CLXXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIX. THE METABOLIC PRODUCTS OF *PENICILLIUM CRATERIFORME* GILMAN AND ABBOTT.

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SPICULISPORIC acid ($C_{17}H_{28}O_6$, the γ -lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecanoic acid) was first isolated by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, when grown on Czapek-Dox solution with glucose as sole source of carbon. They also determined its structural formula. Later, Birkinshaw and Raistrick [1934] showed that this acid together with a related acid, minioluteic acid, were formed when *P. minioluteum* Dierckx was grown on the same medium. Our colleague, Mr G. Smith, recently noticed that when *P. crateriforme* Gilman and Abbott [1927] was grown on Czapek-Dox medium in test-tubes, the metabolism solution soon gave a characteristic reaction in that a heavy precipitate was formed on addition of ferric chloride solution. After following the usual procedure of growing the mould in flasks, we have isolated the metabolic product responsible for this reaction and have found that it is in fact spiculisporic acid. Although *P. crateriforme* is, according to Thom [1930], more closely related morphologically to *P. minioluteum* than to *P. spiculisporum*—all being members of the Biverticillata-Symmetrica division of species of *Penicillium*, but only the first two species being placed in the Funiculose series—yet no trace of minioluteic acid, or of any other similar complex aliphatic acid, could be isolated from its metabolism solution. As regards metabolism of glucose, therefore, *P. spiculisporum* and *P. crateriforme* are very closely related biochemically, the yield of spiculisporic acid being of the same order of magnitude for each species, *viz.* about 0.16 g. per litre of Czapek-Dox medium, but the first-named mould appears to metabolise glucose much faster than the latter. Succinic acid is also a metabolic product of both moulds. *P. crateriforme* synthesises from glucose a complex carbohydrate which is being further investigated.

EXPERIMENTAL.

A number of 1 litre conical flasks, each containing 350 ml. of Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4 , 1 g.; KCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; water, to 1000 ml., were sterilised and sown with a spore suspension of *P. crateriforme* Gilman and Abbott and incubated at 24°. The organism was purchased from the Centraalbureau voor Schimmelcultures at Baarn, Holland, in November 1933 and had been received by them directly from Abbott.

Growth was slow and in most flasks the initial thin mycelial felt was just complete in 6 days. Thereafter, successive layers of greyish, slimy mycelium developed and in turn fell to the bottom of the flask. In the later stages of metabolism the surface mycelium became faintly pink and in some flasks became brick-red at the edge. One flask was removed after each of the following periods of incubation: 6, 12, 20, 28, 49, 57 and 84 days. The following estimations and tests were carried out on the filtered metabolism solution: p_H ; titratable acidity; apparent % glucose by polarimeter; % glucose by the Shaffer-Hartmann method; bromine absorption value by Koppeschaar's method; and reactions with the following reagents (mostly in dilute solution) added drop by drop to 2 ml. of the metabolism solution: the sulphates of Cu, Zn, Cd, Ni, Co, Mn^{++} ; the chlorides of Mg, Ba, Fe^{+++} , and Hg^{++} ; the nitrates of Ag, Li and Sr, and the acetates of U, Ca and Pb (normal and basic); sodium hypochlorite; bromine water; dilute potassium permanganate; Brady's 2:4-dinitrophenylhydrazine reagent; dilute HCl; and ethyl alcohol (4 vols.).

The only striking reactions which developed during the course of metabolism were the formation of precipitates with HCl (slight), $FeCl_3$ (especially good greyish brown precipitate), $CuSO_4$ (bluish green, much less copious than the $FeCl_3$ precipitate), and ethyl alcohol (precipitation of a polysaccharide). No reactions were given with the other reagents. The salient results are summarised in Table I, from which it will be seen that glucose is slowly metabolised during some 7 weeks; the p_H , after an initial fall, remains constant at about 3.5; the titratable acidity is never very great; the bromine absorption value is negligibly small; no appreciable amount of highly optically active material is to be found in the metabolism solution when all the glucose has been metabolised, although evidence of the formation of a polysaccharide was obtained.

Isolation of spiculisporic acid from the Ba and Fe precipitates. After 49 days' incubation, the precipitate given by addition of excess of 2N $FeCl_3$ to some 250 ml. of filtered metabolism solution was collected, washed with water and triturated with 2N H_2SO_4 , the acid solution being then extracted twice with ether. The oil obtained by evaporation of the dried ether extract became solid when treated with boiling light petroleum (B.P. 40–50°) and was then crystallised from water to yield a small amount of almost colourless, shining plates, melting at 140–143° alone, and at 140–145° when mixed with authentic spiculisporic acid (M.P. 145–146°).

In order to isolate a quantity of the metabolic product sufficient for complete identification, 13 flasks were removed after 62 days' incubation, and to the total filtered metabolism solution was added, first of all, an excess of 6 % barium nitrate solution. The relatively slight precipitate (2.66 g.) which should contain barium minioluteate if the metabolism solution contained minioluteic acid [see Birkinshaw and Raistrick, 1934] appeared to consist chiefly of $BaSO_4$, and was triturated with excess of 2N HCl and then extracted three times with much ether, the undissolved part not being filtered off before extraction. Evaporation of the dried ether extract yielded 0.1 g. of an oil which later partly solidified. After three crystallisations from water, shining plates of almost pure spiculisporic acid separated, M.P. 142–144°; mixed with authentic spiculisporic acid (M.P. 145–146°), the M.P. was 142–146°. No other crystalline product could be isolated by slow evaporation of the various aqueous mother-liquors.

The filtrate from the barium precipitate was treated with excess of ferric nitrate solution and, after standing overnight, the copious greyish brown precipitate was filtered off, washed with water and triturated with 2N H_2SO_4 , the resulting solution being extracted three times with much ether. The filtrate

Table I.

Period of incubation Days	p_H	Titratable acidity per 10 ml. ml. N/10 NaOH	Apparent glucose by polarimeter %	Glucose by Shaffer-Hartmann %	Bromine absorption value mg. per ml.	Physical properties of metabolism solution	Precipitates with			
							HCl	$FeCl_3$	$CuSO_4$	$EtOH$ (4 vols.)
6	4.3*	0.4	4.64†	—	0.09	Colourless	No precipitate	No precipitate	Slight gelatinous precipitate	Faint turbidity
12	3.7	1.3	4.16	4.23	0.17	Do.	Definite flocculent precipitate	Do.	Slight precipitate	Slight turbidity
20	3.5	1.7	3.32	—	—	Colourless. Definitely more viscous than control	Slight colourless amorphous precipitate	Moderate greyish amorphous precipitate	Moderate bluish green precipitate	Marked turbidity
28	3.3	—	3.0	3.19	0.41	Do.	Do.	Good greyish amorphous precipitate	Do.	Do.
49	—	—	1.02	—	0.5	Colourless. Definitely not as viscous as after 28 days	Do.	Do.	Only turbidity	Marked turbidity. Precipitate settles on standing
57	3.4	—	1.66	—	—	Do.	Do.	Do.	Do.	Do.
84	3.7	3.55	0.03	0.11	—	Do.	Moderate precipitate	Heavy precipitate	Moderate precipitate	Do.

* Initially 4.2.

† 5.08 for unsown medium.

from the Fe precipitate gave no further precipitate with lead or copper nitrate solutions. Evaporation of the dried ether extract yielded 2.0 g. of a pasty solid which, after three crystallisations from about 40 ml. H_2O , yielded almost colourless slender needles (0.32 g.), m.p. 141–143°. Mixed with spiculisporic acid, the m.p. was 142–146°.

0.3019 g. of this product, dissolved in 20.0 ml. absolute alcohol in a 1 dm. tube gave a rotation of -0.23° with the mercury green light. Hence, $[\alpha]_{5461} - 15^\circ$; Clutterbuck *et al.* [1931] give for spiculisporic acid $[\alpha]_{5461} - 14.76^\circ$. The above alcoholic solution was titrated in the cold using phenolphthalein as indicator. 18.05 ml. $N/10$ NaOH were required; equivalent = 167 (theoretical, 164). For final confirmation of the identity of the product with spiculisporic acid, 0.2605 g. of the acid recovered from the above titration (m.p. 139–142°) was dissolved in 15.9 ml. $N/10$ NaOH (*i.e.* just enough for neutralisation) made up to 20 ml., and the rotation of resulting solution observed in a 2 dm. tube. Found: $\alpha + 0.48^\circ$, hence $[\alpha]_{5461} + 18^\circ$; Clutterbuck *et al.* [1931] give $[\alpha]_{5461} + 14.98^\circ$ for the sodium salt.

No crystalline product other than spiculisporic acid was isolated by slow evaporation of the various aqueous mother-liquors.

Estimation of yield of spiculisporic acid. Twenty-nine flasks were removed after 90 days' incubation (residual glucose, nil). No spiculisporic acid could be isolated by extraction of the dried mycelium with boiling water. The filtered metabolism solution was acidified with HCl and the dark, slimy precipitate collected and repeatedly extracted with boiling water until nothing further crystallised from the extract when placed in the cold-room overnight; yield, 1.6 g. of nearly pure spiculisporic acid $\equiv 0.16$ g. per litre.

Isolation of succinic acid from the metabolism solution. The acid filtrate from the precipitate of crude spiculisporic acid was evaporated to small bulk *in vacuo* at 50° and then extracted many times with ether. Evaporation of the dried ether extract yielded a dark-coloured oil which on long standing deposited some crystalline material, which was collected by draining on a porous tile and then taken up in alcohol; the crystalline material left after slow evaporation of the filtered alcoholic solution was sublimed at 110 – 140° in a high vacuum. The crystalline sublimate melted at 187 – 189° , alone or mixed with authentic succinic acid (m.p. 188 – 189°).

SUMMARY.

The metabolic products of *Penicillium crateriforme* Gilman and Abbott, when grown at 24° on Czapek-Dox solution containing glucose as sole source of carbon, have been investigated. Spiculisporic acid ($\text{C}_{17}\text{H}_{28}\text{O}_6$, the γ -lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecanoic acid), succinic acid and a complex polysaccharide, which is being further investigated, were isolated.

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