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PRODUCTION OF PENTOSE INTERMEDIATES DURING GROWTH OF *NOCARDIA OPACA* AND OTHER SAPROPHYTIC SOIL NOCARDIAS AND MYCOBACTERIA

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SUMMARY

1. During growth in simple media *Nocardia opaca* (strain T_{18}) and some other saprophytic soil nocardias and mycobacteria liberate quantities of sedoheptulose and ribulose which can be detected by applying as little as 10–20 μl of medium directly to the paper chromatogram. An unknown substance which gave a spectrum similar to that of pentulose in the cysteine–carbazole reaction was also formed but this was neither ribulose xylulose, nor erythro-3-pentulose.

2. Liberation of the pentose cycle components occurs more quickly and generally in greater amounts when the medium is kept at about pH 7.0 (*e.g.* by addition of calcium carbonate, by using an increased phosphate buffer concentration, by using sodium nitrate rather than ammonium sulphate as a nitrogen source or by using sodium gluconate as a source of carbon). There seems no reason to suppose that calcium ion has any specific effect on the system.

3. Sedoheptulose and ribulose from the above cultures were identified and estimated by paper chromatography and by application of the cysteine–sulphuric acid and orcinol reactions respectively to larger quantities isolated by column chromatography. Dihydroxyacetone (characterised as the diacetate) was obtained only when the cultures were kept at about pH 7.0 (*i.e.* by addition of calcium carbonate). This and the failure to produce dihydroxyacetone from sodium gluconate suggests that the triose is mainly a product of the Emden-Meyerhof-pathway. Aldolase (with fructose diphosphate as a substrate) is present in cell-free extracts of the bacteria.

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INTRODUCTION

The significance of the pentose cycle in the life processes of many micro-organisms has hitherto been established by demonstrating that the enzymes required for the various stages exist in the cell-free extracts[1,2], rather than by the isolation of intermediates during growth. However, MORTENSON AND WILSON[3] showed that dried cells of *Azotobacter vinelandi* contain very small amounts of sedoheptulose and ribose phosphates, GHIRETTI AND GUZMAN-BARRON[4] found similar small amounts of sedoheptulose, ribulose and ribose in the medium when washed cells of *Corynebacterium creatinovorans* were incubated with glucose, and GODIN[5] found gluconic acid and dihydroxyacetone with traces of arabinose and ribose during the breakdown of glucose (in the presence of calcium carbonate) by preformed mats of *Penicillium brevi-compactum*.

Our organism, a pleomorphic paraffin- and fat-decomposing soil nocardia (*N. opaca* strain T_{16} see WEBLEY[6]), when growing on certain substrates produces considerable quantities of dihydroxyacetone, ribulose and sedoheptulose in liquid medium. The intermediates are present in such amounts that they can be demonstrated by applying as little as 10–20 μl of medium directly to the paper chromatogram.

MATERIALS AND METHODS

Organisms. *N. opaca* strain T_{16} was mainly used. Some other soil nocardias and mycobacteria were tested as indicated in the text and also a strain of *Acetobacter suboxydans*.

Growth. The medium contained KH_2PO_4, 0.054 g; $MgSO_4.7H_2O$, 0.025 g; $(NH_4)_2SO_4$, 0.075 g; $FeCl_3$, trace; yeast extract (Difco) or sodium glutamate, 0.2 g; and tap water to 100 ml. The pH was adjusted to 6.8. Calcium carbonate (1% w/v) was used where indicated and was moistened with water and sterilised separately. The substrates were used at a final concentration of 4% (w/v). The medium was put up in 10-ml lots in medical "flats" for paper chromatographic determination of the intermediates.

For isolation 200-ml lots in 1-l Pyrex culture flasks (Joblin Cat. No. 1410) were used. Both "flats", and culture flasks were incubated with mechanical shaking[7] at 25°. They were inoculated with 2 drops of suspensions of the organisms prepared from agar plates. Samples were removed aseptically during growth when required.

Paper chromatography. Generally No. 1 Whatman paper was used and run at room temperature in butanol saturated with water. The dried papers were sprayed with phloroglucinol–acetic acid–HCl[8] to reveal sedoheptulose and ribulose or with aniline phthalate[9] to reveal dihydroxyacetone. The orcinol–trichloroacetic acid–aniline phosphate spray described by DICKENS AND WILLIAMSON[10], was also used in attempts to reveal the pentulose group of sugars (ribulose, erythro-3-pentulose, and xylulose) which have similar R_G values. For quantitative work about 50 spots (total volume 0.14 ml) of the centrifuged medium were applied to each paper and the areas associated with each sugar were cut out after location with marker strips, and extracted by immersion for 30 min in water (5 ml or 10 ml according to the intensity of the spots), and samples analysed.

Ribulose. The DISCHE[11] modification of the orcinol reaction gives a characteristic peak at 540 mμ with ribulose[12]. Our method used these reagents but with a heating

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time of 30 min (intermediate between the times adopted by the above authors). The absorption was read with an E.E.L. colorimeter with a green filter (ORG1). Standards were prepared from pure (syrupy) ribulose obtained from diacetone ribulose by the method of LEVENE AND TIPSON[13] followed by separation from the accompanying aldopentose by bromine oxidation[12] and final purification on a cellulose column. The range covered was 5–40 μg.

Sedoheptulose. This was determined by the cysteine–sulphuric acid reaction as described by NEWBURGH AND CHELDELIN[14]. The absorption was read at 505 mμ with a Unicam S.P. 500 spectrophotometer and a correction was made for the presence of glucose, which was never completely separated from sedoheptulose on the paper chromatogram, by making a second reading at 415 mμ.

The interference from the glucose substrate was much greater in the earlier stages of growth, and in some cases prevented any determination being made. An empirical nomogram enabled the corrected amount of sedoheptulose to be obtained conveniently and was checked with mixtures of known amounts of glucose and sedoheptulose. A pure sample of the latter sugar was obtained by column chromatography on cellulose of the crude material prepared from *Sedum rupestre* (*cf.* UJEJSKI AND WAYGOOD[15]). A derivative from this (dibenzylidenesedoheptulosan) had m.p. 245° in agreement with the values recorded.

Dihydroxyacetone. Obtained from L. Light & Co. Colnbrook, England; paper chromatography showed that this was practically pure monomer.

RESULTS

Identification of the intermediates

The organism was grown in 200 ml of the medium (with calcium carbonate) using glycerol as a substrate. With glucose as substrate it would have been more difficult to obtain a pure sample of sedoheptulose by chromatography. After 17 days incubation at 25° on the shaking machine the cells and insoluble calcium salts were removed at the centrifuge. The supernatant solution was deionised with columns (40 × 1.5 cm diam.) of ion exchange resins (Amberlite IR 120 (H) and IR 4B). The effluent was evaporated *in vacuo* at 30° and fractionated on a cellulose column (60 × 2.8 cm) with water-saturated butanol as a solvent. The bulked fractions were dried over P_2O_5 and weighed after removing any water-insoluble material in the usual way. The yields were—dihydroxyacetone, 3 g; ribulose, 99 mg; ribulose + pentulose, 110 mg; pentulose, 8 mg; sedoheptulose + pentose (arabinose?), 40 mg; and sedoheptulose 190 mg.

Dihydroxyacetone

A portion (0.4 g) was treated with pyridine and acetic anhydride and the acetate extracted with chloroform and isolated as a syrup which crystallised on cooling in ice. After three recrystallisations from light petroleum (b.p. 40°–60°) the product (0.12 g) had m.p. 46° not changed on admixture with authentic material. The infrared spectrum was identical with that of di-O-acetyl-dihydroxyacetone. The original fraction and authentic dihydroxyacetone had identical R_F values on the paper chromatogram and gave the same colour with the aniline phthalate and phloro-

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glucinol sprays. On keeping for some weeks the syrupy fractions tended to polymerise and produce trails on the paper chromatogram.

Sedoheptulose

The cysteine–sulphuric acid reaction was carried out on equal amounts of the fraction and of authentic material. The products had identical spectra over the range 400–625 mμ and showed the characteristic peak at 505 mμ[14]. The R_F on the paper chromatogram and the colour given with the phloroglucinol and orcinol[16] sprays was identical for the fraction and authentic sedoheptulose.

Ribulose

For this the organism was grown on glucose (with calcium carbonate) since the pentulose which contaminated the ribulose fraction obtained from the column above appeared only with glycerol as a substrate and it was thought preferable to avoid this difficult separation. A pure specimen of ribulose was obtained by spotting several papers with the centrifuged medium and running in the usual way using marker spots to locate the ribulose. The orcinol reaction was carried out with samples of the eluate and with an authentic specimen of ribulose. The spectra were identical over the range 400–700 mμ with peaks at 540 and 670 mμ.

Pentulose

The unknown pentulose from cultures of *N. opaca* on glycerol gave a spectrum in the cysteine–carbazole reaction which was very similar to that of ribulose with maxima at 540 and 390 mμ. There was no increase in the optical density (at 540 mμ), at 3 h as compared with that recorded at 15 min so that the pentulose cannot be xylulose[17]. The chromatographic characteristics distinguished the sugar from ribulose (R_G 0.245 and 0.255 and a pink-purple colour instead of a green colour with the phloroglucinol spray respectively). The pentulose gave a pink colour identical with that given by ribulose with the orcinol–trichloroacetic acid–aniline phosphate spray whereas DICKENS AND WILLIAMSON[10] indicate that the erythro 3-ketopentulose obtained by ASHWELL AND HICKMAN[18,19] gives an orange colour with the reagent.

Intermediate production with varying cultural conditions

Effect of $CaCO_3$ (Table I)

Calcium carbonate is required for intermediate production in the earlier stages of growth. In its absence about half the ribulose concentration was obtained and this only in the later stages. With medium containing glucose and calcium carbonate the pH is maintained at the initial value of about 6.8 throughout the experiment. In the absence of calcium carbonate the pH drops to approx. 4.0 within 2 days (see also Table II for estimations of substrate usage). The effect is not so marked with glycerol as a substrate since, even in the absence of calcium carbonate, the pH does not drop below 6.0 during growth. The fall in pH is presumably linked with acid produced from the ammonium sulphate and also with acids produced from the substrate.

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TABLE I

INTERMEDIATE PRODUCTION WITH GLUCOSE OR GLYCEROL AS SUBSTRATES IN THE PRESENCE AND ABSENCE OF CALCIUM CARBONATE

The bacteria were grown and samples withdrawn aseptically for determinations, as indicated in the text. The concentrations of sedoheptulose (S) and ribulose (R) are given in mg/ml; —signifies that although the intermediate (sedoheptulose) was present, the mixture contained glucose in quantity sufficient seriously to interfere with the determinations.

Substrate	*Growth (days)*							
	5		*8*		*13*		*17*	
	S	*R*	*S*	*R*	*S*	*R*	*S*	*R*
Glucose	nil	nil	nil	nil	—	0.6	—	1.3
Glucose + $CaCO_3$	nil	0.9	0.1	1.6	0.2	2.1	0.25	3.0
Glycerol	nil	nil	0.2	nil	0.2	0.7	0.25	1.0
Glycerol + $CaCO_3$	nil	nil	0.2	0.9	0.25	1.0	0.25	1.3

TABLE II

INTERMEDIATE PRODUCTION ON NORMAL AND MODIFIED MEDIUM (pH CONTROLLED) IN THE PRESENCE AND ABSENCE OF CALCIUM CARBONATE

The bacteria were grown (as indicated in text), on the normal medium (with glucose as a substrate) and also on a similar medium in which $(NH_4)_2SO_4$ was replaced by an equivalent molar concentration of $NaNO_3$. In this medium the pH remained at the initial value of 6.8 during growth in contrast to the usual media (without $CaCO_3$) where the pH dropped to 3.5–4.0 in 2–4 days. The intermediate concentrations are represented as in Table I. Glucose (G) was determined by a specific method using glucose oxidase (HUGGETT AND NIXON, 1957).

Growth (days)	*Medium*											
	Normal			*Normal + $CaCO_3$*			*Modified*			*Modified + $CaCO_3$*		
	G	*S*	*R*	*G*	*S*	*R*	*G*	*S*	*R*	*G*	*S*	*R*
3	31	nil	nil	13	nil	0.6	16.5	nil	0.3	17.5	nil	0.3
6	31	nil	nil	2	0.8	0.8	1.4	nil	0.8	5.0	nil	0.4
8	26	nil	0.1	0.1	0.9	1.1	nil	nil	0.8	1.0	0.5	1.3
12	26	—	1.3	nil	0.9	1.1	nil	0.5	1.2	nil	0.6	1.3
17	26	—	1.6	nil	0.9	1.2	nil	0.5	1.2	nil	1.5	1.5

TABLE III

INTERMEDIATE PRODUCTION ON NORMAL MEDIUM AND ON HIGHLY BUFFERED MEDIUM IN THE PRESENCE AND ABSENCE OF CALCIUM CARBONATE

The bacteria were grown (as indicated in text) on the normal medium and on similar medium in which the phosphate buffer concentration had been increased to 0.017 *M*. The intermediate concentrations are as represented in Table I.

Growth (days)	*Medium*							
	Normal		*Normal + $CaCO_3$*		*High buffer*		*High buffer + $CaCO_3$*	
	S	*R*	*S*	*R*	*S*	*R*	*S*	*R*
7	—	0.4	—	1.5	0.3	1.1	0.3	1.9
9	—	0.9	—	1.6	0.4	0.9	0.3	2.1
11	—	1.3	—	1.8	0.7	1.6	0.5	0.6

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Effect of replacing NH_3 by NO_3 in the medium (Table II)

Here a modified medium, in which the pH remains at the initial value of about 6.8 during growth, is compared with the normal medium. With the modified medium calcium carbonate had little or no effect on intermediate production. With the normal medium the pH fell rapidly in the absence of calcium carbonate and the initial glucose concentration (approx. 50 mg/ml) fell only to 26 mg/ml in 8 days. Where the pH was controlled, (as in the modified medium and with calcium carbonate) the glucose concentration was reduced practically to zero in that time.

Attempts to buffer the medium with high concentrations of phosphate (Table III)

Here the pH was controlled by raising the (phosphate) buffer concentration in the medium to give a final concentration of 0.017 *M*. There is a significant improvement in intermediate production during the initial growth period in the more highly buffered as compared with the normal medium. In the later stages of growth the more highly buffered medium also became acid.

Effect of replacing $CaCO_3$ by $MgCO_3$ and Amberlite resin (Table IV)

Here the pH was controlled by adding either calcium carbonate, magnesium carbonate or Amberlite IR4B resin. Since the fresh resin was either toxic or removed material essential for growth from the medium it was necessary to autoclave and wash it with sterile water and then preincubate (2 days) with a suitable quantity of medium. This was then replaced aseptically with fresh medium before inoculation. The results show that calcium carbonate is the most effective agent for inducing the early production of the intermediates and that magnesium carbonate and ion-exchange resin are about half as effective. The addition of calcium ion to the resin-containing medium had no effect on intermediate production. A separate experiment showed that calcium ion was only partly removed from solution by the resin. For this purpose samples were removed from the calcium chloride medium immediately after adding the resin and again after incubating for 5 days. The calcium content of the samples (initial 48.6 mg/ml, final 28.5 mg/ml) was estimated with a flame photometer by the Department of Spectrochemistry (DR. A. M. URE).

TABLE IV

EFFECT OF VARIOUS ADDITIONS ON INTERMEDIATE PRODUCTION FROM GLUCOSE

The bacteria were grown (as indicated in text) with glucose as a substrate and with the various additions. The intermediate concentrations are as represented in Table I.

Addition	*Growth (days)*			
	5		*10*	
	S	*R*	*S*	*R*
Calcium carbonate (1 %)	1.2	1.5	1.8	2.7
Magnesium carbonate (1 %)	0.3	0.7	nil	0.9
Amberlite IR4B resin (20 %)	0.5	0.7	0.5	0.6
Amberlite IR4B resin (20 %) + calcium chloride (0.5 %)	nil	0.5	0.2	0.7
No addition	nil	0.25	nil	1.2

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Production of intermediates from various substrates (Table V)

Calcium carbonate has no effect with sodium gluconate as a substrate. This would be expected if the acid is converted to neutral sugars and the Na^+ liberated to neutralise any CO_2 or acids formed, thus preventing any fall in pH.

TABLE V

INTERMEDIATE PRODUCTION FROM VARIOUS SUBSTRATES IN THE PRESENCE AND ABSENCE OF CALCIUM CARBONATE

The bacteria were grown (as indicated in text) on the normal medium but with various sources of carbon (4% concn.): The intermediate concentrations are as represented in Table I.

Carbon source	Growth (days)							
	2		4		6		9	
	S	R	S	R	S	R	S	R
Glucose	nil	nil	nil	nil	nil	nil	—	1.0
Glucose (+ $CaCO_3$)	—	0.8	—	1.2	0.4	1.1	0.7	1.8
Fructose	nil	nil	—	0.37	—	0.6	—	0.8
Fructose + $CaCO_3$	nil	nil	—	0.75	—	0.8	—	1.4
Gluconic acid	0.4	0.8	2.3	1.4	—	—	2.1	nil
Gluconic acid + $CaCO_3$	0.4	0.5	nil	1.0	2.2	0.3	1.8	nil
Sorbitol	—	0.7	—	1.5	0.8	2.6	0.9	3.0
Sorbitol + $CaCO_3$	—	0.5	—	1.1	1.1	1.5	1.6	1.6
Mannitol	nil	0.5	0.3	0.8	0.3	0.8		
Mannitol + $CaCO_3$	nil	0.5	0.3	1.3				

*Intermediate production with other organisms**

The organisms were grown on the medium (10 ml) containing glucose and calcium carbonate. Intermediate production was followed by paper chromatography of samples taken at convenient intervals using heavy spotting (10 times) to detect small quantities of product.

With these conditions *Mycobacterium lacticola* (NCTC 1472), *N. opaca* (strain T_1) and *N. caprae* (strain T_3) gave the intermediates at periods ranging from 4–12 days. *Nocardia* sp. (strain P_2) and *M. phlei* (strains R 36 and W 23) gave the intermediates at periods varying from 12–32 days and *M. rubropertinctum* (NCTC 4220), *M. phlei* (strain R 8A). *N. salmonicolor* (strains 2, 22, $\frac{14}{1}$ and $\frac{8}{1}$), and *N. ruber.* (3 strains) did not give any intermediates.

Acetobacter suboxydans (NCIB, 5595) gave a large amount of dihydroxyacetone but not the other intermediates.

Aldolase content of cell-free extracts

The organism was grown on the usual medium but to obtain good growth in the absence of calcium carbonate, the pH was kept at about 7.0 by using sodium nitrate instead of ammonium sulphate as a source of nitrogen. The organism was harvested at the centrifuge and washed twice with distilled water. Grinding was carried out

* The strains of *N. opaca*, *N. salmonicolor*, *N. caprae* were isolated by ERIKSON[20]. *Nocardia* sp. (strain P_2) was obtained from DR. TRECCANI, University of Milan and the strains of *M. phlei* from DR. RUTH GORDON, Institute of Microbiology, Rutgers University, U.S.A.

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as described by McILWAIN[21] (extracting with 0.1 *M* phosphate buffer pH 7.0) but using H fine Aloxite (Carborundum Co. Ltd., Manchester) three times the wet weight of the cells. After removing alumina and most of the cell debris at 5000 rev./min the extract was clarified at 50,000 × *g* in a refrigerated centrifuge (M.S.E. Super Speed 20, M.S.E. Ltd., Spenser Street, London W.1).

From 4 g wet weight of bacteria we normally obtained about 3.5 ml of clarified extract containing about 25–50 mg of protein (precipitated by trichloroacetic acid).

The aldolase activity was measured using the "Biochemica Boehringer Test Combination" in which the decrease in optical density at 340 mμ due to the glycerophosphate dehydrogenase mediated oxidation of DPN-H by dihydroxyacetone phosphate is measured, the triose being formed from fructose diphosphate in the presence of aldolase and added triosephosphate isomerase.

A considerable oxidation of DPN-H in the absence of fructose diphosphate was observed and it was necessary to dialyse the extract for 24 h against several changes of 0.1 *M* Tris buffer (pH 7.4) at 2° to remove this interference. The dialysed extract had about 40 BRUNS[22] aldolase units in reasonable agreement with the value obtained (in a different extract) by measuring the increase in alkali-labile organic phosphate (32 BRUNS units), by the method of SIBLEY AND LEHNINGER[23].

DISCUSSION

A considerable production of intermediates takes place in ageing cultures of *N. opaca* (strain T_{16}) but intermediate production can be induced in the early stages of growth by various additions to or modifications of the medium. The effect of these changes is to prevent the medium going acid but a neutral condition is not essential for intermediate production since the ageing cultures, (without pH control) may have a pH of about 4.0. One difference however, between the intermediates liberated in the presence of $CaCO_3$ as compared with those produced by ageing is that under the latter conditions dihydroxyacetone does not appear.

AXELROD, BANDURSKI, GREINER AND JUNG[24] have indicated that calcium may be involved in the pentose-heptulose stage of the hexose monophosphate pathway and SLATER AND CLELAND[25] have shown that calcium ion is the cause of abnormal permeability of rat heart sarcosomes (causing the ATP concentration and therefore phosphorylation to diminish). With our organism and using the normal medium (containing ammonium sulphate), calcium carbonate is the most effective agent for inducing early intermediate production (magnesium carbonate and Amberlite ion-exchange resin are only about 50 % as effective). However, calcium ion has no effect when added to the resin-containing medium. Furthermore, intermediate production using a medium modified so that the pH remains practically constant throughout the growth period (by replacing ammonium sulphate in the normal medium with sodium nitrate or by using a more concentrated phosphate buffer) is practically as good as that with the normal medium in the presence of calcium carbonate (Tables II and III respectively). With sodium gluconate as a substrate intermediate production is not affected by the presence or absence of calcium carbonate and the pH remains practically constant throughout the growth period (Table V). Dihydroxyacetone accompanied the other intermediates when the bacteria were grown on the substrates indicated in Table V (in the presence of calcium car-

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bonate) with the sole exception of sodium gluconate. This may indicate that it is produced by the Emden-Meyerhof pathway, which may exist in the organism, since cell-free extracts exhibited aldolase activity with fructose 1–6 diphosphate as a substrate.

In the hexose monophosphate pathway it is now recognised that xylulose (as the 5-phosphate) is required as a substrate for transketolase (which must be highly active in our organism since sedopheptulose is formed in quantity) but this pentulose was detected neither on paper chromatograms of the medium from our organism nor in the concentrated fractions from chromatographic columns. Presumably the phosphoketopentose epimerase activity is low here as in the case of horse erythrocytes[10]. These latter, however, have a very weak aldolase activity in contrast with that of our organism which therefore does not fit well into the pattern suggested by HORECKER, HURWITZ AND SMYRNIOTOS[24], who report that there is a tightly bound association of aldolase and phosphoketopentose epimerase (from *Lactobacillus pentosus*).

It is possible that the Emden-Meyerhof scheme for the conversion of triose to acetylphosphate is by-passed in our organism as with *L. pentosus*[25] and *Leuconostoc mesenteroides*[26] by a direct phosphorolytic cleavage of xylulose-5-phosphate to acetyl phosphate and triose (mediated by phosphoketolase).

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