constituent amino-acids, which are then used for synthesising the proteins specific for the organism. It is as yet too early to speculate on the actual degradation products which are the precursors of the specific polysaccharides, although obviously they must be of a relatively simple nature, since they are derived from such a variety of substrates.

SUMMARY.

Luteic acid is elaborated by *Penicillium luteum* Zukal when grown in a synthetic medium, containing as sole source of carbon any of the following substances: glucose, fructose, galactose, mannose, xylose, arabinose and glycerol. Indications of a similar product were obtained with succinic and citric acids as substrates. Since luteic acid gives malonic acid and glucose on acid hydrolysis, proof has been afforded of the conversion by this organism of the hexoses fructose, galactose and mannose, of the pentoses xylose and arabinose and of other carbon compounds into glucose.

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