CXCIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIV. A NOTE ON THE MECHANISM OF THE PRODUCTION OF PHENOLIC ACIDS FROM GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* DIERCKX.

BY ALBERT EDWARD OXFORD AND HAROLD RAISTRICK.

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

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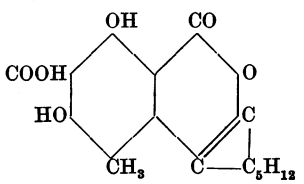
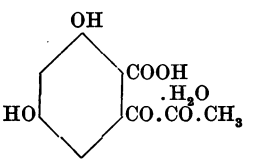
IT was shown in Part XXIV of this series [Clutterbuck *et al.*, 1932] that when most species and strains in the *P. brevi-compactum* Dierckx series are grown on synthetic media containing glucose, or glucose and tartaric acid, as sole source or sources of carbon, five phenolic acids are present in the metabolism solution when all the glucose originally present has been utilised by the mould. The empirical formulae of these five acids are, in descending order of complexity, mycophenolic acid, $C_{17}H_{20}O_6$ (I), $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV), and 3:5-dihydroxyphthalic acid, $C_8H_6O_6$ (V). Their respective constitutional formulae have been either completely or almost completely elucidated [see Clutterbuck and Raistrick, 1933; Oxford and Raistrick, 1932; 1933].

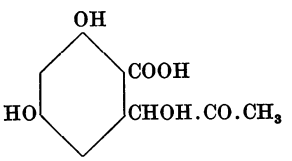
Monomethyl ether of



(I)

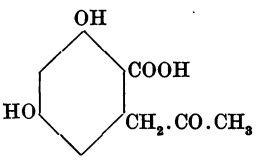


(II)

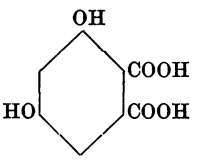


(or tautomeric form)

(III)



(IV)



(V)

We may note here that all five acids are derivatives of resorcinol, and hence it seemed unlikely that all would prove to be stable end-products formed from glucose by five separate series of reactions, a more likely hypothesis being that some of these acids are intermediates in the formation of others. The present work was undertaken to discover the exact order of appearance of the phenolic metabolic products and the nature of the stable end-products of metabolism.

Experimental.

The strain of *P. brevi-compactum* Dierckx chosen (Catalogue Number M 3 (1)) was isolated by the late J. H. V. Charles in 1931 from mouldy Italian maize, and in that year gave relatively good yields of all five metabolic products when grown on Raulin-Thom medium [see Clutterbuck *et al.*, 1932, p. 1451]. 105 one-litre flasks each containing 350 cc. of Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate; 4 g. di-ammonium hydrogen phosphate, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4, 7H_2O$, 0·07 g.; $FeSO_4, 7H_2O$, 0·07 g.; water to 1500 cc., were sterilised and to each were added 3 cc. of a well-shaken suspension of spores in sterile distilled water (total volume, 350 cc.) prepared from 4 beer wort agar slopes of the above organism which had been incubated for 36 days. The flasks, after inoculation, were incubated at 24°, and rapid growth took place, a complete felt of mycelium being formed in every flask after 3 or 4 days' incubation. 26 flasks were removed after 8 days when the addition of ferric chloride to a test portion of the metabolism solution first showed definitely that phenols were present. The mycelium and the filtered metabolism solution were dealt with separately, the following tests and estimations being carried out on a small average sample of the latter: coloration with ferric chloride; approximate p_H; residual glucose by polarimeter; bromine absorption value in mg. per cc. (by Koppeschaar's method). The metabolism solution was then evaporated to 400 cc. *in vacuo* below 50° and worked up exactly as described by Clutterbuck *et al.*, 1932], except that the ether extraction at p_H 5·3 was omitted. The mycelium was dried, powdered and extracted in an all-glass Soxhlet apparatus first with boiling light petroleum (B.P. 50–60°) for 2 days, and then with ether for 2 days. It was found that when the mycelium contained mycophenolic acid, part of this acid was removed by light petroleum (in which it is very sparingly soluble) and crystallised out from the extract, the remainder being subsequently extracted by ether. The ethereal extract was evaporated, the residue was dissolved in a little alcohol and the mycophenolic acid isolated *via* the insoluble dipotassium salt.

The remaining flasks were taken off at the following intervals: 25 after 11 days; 20 after 15 days; 20 after 22 days (when all the glucose had been metabolised) and the remaining 14 after

Table I.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		Tests on metabolism solution					
Period of incubation (days)	Glucose metabolised (g.)	p_H	Bromine absorption value (mg. per cc.)	Coloration with ferric chloride	Tartaric acid recovered (g.)	Wt. of dried mycelium (g.)	Wt. of $C_{17}H_{20}O_6$ (I) (mycophenolic acid) (g.)
8	202 (54 %)	4	1·82	Not very intense brown	3·0	66	1·67 (63 % in mycelium)
11	269 (72·5 %)	4	2·80	More intense brown	0·8	83	2·28 (61 % in mycelium)
15	333 (90 %)	Between 4 and 5	3·86	Brownish-purple	0	87	2·67 (62 % in mycelium)
22	371 (100 %)	Between 4 and 5	4·64	Deep blue-crimson	0	78	2·85 (60 % in mycelium)
56	371 (100 %)	About 8	5·07	Deep blue-crimson	0	61	3·49 (all in metabolism solution; none in mycelium)

Notes: (*a*) Recrystallised from water, M.P. 120–132° (efferv.). Converted into 2:4-dinitrophenyl-
(*b*) The p_H 7 fraction weighed 3·3 g. of which 1·26 g. was $C_{17}H_{20}O_6$ (I). The rest (2·04 g.)

56 days. The last group of flasks was therefore incubated for 34 days after all the glucose had disappeared, the mycelial felts being partly waterlogged at the expiry of this period, and the metabolic products found in this final metabolism solution were, therefore, stable or relatively stable end-products of metabolism.

The results are summarised in Table I concerning which the following observations may be made.

(*a*) The data for each period of incubation are calculated on the basis of 20 flasks containing initially 371 g. glucose, 19 g. tartaric acid and 19 g. ammonium tartrate, the initial p_H of the medium being about 4.

(*b*) By tartaric acid recovered (column 6) is meant the weight of tartaric acid corresponding to the ether-insoluble part of the material precipitated during the evaporation of the metabolism solution to small bulk. This material gave strong reactions for potassium and for tartrates, and its very sparing solubility in water indicated that it consisted largely of potassium hydrogen tartrate.

(*c*) The weights of $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ recorded in columns 9, 10 and 12 were isolated from the final fraction extracted by ether at a reaction acid to Congo red, the total weight of this fraction being given in column 13. The much smaller fraction extracted at p_H 7 was worked up for mycophenolic acid only but undoubtedly contained small amounts of the C_{10} compounds. This unavoidable loss accounts in part for the discrepancy between the weights recorded in columns 14 and 15.

The following points arise from a consideration of the results in Table I.

(*a*) Utilisation of glucose and utilisation of tartaric acid by *P. brevi-compactum* take place concurrently. Although the presence of tartaric acid is necessary for good colour reactions in flasks it is not metabolised preferentially.

Table I (*cont.*).

(9)	(10)	(11)	(12)	(13)	(14)	(15)
Wt. of $C_{10}H_{10}O_6$ (III) (g.)	Wt. of $C_{10}H_{10}O_7$ (II) (g.)	Wt. of $C_{10}H_{10}O_5$ (IV)	Wt. of $C_8H_6O_6$ (V) (g.)	Wt. of "acid to Congo" fraction (g.)	Total wt. of crystalline phenols isolated (g.)	Total wt. of crude phenols from which (14) was isolated (g.)
2·41	Not detected with certainty but probably present	Trace detected with certainty, M.P. 146–150° efferv. remelts at 200–220°	Trace	3·56	4·08	6·0
3·69	0·13 (*a*)	Not looked for	0·03	5·79	6·13	9·32
4·05	1·3	Detected with certainty	Not worked up	7·97	8·02	12·42
3·65	3·0	None	0·11	10·55	9·61	14·31
None	5·14	None	0·30	10·20	8·93 (*b*)	15·73 (*b*)

hydrazone, M.P. 210–212° decomp. after crystallisation from ethyl acetate-light petroleum.
was necessarily lost in the estimation of $C_{17}H_{20}O_6$ (I).

(*b*) For some reason at present unknown the ferric chloride coloration given by the metabolism solution in the early stages of growth is of no service in predicting which phenolic substances are present.

(*c*) As long as the p_H of the metabolism solution is well on the acid side of neutrality, the greater part of the mycophenolic acid remains in the mycelium. This is due to the fact that mycophenolic acid is almost insoluble in cold water but is readily soluble in dilute aqueous alkali. The other phenolic metabolic products are, on the other hand, very much more soluble in cold water.

(*d*) The stable end-products of metabolism are mycophenolic acid (I), and the acids $C_{10}H_{10}O_7$ (hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone II) and $C_8H_6O_6$ (3:5-dihydroxyphthalic acid V), but the final yield of the last named is small compared with the yields of the other two acids. The acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol III) is definitely an intermediate product of metabolism since its maximum yield (relatively very considerable) is reached on about the fifteenth day, after which, during the next 40 days, it is completely metabolised. The acid $C_{10}H_{10}O_5$ (3:5-dihydroxy-2-carboxybenzylmethylketone (IV)) is never at any time present in other than trifling amount and appears not to be a stable end-product of metabolism.

Discussion and Summary.

The results given in Table I definitely prove four series of facts, (*a*), (*b*), (*c*) and (*d*), and give fairly clear indication of another series (*e*).

(*a*) The yield of mycophenolic acid (I) increases continuously during the whole course of metabolism. A surprisingly large proportion, however (almost 50 %), of the total amount of mycophenolic acid (I) produced arises in the very early stages of metabolism (8 days after inoculation) and before growth of the organism is complete as is indicated by the weight of mycelium at this stage. Only a small proportion of the total yield of mycophenolic acid (less than 20 %) arises during the 34 days' incubation following the complete disappearance of glucose from the metabolism solution.

(*b*) The rate of production of the acid $C_{10}H_{10}O_7$ (II) is very different from that of mycophenolic acid. In the early stages of metabolism the presence of the acid $C_{10}H_{10}O_7$ could not be detected with certainty, and even after 11 days' incubation when 72·5 % of the glucose had been metabolised and growth of the mould had almost reached a maximum, only about 2·5 % of the final yield of the acid $C_{10}H_{10}O_7$ had been produced. On the other hand, about 40 % of the total amount of the acid $C_{10}H_{10}O_7$ produced arises towards the end of the incubation period, *i.e.* during the 34 days' incubation following the complete disappearance of glucose from the medium.

(*c*) The yield of the acid $C_{10}H_{10}O_6$ (III) increases rapidly to a maximum which is reached after an incubation period of 15 days, by which time 90 % of the glucose has disappeared, and then decreases in amount until this acid has disappeared completely from the medium after 56 days' incubation. Like mycophenolic acid (I) and unlike the acid $C_{10}H_{10}O_7$ (II) this acid is produced in largest amount (about 60 %) in the very early stages of metabolism, *i.e.* after 8 days' incubation.

(*d*) 3:5-Dihydroxyphthalic acid (V) is never present in large amounts but increases steadily during the whole course of metabolism, and at least 60 % of the total yield is produced after all the glucose has disappeared from the medium.

(*e*) The acid $C_{10}H_{10}O_5$ (IV) is present in very small amounts in the early stages of metabolism, but is absent in the later stages, none being detected after 22 days' incubation.

It must be admitted that the above results were entirely unexpected and are not easy of explanation, though it is hoped to obtain further light on the subject by a continuation of the work on somewhat different lines.

In view of the facts that mycophenolic acid (I), the acids $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV) and 3:5-dihydroxyphthalic acid (V) are all resorcinol derivatives, and that all have carbon side-chains in the 1 and 2 positions, it was natural to assume that one series of reactions or one set of precursors would be common to them all. The results presented do not offer conclusive evidence that this is the case.

We may, however, conclude with reasonable certainty that 3:5-dihydroxyphthalic acid (V) is an oxidation product of one or more of the other metabolic products.

Further, the fact that the acid $C_{10}H_{10}O_6$ (III) is produced in the early stages of growth and disappears completely in the later stages, while the acid $C_{10}H_{10}O_7$ (II) increases in amount throughout the whole course of metabolism and is produced in largest proportions during the later stages, renders it reasonably certain that the acid $C_{10}H_{10}O_7$ (II) arises by direct oxidation of previously formed $C_{10}H_{10}O_6$ (III). This change is indeed one which, as has already been shown [Oxford and Raistrick, 1933], can be readily brought about by purely chemical means. It is surprising to find that there should be a progressive accumulation of the acid $C_{10}H_{10}O_7$ (II), which is an α-diketone and which, certainly on purely chemical grounds, and probably on general biochemical grounds, might be expected to be readily oxidised by an organism whose available supplies of energy in the form of glucose have been exhausted.

The origin of the acid $C_{10}H_{10}O_5$ (IV) is not clear since the small yield and the difficulties of isolation render its estimation very difficult. It seems unlikely that the side-chain $-CH_2.CO.CH_3$ in this acid would be oxidised to $-CHOH.CO.CH_3$ in $C_{10}H_{10}O_6$ (III) more rapidly than the latter side-chain is oxidised to $-CO.CO.CH_3$ in $C_{10}H_{10}O_7$ (II), as must be the case if $C_{10}H_{10}O_5$ (IV) is the precursor of the other C_{10} acids. However, the ease with which it can be prepared *in vitro* from the acid $C_{10}H_{10}O_6$ (III) [Oxford and Raistrick, 1933] and the fact that it is present in the metabolism solution when the yield of the acid $C_{10}H_{10}O_6$ (III) is at a maximum, whereas it is absent when the acid $C_{10}H_{10}O_6$ (III) has disappeared and the yield of the acid $C_{10}H_{10}O_7$ (II) is at a maximum, indicate that it may be formed by the mould by reduction of the acid $C_{10}H_{10}O_6$ (III) and not by reduction of the acid $C_{10}H_{10}O_7$ (II).

It is tempting to postulate that the acid $C_{10}H_{10}O_6$ (III) is also the precursor of mycophenolic acid (I). The results presented do not exclude this possibility, but a consideration of the constitutional formulae assigned to these two acids shows that if this hypothesis is to be entertained certain assumptions must be made for which there are, so far as we are aware, no existing analogies in microbiological chemistry. Among other chemical changes necessary to convert $C_{10}H_{10}O_6$ (III) into mycophenolic acid (I) methyl and carboxyl side-chains must be introduced into the benzene nucleus. If, however, the possibility were admitted of introducing a —CHO group into the benzene nucleus by a microbiological process, then the introduction of two —CHO groups in the 4 and 6 positions into the molecule of the acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol, III) followed by the reduction of the —CHO group in the 6-position to CH_3, and the oxidation of the —CHO group in the 4-position to —COOH, immediately leads to the substituted aromatic nucleus shown to be present in mycophenolic acid (I).

The possibility that mycophenolic acid (I) is the precursor of the three

C_{10}-acids, which might be supposed to be formed from it by oxidation, demethylation and decarboxylation, is rendered improbable by the fact that even after all the glucose has been metabolised the yields of both mycophenolic acid (I) and the acid $C_{10}H_{10}O_7$ (II) continue to increase. It must be remembered, however, that after the glucose has completely disappeared the mycelium decreases appreciably in weight, and hence the possibility cannot be ruled out entirely that the substances produced from the mycelium during this period might give rise to further amounts of mycophenolic acid (I) and/or the acid $C_{10}H_{10}O_7$ (II). Further, in view of the relatively large final yield of mycophenolic acid, this possibility would involve the assumption that, after all the glucose has been utilised, the mould loses to a very large extent its former power of oxidising mycophenolic acid.

Finally it must be pointed out that we have failed up to the present to find any indication as to what are the steps involved during the initial formation from glucose of the resorcinol nucleus common to all the five phenolic metabolic products of *P. brevi-compactum*, since investigation of the metabolism solution in the earliest stages of growth failed to reveal any products of metabolism other than the five phenolic metabolic products which we have described previously.

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