

## CCXXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

### XXV. 3:5-DIHYDROXYPHTHALIC ACID, A NEW PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* AND RE- LATED SPECIES.

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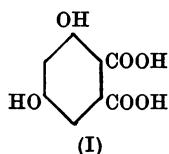
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IN Part XXIV of this series [Clutterbuck, Oxford, Raistrick and Smith, 1932] it was shown that out of 15 species in the *P. brevi-compactum* series submitted to examination, 14 metabolised glucose into a mixture of phenolic acids from which were isolated four new substances with the empirical formulae  $C_{10}H_{10}O_5$ ,  $C_{10}H_{10}O_6$ ,  $C_{10}H_{10}O_7$ , and  $C_8H_6O_6$  respectively. The last-named was detected with certainty in 10 cases only, but since the yield of this substance actually isolated was never greater than 0.03 % of the sugar metabolised, being always much smaller than that of any of the other metabolic products, and since the method for its isolation and separation from tarry and other impurities inevitably led to considerable losses, it appears quite likely that it was in fact produced in all 14 instances. This paper deals with the constitution of this compound  $C_8H_6O_6$ , which, remarkably enough, is isomeric with puberulic acid [Part XXIII, Birkinshaw and Raistrick, 1932], a metabolic product of *P. puberulum* Bainier and *P. aurantio-virens* Biourge, the constitution of which is not yet completely elucidated, although, in view of what follows later it is of interest that these authors consider that it is probably not a dihydroxybenzenedicarboxylic acid (Part XXIII, 1932, pp. 447-9).

It was recorded in Part XXIV (p. 1455) that the new compound  $C_8H_6O_6$  loses nearly 1 molecule of water (together with some  $CO_2$ ) at its first m.p. (188-190°), titrates sharply as a dibasic acid, and gives a stable deep red coloration with ferric chloride and a magenta coloration with bleaching powder solution. It has since been found that complete methylation with diazomethane yields a crystalline tetramethyl derivative which is insoluble in cold alkali and gives no coloration with ferric chloride. These facts not only make it probable that  $C_8H_6O_6$  is a dihydroxybenzenedicarboxylic acid, but also point clearly to one particular acid in that series, since the only dihydroxy-

benzenedicarboxylic acid which would behave as a true phthalic acid on heating and also exhibit the colour reactions of  $\beta$ -resorcylic acid is the unknown 3:5-dihydroxyphthalic acid (I). The dimethyl and diethyl ethers of this acid



and their anhydrides are, however, known [Fritsch, 1897], having been synthesised by a series of reactions leaving no doubt as to their constitution. Asahina and Asano [1932] have also synthesised 3:5-dimethoxyphthalic anhydride by the same method, for comparison and identification with an oxidation product of dimethylolivetonic acid, the latter being derived, by methylation and opening of a lactone ring, from olivetonide, one of the products of the fission (by dehydration) of the lichen acid olivetoric acid extracted from the lichen *Alectoria divergens*.

The dimethyl ether of our mould metabolic product was readily obtained by hydrolysis of the tetramethyl derivative by hot alkali, and was found to crystallise from water with  $1\text{H}_2\text{O}$  (lost below  $100^\circ$ ) and to lose water of constitution at  $157\text{--}158^\circ$ , to yield the corresponding anhydride, m.p.  $147\text{--}149^\circ$ . All these facts are in complete agreement with those recorded by Fritsch [1897] for 3:5-dimethoxyphthalic acid (m.p.  $158^\circ$ ) and anhydride (m.p.  $147^\circ$ ). The fully ethylated derivative was an oil which lost only one ethyl group when hydrolysed by hot dilute alkali, but the resulting monoethyl ester of the diethyl ether lost the elements of ethyl alcohol at about  $180^\circ$  to yield an anhydride, m.p.  $133\text{--}134^\circ$ , a value quite close to  $130^\circ$ , the recorded m.p. of 3:5-diethoxyphthalic anhydride. The identity of our product  $\text{C}_8\text{H}_6\text{O}_6$  with 3:5-dihydroxyphthalic acid was finally placed beyond doubt by direct comparison of the anhydride of its dimethyl ether with an authentic specimen of synthetic 3:5-dimethoxyphthalic anhydride very kindly supplied by Prof. Asahina, when a mixed melting-point showed no depression. It is of some interest that this is the first instance in which a constitutional relationship has been definitely established between a mould metabolic product and a group of lichen acids, the structure proposed by Asahina and Asano [1932, p. 477] for olivetoric acid involving the union by loss of  $\text{H}_2\text{O}$  of two fragments, each of which is a 2:4-dihydroxybenzoic acid with a long side chain in the 6-position, and theoretically convertible into 3:5-dihydroxyphthalic acid by oxidation of that side chain. Orsellinic acid, the fundamental unit in the structure of many lichen acids, e.g. lecanoric acid, evernic acid, gyrophoric acid, has a similar structure, the side chain in the 6-position being a methyl group. This constitutional relationship is important since it supports the view that it is the fungal part of the fungus-alga symbionts constituting the lichen which is responsible for the formation of the lichen acids.

## EXPERIMENTAL.

*Properties of the new metabolic product C<sub>8</sub>H<sub>6</sub>O<sub>6</sub> (3:5-dihydroxyphthalic acid).*

The general properties of this compound are given in Part XXIV [Clutterbuck, Oxford, Raistrick and Smith, 1932, p. 1455] but are briefly as follows: m.p. 188–190° (efferv.) remelting at 206–210°; crystallises from ethyl acetate-light petroleum in well-formed hexagonal tablets with slow, and in flint-shaped leaflets with rapid, crystallisation. It is readily soluble in cold water and in most organic solvents except light petroleum and benzene. With aqueous or alcoholic ferric chloride it gives a stable deep red colour with perhaps a trace of purple. It gives no precipitate with bromine water. With bleaching powder solution and a dilute solution of the acid in water, a transient magenta colour is obtained. No precipitate is obtained with 2:4-dinitrophenylhydrazine in 2*N* HCl.

*Dimethyl ester of the dimethyl ether (dimethyl 3:5-dimethoxyphthalate).* 0.4492 g. of the pure substance, which had been recrystallised several times from ethyl acetate-light petroleum, was suspended in dry ether and treated with a large excess of diazomethane prepared from nitrosomethylurethane (4.2 cc.). A vigorous effervescence took place and the solution was allowed to stand for several hours. A few drops were then tested for free phenols by dilution with alcohol and addition of a few drops of dilute ferric chloride solution, when a moderately intense but transient purple coloration was obtained. A further quantity of ethereal diazomethane was therefore added (from 5 cc. nitrosomethylurethane) and the reaction mixture allowed to stand for 2 hours, the ferric chloride test being then negative. The ether was evaporated off and the colourless, crystalline residue (0.6145 g.) was recrystallised from aqueous alcohol, giving colourless, hair-fine needles, m.p. 92–94°, having a very pronounced ethereal odour.

Micro-analysis (Schoeller): loss at 80° in high vacuum (probably due to slow sublimation) 7.54, 5.87%. Found on material dried at 80° in high vacuum: C, 56.65, 56.76%; H, 5.64, 5.71%; OCH<sub>3</sub>, 47.18, 48.08%; mol. wt. 250, 268. C<sub>8</sub>H<sub>2</sub>O<sub>2</sub>(OCH<sub>3</sub>)<sub>4</sub> requires C, 56.67%; H, 5.55%; OCH<sub>3</sub>, 48.83%; mol. wt. 254.

*Dimethyl ether (3:5-dimethoxyphthalic acid).* The crude tetramethyl derivative (0.43 g.) was hydrolysed by suspending it in a mixture of 20.0 cc. *N* NaOH and 20 cc. ethyl alcohol and boiling the whole under reflux for 1 hour. The undissolved part (0.03 g.) was then filtered off, and the filtrate exactly neutralised by *N* H<sub>2</sub>SO<sub>4</sub> (16.6 cc.), indicating that 3.4 cc. of *N* NaOH had been used up (theoretical, 3.15 cc.). More sulphuric acid was then added and the liquid extracted several times with ether. Evaporation of the dried extract yielded a crystalline deposit which crystallised from boiling water (charcoal) in nearly colourless pointed prisms (0.15 g.), m.p. 157–158° (efferv.), with slight softening previously at about 100°. The melt was taken to 170° and maintained at this temperature till effervescence ceased. It reset immediately on removal from the bath and then re-melted at 143–148°.

Micro-analysis (Schoeller): On hydrated material; C, 49.44, 49.21 %; H, 5.17, 4.98 %; loss at 80° *in vacuo*, 7.45 %.  $C_8H_4O_4(OCH_3)_2$ ,  $H_2O$  requires C, 49.16 %; H, 4.96 %;  $H_2O$  7.38 %. On material dried at 80° *in vacuo*: C, 52.44, 52.22 %; H, 4.81, 4.61 %;  $OCH_3$ , 27.41, 28.24 %.  $C_8H_4O_4(OCH_3)_2$  requires C, 53.08 %; H, 4.45 %;  $OCH_3$ , 27.41 %.

0.1474 g. of the hydrated material required 12.17 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 121, the theoretical for  $C_8H_4O_4(OCH_3)_2$ ,  $H_2O$  titrating as a dibasic acid, being 122. This ether gives no coloration with ferric chloride either in aqueous or alcoholic solution. It is moderately soluble in cold water and is so readily soluble in cold alcohol that purification by crystallisation from aqueous alcohol, as recommended by Fritsch [1897] leads to considerable loss of material.

*Anhydride of the dimethyl ether (3:5-dimethoxyphthalic anhydride)*. The above ether was heated at 170–180° in an oil-bath until effervescence ceased, and the residue purified by sublimation *in vacuo*. The sublimate (micro-needles) melted at 147–149°, and when mixed with a specimen of authentic 3:5-dimethoxyphthalic anhydride, supplied by Prof. Asahina and resublimed by us (m.p. 146–148°) had m.p. 146–149°. Several determinations of the mixed m.p. were carried out.

Micro-analysis (Schoeller): C, 57.64, 57.70 %; H, 3.79, 3.96 %;  $OCH_3$ , 29.84, 29.79 %.  $C_8H_2O_3(OCH_3)_2$  requires C, 57.66 %; H, 3.87 %;  $OCH_3$ , 29.80 %.

*Monoethyl ester of the diethyl ether (monoethyl 3:5-diethoxyphthalate)*. 0.5 g.  $C_8H_6O_6$  were ethylated by exactly the same procedure given above for methylation, except that nitrosoethylurethane was used in place of nitrosomethylurethane. The product (1.06 g.) was a yellow syrup not completely soluble in alcohol, the alcohol-insoluble portion, a colourless amorphous solid, m.p. 70–80°, being readily soluble in light petroleum. The analytical figures for this latter product correspond closely with those for  $(CH_2)_n$ , and the hydrocarbon was doubtless formed by polymerisation and decomposition of diazoethane.

Micro-analysis (Schoeller): C, 84.8 %; H, 14.5 %;  $(CH_2)_n$  requires C, 85.7 %; H, 14.3 %.

The alcohol-soluble ethylation product was hydrolysed by boiling with *N*/2 aqueous-alcoholic NaOH until nearly all had gone into solution. The mixture was then cooled, filtered, acidified with dilute  $H_2SO_4$  and extracted with ether, the extract being dried over anhydrous  $Na_2SO_4$ . Evaporation of the ether yielded a yellow, semi-solid paste, which after two recrystallisations from boiling water yielded colourless, long, silky needles, m.p. 97–99° with no effervescence (according to Fritsch 3:5-diethoxyphthalic acid melts at 182° with effervescence). The substance is soluble in cold sodium bicarbonate solution, and gives no coloration with ferric chloride. The following analytical figures (Schoeller) show conclusively that it is a triethyl derivative of  $C_8H_6O_6$ . Found: C, 59.57 %; H, 6.50 %;  $OC_2H_5$ , 47.06 %;  $C_8H_3O_3(OC_2H_5)_3$  requires C, 59.55 %; H, 6.42 %;  $OC_2H_5$ , 47.88 %.

*Anhydride of the diethyl ether (3:5-diethoxyphthalic anhydride).* The above monoethyl ester showed no tendency to decompose at 140°, but when heated at 180° vigorous effervescence took place, and the odour of ethyl alcohol was perceptible. Heating was continued for 15 minutes after effervescence had ceased, and the residue was sublimed *in vacuo*. The crystalline sublimate had m.p. 133–134°, was insoluble in cold sodium bicarbonate solution, and not very readily soluble in cold caustic potash solution.

Micro-analysis (Schoeller): C, 60.92 %; H, 5.41 %;  $\text{OC}_2\text{H}_5$ , 39.35 %;  $\text{C}_8\text{H}_2\text{O}_3(\text{OC}_2\text{H}_5)_2$  requires C, 60.99 %; H, 5.13 %;  $\text{OC}_2\text{H}_5$ , 38.16 %.

*Attempt to isolate 3:5-dihydroxyphthalic anhydride.* Pure  $\text{C}_8\text{H}_6\text{O}_6$  (0.4 g.) was heated in a stream of nitrogen at 190–200° until a quiet melt was obtained and no more water or  $\text{CO}_2$  was evolved. A trace of material had sublimed and condensed on the cool part of the tube. The solid residue, dark in colour, was readily and completely soluble in saturated  $\text{NaHCO}_3$  solution. This was immediately shaken with ether but it was found that practically nothing was extractable. The solution was acidified and again extracted with ether, the ether extract yielding a solid product which, after crystallisation from chloroform containing a little ethyl acetate, was obtained in the form of needles melting between 210 and 220° with marked softening previously at 190°, and having an equivalent of about 120. The product gave an intense purple-red colour with ferric chloride and was probably a mixture of the original  $\text{C}_8\text{H}_6\text{O}_6$  together with  $\alpha$ -resorcylic acid, but a separation was not possible on account of the small quantity of material available.

#### SUMMARY.

The new mould metabolic product  $\text{C}_8\text{H}_6\text{O}_6$  (m.p. 188–190°) formed from glucose by species in the *P. brevi-compactum* series, has been identified as 3:5-dihydroxyphthalic acid, and hence is constitutionally related to a large group of lichen acids.

#### REFERENCES.

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