VI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XIX. 6-HYDROXY-2-METHYLBENZOIC ACID, A PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM GRISEO-FULVUM* DIERCKX.

By WINSTON KENNAY ANSLOW and HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received November 28th, 1930.*)

In a series of publications which have recently appeared [Raistrick *et al.*, 1931] an account is given of an intensive study, extending over a period of seven years, of the products of the metabolism of glucose, in synthetic media containing no other source of carbon, by a large number of different species of the lower fungi. Several new types of metabolic products were described, many of which were shown to contain a benzene nucleus.

More recently it has been possible to extend this work, and, thanks to the courtesy of Professor Philip Biourge of the University of Louvain, several different species of *Penicillium* have been examined which were not available previously.

One of these species was *P. griseo-fulvum* Dierckx, which was originally described by Dierckx [1901], and, more recently, by Biourge [1923] in his monograph on the genus *Penicillium*. This species bears Biourge's catalogue number 34. In view of the interesting results we obtained with this species, we asked Professor Biourge to re-examine our culture and his report follows.

"Voici assez parfaitement déterminé le *Penicillium* que vous m'avez envoyé pendant les vacances. C'est bien le *Penicillium griseo-fulvum* Dierckx (Biourge No. 34). C'est une espèce peu commune. C'est la troisième fois que je la rencontre, y compris l'observation originale de Dierckx en 1898–1900, dans mon laboratoire. Elle est bien pure. Spores bleu céleste très pâle, et finalement gris-rosé. Revers jaune à jaune-rouge. Coremia tardifs, liquéfaction de la gélatine souvent précoce, odeur 0."

We desire to thank Professor Biourge for his friendly co-operation.

It was found that, when this species of *Penicillium* is grown on a synthetic medium containing glucose as the sole source of carbon, the metabolism solution, after some days, gives an intense purple colour with ferric chloride, very similar in shade to aqueous potassium permanganate solution. In addition the metabolism solution also gives a heavy, pale yellow precipitate with

bromine water. The isolation and identification of the substance responsible for these reactions is the object of the present communication.

Preparation of the metabolic product.

A quantity of a slightly modified Czapek-Dox medium was made up, having the following composition:

$NaNO_3$	2·0 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.
Glucose (pure)	50·0 g.
Dist. water	to 1000 cc.

350 cc. quantities of this medium were placed in 67 1-litre conical flasks, which were plugged with cotton-wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with an emulsion, in the above medium, of spores prepared from a 17-day culture of *P. griseo-fulvum* Dierckx, grown on Czapek-Dox agar at 25°. The flasks were thoroughly shaken and incubated at 25° for 35 days. At the end of this period the surface of the medium was completely covered with a thick mycelial felt, showing areas of pale green sporing patches, the intervening spaces consisting of sterile mycelium. The reverse of the mycelium was brick-red in colour. The contents of all the flasks were now filtered without previous sterilisation, the mycelium pressed and dried *in vacuo* at 50°. The combined filtrates, which were orange-yellow in colour, had the following characteristics.

(1) Glucose (by polarimeter) 0·254 %.

(2) Titratable acidity = 2·13 cc. *N* acid per 250 cc. medium.

(3) Bromine absorption (by Koppeschaar's method) = 3·8 mg. per cc.

(4) The metabolism solution gave a very intense purple colour with aqueous ferric chloride and a heavy pale yellow precipitate with bromine water.

The whole of the filtrate was acidified with 400 cc. of 2*N* H_2SO_4 and shaken with half its volume of ether, by which means the substance responsible for the ferric chloride reaction was completely extracted. The ethereal solution was washed with a little water, filtered and evaporated to dryness. The dry residue, which was brownish in colour and completely crystalline, weighed 26·9 g. corresponding to a yield of 2·42 % of the glucose consumed. It was crystallised from chloroform, from which solvent it separated in long white needles, which were practically pure but were recrystallised several times for analysis.

The dried mycelium was powdered and exhaustively extracted with ether. The ethereal extract on evaporation deposited a quantity of a white crystalline material which melted in a crude state at about 150°, gave a brownish purple

colour with ferric chloride, but is apparently not identical with the product extracted from the metabolism solution. The nature of this product will form the subject of a future communication.

Properties, analysis and derivatives of the metabolic product.

The product extracted from the metabolism solution and crystallised from chloroform has the following properties. It consists of beautiful white needles melting without decomposition at 170–171° and may be readily sublimed unchanged in a high vacuum. It is not very soluble in cold water, but readily dissolves in hot water from which it separates on cooling in long white needles. It is very soluble in ether and alcohol, moderately soluble in hot, but not very soluble in cold, chloroform. Its solution in water or alcohol gives a very intense purple-violet colour with ferric chloride, very similar to that given by salicylic acid. Its aqueous solution gives an immediate precipitate with bromine water, and reacts acid to litmus. It gave the following results on analysis:

C, 62·91 and 63·02 %; H, 5·28 and 5·33 % (theoretical for $C_8H_8O_3$, C, 63·13; H, 5·30 %).

In an estimation of the molecular weight by the Rast camphor method, 0·217 mg. lowered the M.P. of 2·608 mg. camphor 20·2°, corresponding to a molecular weight of 160 (theoretical for $C_8H_8O_3$, 152).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 152·6 (theoretical for $C_8H_8O_3$, assuming this to be a monobasic acid, 152).

Methyl ether. 3 g. of the metabolic product were methylated by shaking with 9 cc. of dimethyl sulphate and an excess of 10 % sodium hydroxide. The mixture was maintained at room temperature for about 2 hours and was then boiled under a reflux condenser until perfectly clear. The solution was cooled, filtered, acidified and extracted with ether. The ethereal solution was evaporated to dryness and the dry residue, which weighed 3·1 g., was crystallised several times from boiling water. The crystallised material consisted of colourless, elongated hexagonal prisms (plates), which melted at 139° and gave no colour with ferric chloride. It gave the following results on analysis:

C, 65·39 %; H, 5·97 % (theoretical for $C_9H_{10}O_3$, C, 65·03 %; H, 6·07 %).

A Zeisel estimation gave 18·66 % OCH_3 (theoretical, 18·68 %).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 166·3 (theoretical for $C_9H_{10}O_3$, assuming this to be a monobasic acid, 166·1).

Acetyl derivative. 2 g. of the metabolic product, 3·3 cc. acetic anhydride and 6·6 cc. pyridine were mixed and incubated in a closed flask at 37° for 4 days. The mixture was cooled, diluted with 100 cc. of water, acidified to Congo red with $2N$ H_2SO_4 and extracted with ether. The crystalline residue from the ether, weighing 2·5 g., was recrystallised several times from boiling

benzene, from which solvent it separated in colourless, elongated prisms, which melted without decomposition at 131°. This product which gave no colour with ferric chloride gave the following results on analysis:

C, 62·29 %, H, 5·23 % (theoretical for $C_{10}H_{10}O_4$, C, 61·83 %; H, 5·19 %).

0·2044 g. titrated with $N/10$ sodium hydroxide to phenolphthalein required 10·40 cc. giving an equivalent of 196·5 (theoretical for $C_{10}H_{10}O_4$, assuming this to be a monobasic acid, 194·1).

On account of the volatility of the metabolic product in steam, it was impossible to carry out a direct estimation of the acetyl groups, but this was done indirectly as follows. An excess of $N/10$ sodium hydroxide was added to the above neutralised solution, the mixture boiled for 3 hours under reflux, cooled and the excess of alkali titrated with $N/10$ hydrochloric acid. Acidity equivalent to 10·98 cc. $N/10$ was produced during the hydrolysis, from which it is evident that the derivative is a monoacetyl compound.

Constitution of the metabolic product.

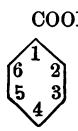
The above results indicate that the metabolic product has the empirical formula $C_8H_8O_3$, contains one hydroxyl group, which is probably phenolic in nature because of the purple colour produced with ferric chloride, and a carboxyl group. Since the material in aqueous solution also gives an insoluble bromine compound with bromine water, it is probably a benzene derivative.

Accepting this assumption for the moment, the metabolic product must be either (i) a hydroxyphenylacetic acid *or* (ii) a hydroxymethylbenzoic acid.

The possibility of the metabolic product being a phenylglycollic acid and having the hydroxyl group in the side chain is ruled out because of the reaction with ferric chloride.

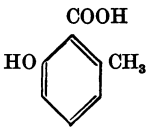
(i) The three possible hydroxyphenylacetic acids, 2-hydroxy-, 3-hydroxy- and 4-hydroxy- are known and melt respectively at 145–147°, 129° and 148°. The metabolic product melts at 171° and this, together with its general properties, proves that it is not a hydroxyphenylacetic acid.

(ii) There are ten possible hydroxymethylbenzoic acids, which have all been described. Writing the formulae for these according to the scheme



the metabolic product cannot be identical with any of the following, since they give no colour with ferric chloride; 5-OH 2-CH_3; 4-OH 2-CH_3; 3-OH 2-CH_3; 5-OH 3-CH_3; 4-OH 3-CH_3; 3-OH 4-CH_3. This leaves the possibility that the metabolic product is one of the four methylsalicylic acids; 6-OH 2-CH_3, 2-OH 3-CH_3, 6-OH 3-CH_3, 2-OH 4-CH_3. All these acids give an intense violet colour with ferric chloride and melt respectively at 168–169°,

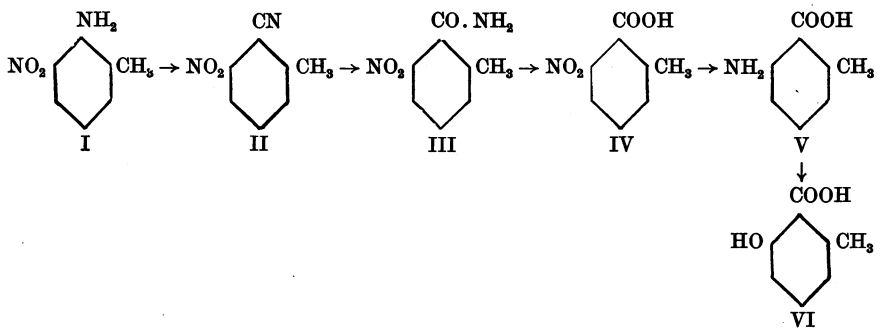
167°, 153° and 177°. (The highest recorded melting point is given in each case.) These melting points are not sufficiently far removed from the melting point of the metabolic product (171°) to be definitive. The melting points of the methyl ethers are, however, very much more widely separated and are respectively 139°, 85°, 70° and 104°. Since the melting point of the methyl ether of the metabolic product is 139°, it appears probable that the metabolic product is identical with 6-hydroxy-2-methylbenzoic acid of the following formula.



This conclusion was confirmed by synthesis.

Synthesis of the metabolic product.

We are indebted to Dr Alexander Robertson for synthetic specimens of 6-hydroxy-2-methylbenzoic acid, of its methyl ether and of its acetyl derivative. These were prepared as follows:



The nitrotoluic acid (IV) was prepared from the nitrotoluidine (I), *via* the nitronitrile (II), and nitroamide (III), according to the methods of Gabriel and Thieme [1919] and Kenner and Witham [1921].

The nitro-acid (IV) was reduced by means of ferrous sulphate and ammonia as used by Gabriel and Thieme. The amino-acid (V) was isolated by extraction with ether, and was converted into the hydroxy-acid (VI) without further purification. A solution of the amino-acid (6 g.) in 90 cc. $N/2$ HCl was cooled to 0°, treated with sodium nitrite (3 g.) and the mixture kept at 0° for 1 hour. 5 % sulphuric acid (40 cc.) was then added and the mixture was rapidly heated to 65° by plunging the vessel into a water-bath at 70–75°, and was maintained at this temperature for ¼ hour. The hydroxy-acid, contaminated with only traces of brown coloured material, quickly separated. Concentrated hydrochloric acid (40 cc.) was added to the cooled reaction mixture, and after 2 hours in the ice-chest the hydroxy-acid was collected, washed with water, and air-dried. Yield 3–3·5 g. It was purified by crystallisation from chloroform.

The synthetic hydroxy-acid gives the same colour reaction with ferric chloride as the metabolic product, has the same crystalline form, and the same melting point (170–171°). A mixture of the natural and synthetic products melts at the same temperature.

The methyl ether and the acetyl derivative of the synthetic hydroxy-acid were prepared by the methods previously described for the corresponding derivatives of the metabolic product.

The melting point of the methyl ether of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 139° in all three cases. Similarly, the melting point of the acetyl derivative of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 131° in all three cases, and hence identity of the metabolic product with the synthetic acid (6-hydroxy-2-methylbenzoic acid) is clearly established.

Identity of bromine compound.

It has been previously mentioned (p. 40) that the metabolism solution gives a heavy pale yellow precipitate with bromine water. In order to prepare a quantity of this material 5 litres of metabolism solution were treated with a slight excess of a saturated solution of bromine in water, allowed to stand overnight, and the precipitate filtered off. It was air-dried and extracted with ether. The ether solution on evaporation left a brownish residue consisting of rosettes of needles. A portion of this was recrystallised from light petroleum and then sublimed in a mercury vapour vacuum, giving a sublimate, at a bath temperature of 50–60°, of pale yellow needles, which melted at 84° and on admixture with a sublimed synthetic specimen of 2:4:6-tribromo-*m*-cresol had a mixed melting point of 83–84°.

It contained 69·6 % Br (theoretical for $C_7H_5OBr_3$, 69·5 %). Since the same substance is produced when bromine water is added to a dilute aqueous solution of the sodium salt of the metabolic product $C_8H_8O_3$, it is evident that this material is responsible for both the purple colour with ferric chloride and for the precipitate with bromine water given by the metabolism solution. This precipitate consists principally of 2:4:6-tribromo-*m*-cresol.

SUMMARY.

6-Hydroxy-2-methylbenzoic acid was isolated and identified as a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx.

REFERENCES.

Biourge (1923). *La Cellule*, **33**, 164.
Dierckx (1901). *Ann. Soc. Sci. Brux.* **25**, 88.
Gabriel and Thieme (1919). *Ber. deutsch. chem. Ges.* **52**, 1081.
Kenner and Witham (1921). *J. Chem. Soc.* **119**, 1452.
Raistrick and co-workers (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**. (In the press.)