LXXXIV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXIX. 2:5-DIHYDROXYBENZOIC ACID (GENTISIC ACID) A NEW PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM GRISEO-FULVUM* DIERCKX.

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IN Part XIX of this series [Anslow and Raistrick, 1931] it was shown that when *Penicillium griseo-fulvum* Dierckx is grown at 24° on Czapek-Dox medium containing glucose (5 %) as sole source of carbon, the metabolism solution, after some days, gives an intense purple colour with ferric chloride. The substance responsible for this coloration was isolated and identified as 2-hydroxy-6-methylbenzoic acid (6-methylsalicylic acid), but at that time no search was made for other phenolic metabolic products, since the above acid was undoubtedly the chief phenolic product formed under these conditions and was produced in good yield (2·42 % of the glucose metabolised).

This work has now been continued, and in order to determine the optimum conditions for the production of phenolic substances by this mould small scale experiments (in test-tubes) have been carried out at 20, 24, 28, 30 and 35°. It has been found that the optimum temperature for sugar utilisation and production of phenols, as measured by the bromine absorption method of Koppeschaar [1876], is 30°. At this temperature also, sporing is much heavier than at 24°. The mould has also been grown on various modified Czapek-Dox media, the temperature of incubation being always 30°. It has been found that when the glucose content of the medium was 8 % the bromine absorption value, in mg. Br per cc. of metabolism solution after 40 days' incubation, was much higher than was obtained with the corresponding metabolism solutions when the media contained initially only 4, 5 or 6 % glucose, the actual value being 6 mg. per cc. as compared with 2·5, 2·7 and 3·8 mg. respectively. The sequence of colours with ferric chloride was also rather different. For example, after 40 days' incubation, the colour obtained with 8 % glucose was no longer purple but dark blue, changing with excess $FeCl_3$ to dark brown, whereas the corrresponding colour for media containing less glucose was still purple. It was therefore evident that 6-methylsalicylic acid was not the only phenolic metabolic product, and in order to arrive at still better conditions for the production of the second phenolic substance experiments were carried out with media containing 8 % glucose, but having different glucose-sodium nitrate ratios. Although, after 40 days, the

bromine absorption values were the same for media with different ratios of glucose to sodium nitrate, the ferric chloride colours were different, *e.g.* when the ratio was 25 : 1 (*i.e.* the ratio in the usual 5 % Czapek-Dox glucose medium), the colour was purple, though when the ratios were 16 : 1 and 32 : 1 the colours were dark brown and dark blue respectively.

The medium chosen for large scale experiments contained therefore 8 % of glucose and 0·25 % of sodium nitrate (ratio = 32 : 1).

Isolation of the new metabolic product responsible for the blue colour with ferric chloride.

A quantity of Czapek-Dox medium, modified as indicated above, was made up, having the following composition: glucose, 80 g.; $NaNO_3$, 2·5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·02 g.; distilled water, 1000 cc. One hundred and ten 1-litre conical flasks, each containing 350 cc. of medium, were sterilised by steam on three consecutive days. They were sown with a suspension of spores (from a 10-day pure culture of *P. griseo-fulvum* Dierckx grown on beer wort agar at 30°) and incubated at 30° for 45 days. An average sample of the filtered metabolism solution contained 1·12 % glucose, the bromine absorption corresponded to 3·7 mg. per cc., and ferric chloride gave a dark brown colour. The metabolism solution was concentrated to 1 litre *in vacuo* below 50°. From the precipitate which separated during evaporation a considerable quantity of crude mannitol was isolated (120 g.). After crystallisation from aqueous alcohol, it melted at 163–164°, and the M.P. was not depressed by admixture with an authentic specimen of mannitol. Further, the purified substance was optically inactive, but when borax (3 g.) was added to a solution of the substance (0·270 g.) in water (25 cc.) and the whole diluted to 50 cc., the observed rotation was + 0·4° in a two dm. tube, using the mercury yellow line $\lambda = 5790$. This corresponds exactly with the value calculated from the tables given by Raistrick and Young [1931] and proved conclusively that the metabolic product was mannitol. The filtrate from the mannitol was made acid to Congo red with dilute sulphuric acid and extracted with an equal volume of chloroform until the extract gave no colour with ferric chloride, three extractions being necessary. In this way all the 6-methylsalicylic acid was removed, since it is moderately soluble in chloroform, while the new metabolic product is almost insoluble in this solvent in the cold. The liquid was then repeatedly extracted with an equal volume of ether until the extract no longer gave a blue colour with ferric chloride. Evaporation of the ether yielded a brown semi-crystalline product which was submitted to a long series of fractional crystallisations from chloroform-light petroleum. Two crystalline acids were isolated, the less soluble subliming at about 200° and melting at 272–274° in a closed M.P. tube; the more soluble having M.P. about 195°.

Identification of the less soluble acid as fumaric acid.

This product gave no colour with ferric chloride and was identified as fumaric acid, a mixed melting-point with authentic fumaric acid (M.P. 286°) being about 280° in a closed M.P. tube. Micro-analysis (Schoeller): C, 41·50, 41·62; H, 3·53, 3·52 %. $C_4H_4O_4$ requires C, 41·38; H, 3·48 %.

0·0100 g. required 8·8 cc. $N/50$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 57, the theoretical value for $C_4H_4O_4$ titrating as a dibasic acid being 58.

Purification and properties of the second phenolic metabolic product.

The second and more soluble metabolic acid mentioned above gave a deep blue colour with ferric chloride and was purified by sublimation followed by crystallisation from ethyl acetate-light petroleum or from toluene. It formed colourless needles, M.P. 197–198°, but the melting-point can with difficulty be raised to 202° after repeated crystallisations from ethyl acetate-light petroleum, involving much loss of material. Micro-analysis (Schoeller): C, 54·85, 54·91; H, 4·04, 4·09 %; Mol. wt., 139, 158. $C_7H_6O_4$ requires: C, 54·52; H, 3·92 %; Mol. wt., 154.

0·1910 g. required 12·3 cc. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 155, the theoretical for $C_7H_6O_4$ titrating as a monobasic acid being 154.

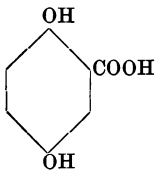
The new metabolic product is readily soluble in the cold in water, alcohol, ether and ethyl acetate. It is sparingly soluble even in hot chloroform, moderately soluble in hot toluene and almost insoluble in light petroleum.

To obtain more of the new metabolic product 330 1-litre flasks containing the same quantity of medium were sown as mentioned above. The time of incubation was extended to 65–70 days. After that period the average glucose content was 0·6 %, the average bromine absorption corresponded to 4 mg. per cc. and the metabolism solution now gave a dark blue colour with ferric chloride. The metabolism solution was concentrated *in vacuo* to 6 litres and treated exactly as described above. The chloroform extracts yielded a brown crystalline material (crude 2-hydroxy-6-methylbenzoic acid; yield 61·2 g., corresponding to 0·72 % of the glucose metabolised). The ether extracts after drying over anhydrous Na_2SO_4 yielded on evaporation a dark brown semi-crystalline material (114·1 g., *i.e.* 1·34 % of the weight of glucose metabolised). This product was boiled several times with toluene, which does not dissolve the dark brown impurity, and the new metabolic product crystallised from the toluene extracts on cooling. The crude crystalline product (11 g. representing 0·12 % of the weight of glucose metabolised) was recrystallised several times from toluene (charcoal).

The residues obtained by Anslow and Raistrick [1931] by evaporation of the mother-liquors from the crystallisation of 6-methylsalicylic acid were also examined and both fumaric acid and the new phenolic product $C_7H_6O_4$, M.P. 197–198°, were isolated in small amounts.

Identification of the new metabolic product as gentisic acid (2 : 5-dihydroxybenzoic acid).

The ferric chloride reaction (deep blue, slowly fading to brown) suggests that the substance is a derivative of quinol, and its empirical formula, $C_7H_6O_4$, and the fact that it titrates as a monobasic acid show clearly that it is probably quinolcarboxylic acid (2 : 5-dihydroxybenzoic acid, gentisic acid (I))



(I)

A mixed melting-point with a specimen of synthetic gentisic acid, M.P. 197–198°, prepared from quinol and $KHCO_3$ by the method of Brunner [1907] showed no depression, and the identity of the new metabolic product with gentisic acid was settled beyond all doubt by the preparation of the following derivatives, all of which were identical (by M.P. and mixed M.P.) with the corresponding derivatives prepared by parallel experiments carried out on synthetic gentisic acid: (*a*) the methyl ester, M.P. 85–86°, (*b*) the monomethyl ether (5-methoxy-2-hydroxybenzoic acid), M.P. 141–142°, prepared by methylation of gentisic acid with diazomethane and hydrolysis of the resulting phenolic ester with hot alkali, (*c*) the diacetyl derivative, M.P. 119–121°. Finally, (*d*) quinol itself was obtained by heating the acid above its melting-point and resublimation of the crystalline sublimate.

Preparation of derivatives of the new metabolic product (gentisic acid).

(*a*) *Methyl ester* [*cf.* Graebe and Martz, 1905]. A solution of the pure acid (0·5 g.) in methyl alcohol (10 cc.) containing 3 % HCl was boiled under reflux for 3 hours, diluted with water, neutralised by addition of $NaHCO_3$ and extracted with ether. The residue left after evaporation of the dried ether extract was crystallised from chloroform-light petroleum to yield colourless prisms, M.P. 85–86°, which gave a blue colour with ferric chloride. A mixture of the above product with authentic methyl gentisate (M.P. 85–86°) also melted at 85–86°.

Micro-analysis (Schoeller): C, 57·34, 57·15 %; H, 4·86, 4·83 %; OCH_3, 18·13, 18·20 %; mol. wt. (in camphor), 178, 186. $C_7H_5O_3(OCH_3)$ requires C, 57·12; H, 4·79 %; OCH_3, 18·45 %; mol. wt., 168.

(*b*) *Methylation by diazomethane and hydrolysis of the methylation product; formation of 5-methoxy-2-hydroxybenzoic acid.* To the pure metabolic product (0·5 g.) was added ethereal diazomethane prepared from nitrosomethylurethane (10 cc.). After 24 hours the ether was removed, and the pale brown oily residue (0·60 g.) was found still to give a blue colour with ferric chloride. As was anticipated, the phenolic hydroxyl group *ortho* to COOH had escaped methylation, and the product was doubtless essentially the methyl ester of 5-methoxy-2-hydroxybenzoic acid. The crude ester was hydrolysed by boiling for 1 hour with a mixture of alcohol (20 cc.) and *N* NaOH (20 cc.). The resulting solution was made acid to Congo red by addition of dilute sulphuric acid and extracted with ether. The dark-coloured residue remaining after evaporation of the dried ether extract was crystallised from water (charcoal) to yield light, colourless needles, M.P. 141–142°, an aqueous solution of which gave a deep blue colour with ferric chloride and reacted acid to litmus.

Micro-analysis (Schoeller): C, 57·03, 57·11; H, 4·81, 4·81; OCH_3, 18·72, 18·17 %. $C_7H_5O_3(OCH_3)$ requires C, 57·12; H, 4·79; OCH_3, 18·45 %.

The melting-point of 5-methoxy-2-hydroxybenzoic acid is variously given in the literature as 141, 142 and 143·5°. When prepared from synthetic gentisic acid by the above method it melted at 141–142° and a mixture of the synthetic compound with the above monomethyl ether of the metabolic product also melted at 141–142°.

(*c*) *Diacetyl derivative* (2:5-diacetoxybenzoic acid). A solution of the pure metabolic product (0·5 g.) in pyridine (5 cc.) and acetic anhydride (5 cc.) was kept at 37° for 2 days. The dark-coloured liquid was then poured into water (100 cc.). The solution was acidified with dilute sulphuric acid and extracted with ether. The residue left after evaporation of the ether was crystallised from water and yielded colourless crystals, M.P. 116–117°, which gave no colour with ferric chloride. The yield was very small.

Micro-analysis (Schoeller): C, 55·00; H, 4·30 %. $C_{11}H_{10}O_6$ requires C, 55·44; H, 4·23 %.

Synthetic 2:5-diacetoxybenzoic acid was prepared by boiling a solution of gentisic acid in excess of acetic anhydride together with some anhydrous sodium acetate for 3 hours, according to the method of Hemmelmayr [1909]. It formed colourless crystals, M.P. 118–120°. A mixture of this synthetic compound with the above diacetyl derivative of the metabolic product melted at 116–119°.

A sample of the diacetyl derivative of the metabolic product was also prepared by boiling 0·5 g. of the latter with anhydrous sodium acetate (0·5 g.) and acetic anhydride (2 cc.) for 30 minutes.

The reaction mixture was diluted with water (100 cc.), acidified with dilute sulphuric acid and the precipitate collected and fractionally crystallised from water. A crystalline product was obtained, which had M.P. 119–121°, gave no colour with $FeCl_3$ and was identical with synthetic 2:5-diacetoxybenzoic acid.

Micro-analysis (Schoeller): C, 55·42, 55·61; H, 4·28, 4·27 %; mol. wt. (in camphor), 213, 207. $C_{11}H_{10}O_6$ requires C, 55·44; H, 4·23 %; mol. wt., 238.

(*d*) *Decomposition by heat with formation of quinol.* The pure metabolic product (a few mg.) was heated in a small test-tube at 250–275°. The sublimate which collected on the cool part of the tube consisted of colourless plates and needles. The experiment was repeated several times, and the combined sublimates were resublimed at 150°. The final product gave a faint and transient blue colour with ferric chloride, reduced Fehling's solution on heating, and melted at 166–167°. A mixture of this substance with a specimen of authentic quinol (M.P. 168–169°) melted at 166–169°.

Micro-analysis (Schoeller): C, 64·74; H, 5·35 %; mol. wt. (in camphor), 128, 120. $C_6H_6O_2$ requires C, 65·42; H, 5·49 %; mol. wt., 110.

Discussion.

It is thus seen that from the metabolism solution obtained when *P. griseo-fulvum* Dierckx is grown under the conditions described in a medium containing glucose as sole source of carbon four metabolic products have been isolated, *viz.* 6-methylsalicylic acid, gentisic acid, fumaric acid and mannitol. Since the sequence of ferric chloride colours given by the metabolism solution during the growth of the mould indicates that 6-methylsalicylic acid is the first phenolic product to appear, it seems quite possible that gentisic acid is an oxidation product of the above acid. Salicylic acid itself can be oxidised to gentisic acid by potassium persulphate [Graebe and Martz, 1905], and if the mould does indeed oxidise 6-methylsalicylic acid to gentisic acid, a hydroxyl group must be introduced, the methyl group in the former must be oxidised to carboxyl, and one carboxyl group must be eliminated as CO_2. It must be admitted, however, that we have not been able to isolate from the metabolism solution any of the possible intermediates in this oxidation.

This is the first recorded instance of the isolation of free gentisic acid from any natural source. It has, however, been obtained by potash fusion from the colouring matter gentisin (the methyl ether of 1 : 3 : 7-trihydroxyxanthone) extracted from the gentian root (*Gentiana lutea*).

There are a few instances in the literature of the production of fumaric acid by fungi, *i.e.* by *Rhizopus nigricans* [Ehrlich, 1911; Butkewitsch and Fedoroff, 1929], and by *Aspergillus fumaricus* [Wehmer, 1918; 1928], although, as far as we are aware, it has not previously been detected among the metabolic products of species of *Penicillium*.

Summary.

The metabolism of glucose by *Penicillium griseo-fulvum* Dierckx has been further examined. A hitherto undescribed mould metabolic product, gentisic acid (2 : 5-dihydroxybenzoic acid), was isolated together with the previously described 2-hydroxy-6-methylbenzoic acid, and an attempt has been made to determine the optimum conditions for the production of gentisic acid.

In addition, mannitol and fumaric acid were isolated, thus affording the first example of the production of fumaric acid by a species of *Penicillium*.

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