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The Synthesis of Cell Constituents from Butane-2,3-diol by *Pseudomonas* sp.

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Juni & Heym (1956) showed that the dissimilation of butane-2,3-diol by micro-organisms proceeds via a cyclic mechanism to acetate. Butane-2,3-diol is oxidized to 2-hydroxybutan-3-one and thence to diacetyl, which, in the presence of thiamine pyrophosphate, is hydrolysed to acetate and an 'active acetaldehyde' complex. The complex undergoes an instantaneous condensation with a further molecule of diacetyl to form 3-hydroxy-3-methylpentane-2,4-dione, which is then subsequently reduced to 2,3-dihydroxy-3-methylpentan-4-one; this is hydrolysed to give a second molecule of acetate and butane-2,3-diol. The net effect of one turn of this cycle is to oxidize butane-2,3-diol to acetate according to the following reaction:

$$\text{Butane-2,3-diol} + 1\tfrac{1}{2}O_2 \rightarrow 2 \text{ acetate} + H_2O$$

Dagley (1958) suggested that the required synthesis of intermediates of the tricarboxylic acid cycle might occur via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The main purpose of this paper is to show that *Pseudomonas* sp. (tentatively identified as *P. fluorescens*), utilizing butane-2,3-diol as sole source of carbon, incorporates [1-^{14}C]acetate into cell constituents consistent with the operation of the tricarboxylic acid and glyoxylate cycles. No evidence has been obtained in support of the direct formation of succinate from acetate (Thunberg, 1920; Glasky, Eicholz & Rafelson, 1958; Glasky & Rafelson, 1957, 1959).

This work has been presented in part to the Biochemical Society (Hullin & Hassall, 1960).

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MATERIALS AND METHODS

Maintenance and growth of the organism. The organism was originally isolated from soil, by S. Dagley, on account of its ability to utilize butane-2,3-diol as sole source of carbon for growth. Stock cultures were kept on agar slopes consisting of (w/v): KH_2PO_4, 0·2%; $(NH_4)_2SO_4$, 0·2%; thiamine hydrochloride, 0·001%; $MgSO_4,7H_2O$, 0·04%; butane-2,3-diol, 0·1%; agar, 2·0%; the pH was adjusted to 7·0–7·2 with NaOH. The organism was subcultured every 10–12 weeks, grown at 30°, and stored at 2°; desiccation of the slopes was prevented by sealing the tubes with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2).

Cells were grown for 18 hr. with forced aeration at 30° in 10 l. flasks, containing 9 l. of medium, after inoculation with 800 ml. of a culture grown overnight in the same medium. This medium contained (g./l.): KH_2PO_4, 5·0; $(NH_4)_2SO_4$, 2·0; $MgSO_4,7H_2O$, 0·4; butane-2,3-diol, 1·8; the pH was adjusted to 7·0–7·2 with NaOH. Cell concentrations were determined turbidimetrically in a Hilger Spekker photoelectric absorptiometer fitted with Ilford filters neutral H. 508 and blue O.B_2. The cells were harvested while still in the logarithmic phase of growth (0·25–0·35 mg. dry wt./ml.) using a Sharples continuous-flow centrifuge.

Manometric studies of oxygen uptake. The conventional Warburg respirometer was used, as described by Umbreit,

Burris & Stauffer (1957), to follow the uptake of oxygen during the oxidation of substrates.

Incorporation of [1-^{14}C]*acetate into cells utilizing butane-2,3-diol.* The procedure of Kornberg (1958) was closely followed for the purification of the [1-^{14}C]acetate and for the subsequent analysis of samples obtained from incubation experiments.

Freshly harvested cells were resuspended (4·3 mg. dry wt./ml.) in medium containing 2·0 mM-butane-2,3-diol, 4·0 mM-phosphate buffer (pH 7·0) and 4·0 mM-NH_4Cl, and aerated in a water bath at 30°. After 15 min., approx. 60–70% of the diol had been utilized, as ascertained from a sample of the medium incubated at the same temperature in a Warburg respirometer. A portion (0·85 ml.) of the reaction mixture was pipetted into a 80 mm. × 120 mm. centrifuge tube containing 3·0 ml. of ethanol and kept in a water bath at 70°. [1-^{14}C]Acetate (50 μc/ml.; 8·3 μc/μmole) (0·96 μmole) was then added and this mixture used as the zero-time sample.

To begin the reaction, 11·5 ml. of the suspension was drawn into a graduated hypodermic syringe (20 ml. capacity), used without the needle, and rapidly transferred to a 50 ml. beaker containing 1·86 ml. of the [1-^{14}C]acetate solution. The addition of the cells under pressure provided adequate mixing so that samples could be withdrawn immediately with a second syringe of 1·0 ml. capacity. These samples were quickly ejected into 3·0 ml. of ethanol, the times of addition to the ethanol being recorded from a stop-clock which had been started when the cells were added to the radioactive solution. The reaction vessel was shaken continuously between sampling of the reactants.

Analysis of samples obtained from incubation experiments. The aqueous-ethanolic suspensions were analysed by two-dimensional chromatography in 90% (v/v) phenol–formic acid–water (500:13:167, w/v/v) (Kornberg, 1958) and butan-1-ol–propionic acid–water (Calvin & Benson, 1949) as described by Kornberg (1958). The radioactive spots were located by radioautography for 10–30 days with Kodak (Kodirex) X-ray film, and the activity assayed by counting, *in situ*, with a mica end-window β-tube (General Electric Co. type EHM 2/5) standing on a mask with an aperture of up to $\frac{5}{8}$ in. square. Radioactive compounds were identified by elution and two-dimensional co-chromatography with authentic unlabelled samples of the compound in two solvent pairs. In addition to the two solvents mentioned above the following were also used: butan-1-ol–pyridine–water (1:1:1, by vol.) (Morrison, 1953) and benzene–ether–90% (v/v)–formic acid–water (30:70:14:10, by vol.) (Weimberg, 1959) for carboxylic acids; butan-1-ol–acetone–water–diethylamine (5:5:5:1, by vol.) (Hardy, Holland & Nayler, 1955) for amino acids.

Unlabelled carrier carboxylic acids were located by spraying with a 1·0% (v/v) solution of aniline and 1·0% (w/v) xylose in 95% (v/v) methanol (Nordmann & Nordmann, 1960), and amino acids by spraying with 0·2% ninhydrin in acetone (Smith, 1953) to which pyridine was added to 2·0% (v/v) immediately before use.

Intensification of radioautographs. Radioautographs with faint spots, occasionally obtained during co-chromatographic identification, were intensified as described by Moses & Edwards (1960).

Preparation of cell extracts. Freshly grown cells were disintegrated in a Hughes (1951) press without abrasive. This crushed material could be stored at −14° for up to a month without noticeable deterioration or change in characteristics. Each 1·0 g. of crushed cells was ground with 0·5 g. of polishing alumina, grade 3/50 (Griffin and George Ltd., Manchester), and 2·5–3·0 ml. of buffer (KH_2PO_4, 2·0 g./l.; adjusted to pH 7·0 with NaOH). This procedure facilitated both the dispersal of the crushed cells and the subsequent clarification of the extract when this was centrifuged at 12 000*g* for 35 min. at 2°. A pink viscous supernatant solution was obtained with a protein concentration of 30–35 mg./ml., as estimated by the method of Sols (1947), with a solution of crystalline bovine serum albumin for the preparation of a standard curve.

Determination of diacetyl. The method used was that of Neuberg & Strauss (1945) as modified by Spencer (1950). It consists in forming the bis-2,4-dinitrophenylhydrazone by heating the sample of diacetyl (0–0·6μmole in 10 ml.) with 5·0 ml. of 0·02% 2,4-dinitrophenylhydrazine (in 2N-HCl) in stoppered tubes at 100° for 60 min. The resulting precipitate was centrifuged (a process facilitated by the addition of a little kaolin), washed successively with 5N-HCl and water, and the colour developed by twice extracting with 5·0 ml. of 0·3% sodium in ethanol. The pooled extracts were diluted to 25 ml. with sodium ethoxide solution, and the extinction was read at 551 mμ.

Determination of 3-hydroxy-3-methylpentane-2,4-dione. This was determined by using acidified ammonium molybdate (Juni & Heym, 1957).

Determination of glyoxylate and α-oxoglutarate. These were determined in the presence of each other by the method of Friedemann & Haugen (1943) as modified by Olson (1959).

Chromatography of 2,4-dinitrophenylhydrazones of keto acids. 2,4-Dinitrophenylhydrazones of keto acids were chromatographed one-dimensionally in butan-1-ol–ethanol–ammonium carbonate buffer (40:11:14, by vol.) (Dagley, Fewster & Happold, 1952). Further characterization was obtained by spraying the chromatograms with 2·0% (w/v) KOH in aq. 90% (v/v) ethanol.

Assay of isocitrate lyase (isocitratase) activity. For each extract a series of dilutions was prepared and each in turn was incubated for 10 min. at 30° in a reaction mixture containing 100 μmoles of DL-isocitrate and 5·0 μmoles of $MgSO_4$ in a total volume of 1·5 ml. of phosphate buffer (KH_2PO_4, 2·0 g./l.; adjusted to pH 7·2 with 5N-NaOH). The reaction was terminated by adding 1·5 ml. of 10% (w/v) trichloroacetic acid, the precipitated protein was removed by centrifuging, and the glyoxylate formed was determined as described above.

Reactions of [1-^{14}C]*glyoxylate in cell extracts.* Reactions were carried out in Warburg respirometers filled with nitrogen, and terminated by quickly removing the flasks from the manometers and adding 3·0 ml. of hot (70°) ethanol. The samples, with washings, were centrifuged, and the supernatant solutions evaporated to dryness at 60° under reduced pressure in an atmosphere of nitrogen. Each desiccate was dissolved in 0·2 ml. of aq. 20% (w/v) ethanol brought to 0·1N with HCl, and 0·05 ml. analysed by chromatography with phenol–formic acid–water. The activities of the glyoxylate and malate spots were assayed, as described above, after locating them by radioautography (2–4 days).

Materials. Sodium DL-isocitrate was prepared by hydrolysis of the lactone by the method of Olson (1959). Glyoxylate, as the monohydrate of the sodium salt, was synthesized as described by Metzler, Olivard & Snell (1954), and

3-hydroxy-3-methylpentane-2,4-dione (obtained in solution) as described by Juni & Heym (1957). Butane-2,3-diol, 2-hydroxybutan-3-one, diacetyl (all redistilled before use), DL-isocitric acid lactone, ATP, NAD, CoA and acetyl phosphate were obtained from L. Light and Co., Colnbrook, Bucks., and bovine serum albumin from Armour Laboratories, Chicago, U.S.A. Isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. Other chemicals used were of AnalaR grade (British Drug Houses Ltd., Poole, Dorset).

RESULTS

Dissimilation of butane-2,3-diol to acetate. Preliminary experiments were designed to confirm that the cycle, proposed by Juni & Heym (1956) for the dissimilation of butane-2,3-diol, operated in *Pseudomonas* sp.

2-Hydroxybutan-3-one and acetate readily replaced butane-2,3-diol as the growth substrate for the organism. No growth was obtained on diacetyl, a result consistent with that of Juni & Heym which, as they suggested, is probably due to the known bacteriostatic effect of diacetyl (Myrvik & Volk, 1954). Suspensions of washed cells, grown on the diol, rapidly oxidized butane-2,3-diol, 2-hydroxybutan-3-one, diacetyl and acetate; cell extracts oxidized the diol and the carbinol at similar rates but diacetyl and acetate only slowly.

The key reaction of the cycle, the synthesis of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl, was shown to occur in cell extracts even under aerobic conditions, The recovery of 3-hydroxy-3-methylpentane-2,4-dione was complete (Table 1), indicating that there was no direct oxidation of diacetyl to acetate. The accumulation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl in cell extracts is believed to be caused by the inhibition of 3-hydroxy-3-methylpentane-2,4-dione reductase at the concentrations of diacetyl used. The extract (10 ml.) for this experiment was prepared in 0·02M-tris buffer, pH 6·8, and dialysed at 2° against three changes of 2 l. of the same buffer for a total of 6 hr. In this way, inorganic phosphate, which interferes in the estimation of 3-hydroxy-3-methylpentane-2,4-dione and which would be carried through from the growth medium, was removed.

Incorporation of [1-^{14}C]*acetate by cells utilizing butane-2,3-diol.* When [1-^{14}C]acetate was added to a suspension of whole cells utilizing butane-2,3-diol, isotope was rapidly and linearly incorporated into the cell constituents soluble in aqueous ethanol; a decrease in the rate was observed after the first minute. Incorporation of ^{14}C into the fraction insoluble in aq. ethanol (protein and lipid) was also observed but this occurred at a much lower rate.

Variation in the percentage distribution of ^{14}C *incorporated from* [1-^{14}C]*acetate into constituents of the soluble fraction.* Analysis of the samples obtained after adding [1-^{14}C]acetate to cells utilizing butane-2,3-diol showed that isotope was incorporated into cell constituents in agreement with the concomitant operation of the tricarboxylic acid and glyoxylate cycles (Fig. 1). The initially high and decreasing percentage of the total activity found in citrate and the rapid accumulation of activity in glutamate

Table 1. *Formation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl by extract of* Pseudomonas *sp. grown on butane-2,3-diol*

The incubation mixture contained: 105 μmoles of diacetyl; 10 μmoles of $MgSO_4$; 2·0 ml. of dialysed cell extract, 33·6 mg. of protein/ml.; 100 μmoles of tris, pH 6·8; water to 10 ml. The reaction mixture was incubated aerobically at 30° with shaking in 1½ in. × 6 in. boiling tubes, and samples (1·0 ml.) were withdrawn from the mixture at the times given and stopped by the addition of 1·0 ml. of 10% (w/v) trichloroacetic acid.

Time (min.)	Diacetyl utilized (μmoles)	3-Hydroxy-3-methylpentane-2,4-dione formed (μmoles)	
		Calc.	Found
2	35·6	17·8	18·0
5	67·1	33·5	32·6
10	85·8	42·9	41·1
20	99·0	44·5	43·2
40	105·0	52·5	50·2

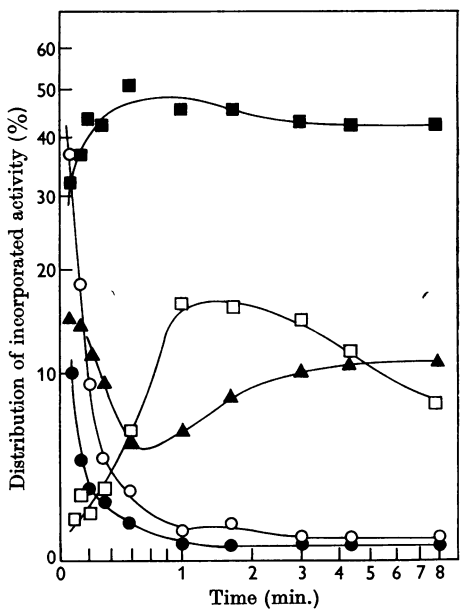

Fig. 1. Variation witb time of the percentage distribution of ^{14}C incorporated from [1-^{14}C]acetate into constituents soluble in aq. ethanol of *Pseudomonas* sp. utilizing butane-2,3-diol. ○, Citrate; ●, malate; ▲, aspartate; □, succinate; ■, glutamate.

were consistent with the action of the condensing enzyme. Similarly, the decreasing percentage activity of malate and the relatively high initial activity in aspartate suggested the entry of acetate into the tricarboxylic acid cycle via the mediation of malate synthase. The low initial activity of succinate, a compound shown to be present in large amounts by the subsequent high ^{14}C content, obviated its direct formation from acetate. Labelled phosphates did not appear until 16 sec. after the addition of [1-^{14}C]acetate and even after 16 min. did not account for more than 5% of the total incorporated activity.

The appearance of activity in β-hydroxybutyrate was noticeable (1·3%) at 6½ sec., and was equal to 11·3% of the total at 1 min. and 12·8% at 4 min. 20 sec. The percentage of the total activity present in β-hydroxybutyrate then decreased concurrently with the formation of an unidentified labelled compound with lipid characteristics. This compound (R_F approx. 0·9–0·95 in both solvents) was probably poly-β-hydroxybutyrate, although this was not confirmed.

The only other compound accounting for more than 5% of the total activity at any one time was glutamine. The incorporation of activity into this compound suggested that it was readily in equilibrium with glutamate. The activities of both β-hydroxybutyrate and glutamine have been omitted from Fig. 1 to preserve clarity of presentation.

Isocitratase activities of cell extracts. The activity of isocitratase was assayed by estimating the glyoxylate produced from isocitrate under standard reaction conditions. Crude extracts of the organism grown on either butane-2,3-diol or acetate rapidly accumulated both glyoxylate and α-oxoglutarate, consistent with the action of isocitratase and isocitrate dehydrogenase respectively. Further, after maximum accumulation of glyoxylate, the concentration of this compound decreased with time and finally it disappeared completely from reaction mixtures. This destruction of glyoxylate was later shown to be due, partially, to its reduction to glycollate (H. Hassall & R. P. Hullin, unpublished work). Dialysis of the extract against buffer (KH_2PO_4, 2·0 g./l.; pH 7·2), as described above, reduced both the production of α-oxoglutarate and the further metabolism of glyoxylate to negligible proportions.

The activity of isocitratase in extracts from cells grown on butane-2,3-diol was identical with that in extracts of cells grown on acetate. This corresponded to a glyoxylate production of 5·76 μmoles/hr./mg. of protein and was approximately 20 times that found when succinate was the growth substrate (Fig. 2).

Synthesis of malate by cell extracts. The presence of malate synthase was confirmed by demonstrating the appearance of activity in malate when cell extracts were incubated with [1-^{14}C]glyoxylate and various unlabelled additions. Although extracts were first dialysed for a total of 6 hr. as described above in all cases, some [^{14}C]glycollate was produced together with traces of other labelled compounds. Nevertheless, certain additions, namely acetate and precursors of acetate with the necessary cofactors, produced a marked effect on the distribution of ^{14}C between malate and glyoxylate (Table 2).

The necessity of CoA for the synthesis of malate was shown by removing the coenzyme from extracts by treatment with Dowex 2 (Cl^- form) (Chantrenne & Lipmann, 1950); the synthesis of malate from glyoxylate, acetate and ATP only occurred if CoA and Mg^{2+} ions were added to the incubation mixtures.

Preliminary experiments suggested that malate synthase, in the organism used, was not under adaptive control. Cell extracts of the organism grown on succinate also exhibited malate-synthase activity, indicating that the development of this enzyme, unlike that of isocitratase, was not necessarily dependent on a C_2 growth substrate, or on a substrate giving rise solely to C_2 compounds.

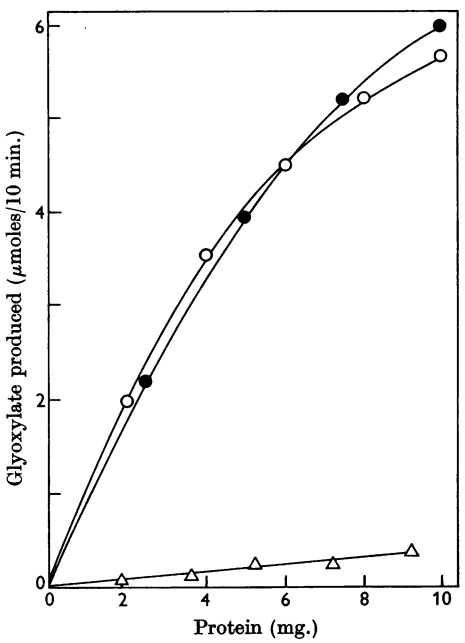

Fig. 2. Isocitratase activities of extracts from cells grown on: ●, butane-2,3-diol; ○, acetate; △, succinate. Each reaction mixture contained 100 μmoles of DL-isocitrate, 5·0 μmoles of $MgSO_4$ and the given amount of extract protein in a total volume of 1·5 ml. of phosphate buffer (KH_2PO_4, 2·0 g./l.; pH 7·2). The mixtures were incubated aerobically at 30° and the reactions terminated after 10 min. by the addition of 1·5 ml. of 10% (w/v) trichloroacetic acid.

Table 2. *Incorporation of* ^{14}C *from* [1-^{14}C]*glyoxylate into malate by cell extract of* Pseudomonas *sp. grown on butane-2,3-diol*

Each incubation mixture contained: 0·4 μmole of sodium [1-^{14}C]glyoxylate (0·05 μc; giving $5·76 \times 10^3$ counts/min. under the conditions of the radio-assay); 20 μmoles of potassium phosphate, pH 7·0; 3·0 mg. of extract protein and additions in a total volume of 1·0 ml.; 0·8 μmole of each addition was used except for NAD (0·2 μmole). The cell extract had previously been dialysed and preincubated for 30 min. with 2·0 units of CoA and 0·1 μmole of $MgSO_4$/mg. of protein. The mixtures were incubated at 30° for 45 min. in an atmosphere of nitrogen and the reactions terminated by the addition of 3·0 ml. of ethanol. The activities of the residual glyoxylate and of the malate were determined as described in the Methods section.

Additions	10^{-3} × Radioactivity (counts/min.)	
	Glyoxylate	Malate
None	5·36	0·22
Acetate	5·34	0·17
ATP	4·98	0·54
Acetate + ATP	0·46	4·18
Diacetyl + ATP	2·24	2·53
2-Hydroxybutan-3-one + ATP	1·82	2·94
Butane-2,3-diol + ATP	2·77	2·09
NAD	5·40	0·20
2-Hydroxybutan-3-one + ATP + NAD	0·26	4·12
Butane-2,3-diol + ATP + NAD	2·40	2·81

DISCUSSION

The results obtained are consistent with the initial dissimilation of butane-2,3-diol to acetate, followed by the synthesis of all cell constituents from this C_2 compound; in the latter respect, organisms grown on butane-2,3-diol behave in a manner identical with those grown on acetate. The implications of microbial growth on acetate as sole carbon source, the necessity of net synthesis of C_4 intermediates of the tricarboxylic acid cycle, and the essential role of the glyoxylate cycle in the metabolism of these organisms, have been discussed fully in recent years (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957; Callely, Dagley & Hodgson, 1958; Collins & Kornberg, 1960; Kornberg, Phizackerley & Sadler, 1960).

We have shown that whole cells of *Pseudomonas* sp. (probably *P. fluorescens*) growing on butane-2,3-diol rapidly incorporate ^{14}C from [1-^{14}C]acetate consistent with the concomitant operation of the tricarboxylic acid and glyoxylate cycles. The distribution of isotope during the first few seconds of incubation period is in agreement with the entry of acetate into the tricarboxylic acid cycle at two sites, namely, via the mediation of the condensing enzyme to give citrate, and of malate synthase to give malate. The low initial activity of succinate and the correspondingly high initial activity of malate indicate that little or no malate is formed via succinate. Thus the direct formation of succinate from acetate (Thunberg, 1920; Glasky, Eicholz & Rafelson, 1958; Glasky & Rafelson, 1957, 1959) plays no obvious part in the synthesis of cell constituents from butane-2,3-diol. The absence of labelled phosphates during the initial incubation periods strongly suggests that carbon dioxide-fixation mechanisms are not operative to any large extent during the metabolism of acetate.

The results obtained using cell extracts are also fully consistent with the operation of the glyoxylate cycle. Isocitratase, an enzyme essential for the functioning of the cycle, and shown by many workers (Campbell, Smith & Eagles, 1953; Smith & Gunsalus, 1955, 1957; Olson, 1954, 1959; Saz, 1954; Saz & Hillary, 1956) to be formed only when there is a need of net synthesis of cell constituents from acetate, was present at high levels of activity during growth on butane-2,3-diol (5·76 μmoles of glyoxylate formed/hr./mg. of protein). Corresponding activities of the enzyme during growth on acetate and succinate were 5·94 and 0·24 respectively.

The presence in cell extracts of malate synthase, also required for the operation of the glyoxylate cycle (Wong & Ajl, 1956), was shown by the incorporation of ^{14}C from [1-^{14}C]glyoxylate into malate. Provided that the necessary cofactors were present, acetate, diacetyl, 2-hydroxybutan-3-one and butane-2,3-diol all increased the amount of ^{14}C incorporated into malate.

The net synthesis of C_4 dicarboxylic acids of the tricarboxylic acid cycle can be accomplished from butane-2,3-diol by the following reactions:

$$\text{Butane-2,3-diol} + 1\tfrac{1}{2}O_2 \rightarrow 2 \text{ acetate} + H_2O$$

$$\text{Acetate} + \text{glyoxylate} + \tfrac{1}{2}O_2 \rightarrow (\text{malate} + \tfrac{1}{2}O_2) \rightarrow \text{oxaloacetate} + H_2O$$

$$\text{Oxaloacetate} + \text{acetate} \rightarrow (\text{citrate}) \rightarrow \text{isocitrate}$$

$$\text{Isocitrate} \rightarrow \text{glyoxylate} + \text{succinate}$$

$$\text{Sum: Butane-2,3-diol} + 2O_2 \rightarrow \text{succinate} + 2H_2O$$

The synthesis of β-hydroxy-[^{14}C]butyrate from [1-^{14}C]acetate is assumed to occur via acetyl-CoA and acetoacetyl-CoA. Both of these are fugitive on chromatograms so that the first labelled product appearing would be β-hydroxybutyrate itself. The relatively high accumulation of this compound is of interest owing to its recent implication in terminal respiration (Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & D'Alessandro, 1961) and its role as a precursor of poly-β-hydroxybutyrate, a storage compound in many micro-organisms (Doudoroff & Stanier, 1959; Macrae & Wilkinson, 1958). The polymer can also serve as a substrate for endogenous respiration in certain aerobes (Macrae & Wilkinson, 1958). A radioactive compound with lipid characteristics, formed from [1-^{14}C]acetate

during the later stages of the incubation, might possibly have been the polymer, although this was not confirmed.

SUMMARY

1. The operation of the cyclic pathway whereby butane-2,3-diol is dissimilated to acetate has been confirmed in *Pseudomonas* sp. Whole cells of the organism growing on butane-2,3-diol rapidly oxidize all members of the cycle. Extracts of these cells catalyse the formation of 3-hydroxy-3-methylpentane-2,4-dione from diacetyl.

2. After brief incubation periods, suspensions of the organism utilizing butane-2,3-diol incorporate ^{14}C from [1-^{14}C]acetate only into intermediates of the tricarboxylic acid cycle, directly associated amino acids and β-hydroxybutyrate. The possible polymerization of this compound to give poly-β-hydroxybutyrate, recognized as a storage compound in some bacteria, is also considered.

3. The distribution of isotope among the initial products is consistent with the entry of acetate into the tricarboxylic acid cycle at two points, namely via the mediation of the condensing enzyme and of malate synthase. Evidence has also been obtained against the direct formation of succinate from acetate; similarly, the absence of phosphates during the early incubation periods suggests that carbon dioxide-fixation mechanisms play very little part in the synthesis of cell constituents from butane-2,3-diol.

4. Extracts of cells grown on butane-2,3-diol exhibit high activities of isocitratase (5·76 μmoles of glyoxylate formed/hr./mg. of protein) equivalent to the activity when acetate is the growth substrate and approximately 20-fold that when succinate is the carbon source. Extracts of the organism grown on butane-2,3-diol rapidly incorporate isotope into malate when incubated with [1-^{14}C]glyoxylate, the necessary cofactors, and one of acetate, diacetyl, 2-hydroxybutan-3-one and butane-2,3-diol.

5. These results indicate that the operation of the glyoxylate cycle is essential for the growth of *Pseudomonas* sp. when butane-2,3-diol is the sole source of carbon for growth.

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