none of the present or earlier results is inconsistent with such a mechanism, which may thus serve as a working hypothesis for future experiments.

SUMMARY

1. The synthesis of 6-amino-2:2-dimethyl-5-oxo-hexahydro-1:4-thiazepine-3-carboxylic acid (cyclic DL-cysteinyl-DL-valine) and its acetyl and phenylacetyl derivatives is described.

2. The monoacetyl and monophenylacetyl derivatives of $\beta\beta$ -dimethyl-lanthionine, *S*-(2-acetamido-2-carboxyethyl)-DL-penicillamine and *S*-(2-phenylacetamido-2-carboxyethyl)-DL-penicillamine respectively have also been prepared.

3. The compounds were tested as penicillin precursors by an isotope-competition method in which washed mycelium of *Penicillium chrysogenum* WIS 51-20 was incubated with L-[carboxy- ^{14}C]valine and the effect of addition of the unlabelled compounds on the specific radioactivity of the penicillin produced was measured.

4. None of the compounds was found to be an intermediate in the conversion of valine into penicillin. Phenylacetyl-L-cysteinyl-D-valine and $\beta\beta$ -dimethyl-lanthionine were also inactive as penicillin precursors.

5. Possible mechanisms of penicillin biosynthesis are discussed.

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The Metabolism of C_2 Compounds in Micro-organisms

1. THE INCORPORATION OF $[2-^{14}\text{C}]\text{ACETATE}$ BY *PSEUDOMONAS FLUORESCENS*, AND BY A *CORYNEBACTERIUM*, GROWN ON AMMONIUM ACETATE

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During the growth of micro-organisms, intermediates of the tricarboxylic acid cycle are utilized to provide the carbon skeletons of synthesized amino acids. When such growth occurs on complex substrates, carbon dioxide-fixing reactions, such as

form oxaloacetate or malate from pyruvate, are thought to replenish the supply of tricarboxylic acid cycle intermediates (Wiame & Bourgeois, 1955). Such reactions are not, however, sufficient to explain the processes by which micro-organisms

can grow on simple substrates, such as ethanol or acetate, as sole carbon sources. The tricarboxylic acid cycle is a means whereby acetate, after reacting with oxaloacetate, is oxidized to carbon dioxide, with the regeneration of oxaloacetate. Any removal of intermediates from the cycle would prevent the regeneration of oxaloacetate, and would therefore cause the cycle to stop. Mechanisms must therefore operate under these conditions whereby the acceptor for C_2 units can be synthesized from acetate.

The present work reports evidence for the operation of such a mechanism in acetate-grown *Pseudomonas fluorescens* KB1 (Kogut & Podoski, 1953) and in a bacterium tentatively identified as *Corynebacterium* sp. The findings are in accordance with the operation in these organisms of the 'glyoxylate by-pass' of the tricarboxylic acid cycle, demonstrated in cell-free extracts (Kornberg & Madsen, 1957*a, b*; Kornberg & Krebs, 1957). This work has been presented in part to the Biochemical Society (Kornberg, 1957*a*).

METHODS

Maintenance and growth of organisms. Cultures of both *Pseudomonas* KB1 (Kogut & Podoski, 1953) and an airborne Gram-positive organism tentatively identified as *Corynebacterium* sp. were maintained on slopes consisting of 25 mm-ammonium acetate, 25 mm-sodium potassium phosphate buffer, pH 7.1, essential salts (2 mg. of $CaCl_2 \cdot 6H_2O$, 0.2 mg. of $MnSO_4 \cdot 4H_2O$, 4 mg. of $MgSO_4 \cdot 7H_2O$ and 0.2 mg. of $FeSO_4 \cdot 6H_2O$ /100 ml. of medium) and 2% (w/v) of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organisms were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of a mixture of 50 mm-ammonium acetate, 50 mm-phosphate buffer, pH 7.1, and essential salts of the same concentration as those used in the solid medium. The flasks were shaken for 10–12 hr. at 30° whilst partially immersed in a Warburg bath. Growth was determined by measurements in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) of the light-scattering of samples of the bacterial suspensions and comparison of the observed reading with a previously constructed calibration curve relating such readings to the dry weight of bacteria/ml. of suspension. Although the medium was adequate to sustain growth of over 1 mg. dry wt./ml., the bacteria were removed whilst still in the phase of logarithmic growth, at densities of 0.2–0.3 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 g.

Manometric experiments. For measurements of the oxidation of acetate and of tricarboxylic acid cycle intermediates, the centrifuged bacteria were suspended in 25 mm-phosphate buffer, pH 7.1, and re-centrifuged. The washed bacteria were resuspended in 25 mm-phosphate buffer, pH 7.1, to a final concentration of 2 mg. dry wt. of bacteria/ml. The rate of O_2 uptake on addition of sub-

strates was measured with Warburg manometers. Flasks (approx. vol., 25 ml.) with single side arms contained 1 ml. of the bacterial suspension, 0.1 ml. of water and 0.5 ml. of 0.2M-phosphate buffer, pH 7.1, in the main compartments, and 20 μ moles of substrates in the side arms. Strips of Whatman no. 40 filter paper and 0.2 ml. of 2N-KOH were placed in the centre wells. The manometers were gassed with air, equilibrated at 30° for 10 min. and the contents of the side arms mixed with those of the main compartments. Readings were taken at 5 min. intervals, and recorded as the total O_2 absorbed in μ l.

Purification of $[2-^{14}C]$ acetate. Sodium $[2-^{14}C]$ acetate, obtained from the Radiochemical Centre, Amersham, Bucks, was found to contain small but significant amounts of propionate and of non-volatile impurities, which increased with time and therefore presumably arose by radiation decomposition. For purification of acetate, the contents of the sealed phials were dissolved in water and chromatographed as strips on Whatman no. 3 paper by the method of Kennedy & Barker (1951). The papers, after chromatography, were heavily sprayed with 0.5M- K_2HPO_4 , air-dried and the acetate area located by radioautography. This area was eluted with 0.05N-NaOH into a 100 ml. Pyrex round-bottomed flask fitted with a B19 standard ground-glass joint. The flask was connected to a large transfer unit (Kornberg, Davies & Wood, 1952) and a receiver attached to the other end. The contents of the flask were frozen in liquid N_2 , the unit was evacuated with an oil pump and the tap closed. The flask contents were now allowed to thaw whilst the receiving flask was cooled in liquid N_2 . Water rapidly distilled over at room temperature into the receiver, leaving a mixture of alkaline acetate and phosphates in the distilling flask. When the volume had been reduced to 0.5–1 ml., the vacuum was released and the receiving flask discarded. The contents of the distilling flask were frozen in liquid N_2 and 0.5 ml. of 5N- H_2SO_4 was pipetted on to the frozen solution. A long thin flask, containing a slight excess of KOH, was connected to the receiving end of the transfer unit, the system evacuated and the tap closed. The receiver was cooled in liquid N_2 and the contents of the distilling flask were allowed to thaw. As the ice melted, labelled acetic acid was liberated and, with accompanying water, rapidly distilled at room temperature into, and condensed in, the cooled receiver. After 20–30 min., distillation was complete and the distilling flask contained only oily H_2SO_4 . At this time, the tap was opened, the receiving flask detached and stoppered. When the contents of this flask thawed, the KOH present neutralized the labelled acetic acid to a pH of approx. 9. The solution was adjusted to pH 7.4 and stored at –12°. Chromatographic analysis showed that acetate was the only volatile acid present, and that the solution, freshly purified or stored at –12° for up to 3–4 weeks, contained no non-volatile impurities.

Bacterial incorporation of $[^{14}C]$ acetate. Bacteria, which had been centrifuged after growth in Carrel culture flasks, were suspended without washing in fresh growth medium containing 25 mm-ammonium acetate, 25 mm-phosphate buffer and essential salts. The concentration of bacteria was adjusted to be 0.1–0.2 mg. dry wt./ml. Of this suspension, 19 ml. was pipetted into the flask of an automatic pipette (Bartley, 1954) and shaken at 30° with partial immersion in a Warburg bath. (Pipettes of this type are available from Exelo Products, W. G. Flaig and Sons, Ltd., 39 Waterloo Road, London, N.W. 2.) Samples (3 ml.) were

removed after 30, 60 and 90 min. and the density of bacteria was measured nephelometrically. When logarithmic growth had been established (mean generation time 1.25 hr.), the flask was removed from the bath, 1 ml. of the [^{14}C]acetate solution (containing $41\mu\text{moles}$ of acetate and giving 1.4×10^7 counts/min. under the conditions of radioassay employed) was added and the top of the pipette replaced. After rapid manual swirling of the contents, to ensure good mixing, samples (1 ml.) were pipetted into thick-walled Pyrex (16 mm. $\times$ 125 mm.) tubes, each containing 3 ml. of absolute ethanol, standing in a bath at 80° . The times of removal of samples were measured with a stop watch, which had been started at the time of addition of the isotopic solution.

Treatment of aqueous ethanolic suspensions. The hot ethanolic (75%) suspensions were left at 50° for 15 min., cooled and centrifuged at 1500 g. The clear supernatant solutions were poured into similar thick-walled Pyrex tubes and the precipitates resuspended each in 1 ml. of 20% (v/v) aqueous ethanol. After re-centrifuging, the supernatant solutions were combined and evaporated to dryness at 60° under a stream of H_2SO_4 -washed N_2 . The precipitates were suspended in 1 ml. of 6N-HCl, the tubes containing these suspensions sealed in an O_2 flame and the tubes left at 110° for 20 hr. After cooling, the tubes were opened and evaporated to dryness at 100° under a stream of H_2SO_4 -washed N_2 .

Chromatographic analysis. The dry material obtained from the ethanol-soluble or ethanol-insoluble portions of the bacterial samples was suspended in 0.5 ml. of 20% (v/v) aqueous ethanol and centrifuged if necessary. Samples (250 $\mu\text{l.}$) of the clear supernatant solutions were applied to the 'origin' positions (3 in. from each side of one corner) of sheets of Whatman no. 4 chromatographic paper (20 in. $\times$ 18 in.). After equilibration the papers were analysed by descending chromatography in phenol-formic acid (500 g. of phenol:13 ml. of 90% HCO_2H :167 ml. of water) in the short direction. The papers were air-dried for 24 hr., equilibrated with the second solvent and chromatographed in the second direction in the butanol-propionic acid-water solvent of Calvin & Benson (1949). Acetate and other volatile compounds were removed by this procedure.

In both directions, the solvents were allowed to flow to within 1 in. from the edge. Good separations were achieved of most of the labelled compounds formed.

Radioautography and radioassay. The air-dried chromatograms were marked at the corners and at the solvent fronts with indian ink, which had been rendered radioactive by addition of waste samples of ^{14}C -labelled fractions, and were placed in lead-backed X-ray holders, 14 in. $\times$ 17 in. (Kodak Ltd.). A sheet of Standard or Blue Brand X-ray film (Kodak Ltd.) was applied to each chromatogram and the closed holders were stored for 3–14 days under a pile of similar holders, efficient contact between films and chromatograms thus being ensured. By superimposing the radioactive-ink markings on the developed films and on the chromatograms, the positions of labelled compounds were accurately located. These labelled areas were outlined in pencil on the chromatograms and their radioactivity was measured by direct radioassay on the papers with a mica end-window β -counter tube (General Electric Co., type 2B2) standing on a Perspex mask into which a square (3.2 cm. $\times$ 3.2 cm.) had been cut. The radioactive areas were thus counted completely without danger of overlapping of spots. Each area was counted for at least 3000 counts total,

giving a standard error of radioassay of less than $\pm 2\%$, and the observed counts were corrected for background radioactivity.

Identification of labelled compounds. The labelled materials, which had been approximately identified in the two solvents used, were eluted, mixed with unlabelled compounds and rechromatographed in the usual and at least two other solvents. Carboxylic acids were thus further analysed by chromatography in butanol-water-diethylamine (Jones, Dowling & Skraba, 1953) and in pentanol-aq. 5M-formic acid (Buch, Montgomery & Porter, 1952). Amino acids were chromatographed in the solvents *B*, *E* and *O* of Hardy, Holland & Nayler (1955). Phosphorylated compounds were analysed by ascending chromatography in ethanol-acetic acid-water (75 ml. of 95% ethanol:2 ml. of acetic acid:23 ml. of water), and by descending chromatography in water-saturated phenol.

Both the position of isotopic material, revealed on film by radioautography and on the chromatograms by the appropriate spray, and the outlines of the markings, had to correspond exactly before identity of the labelled and unlabelled material was taken to be established.

Sprays. Unlabelled carboxylic acids were detected on chromatograms by spraying with 0.04% (w/v) ethanolic bromophenol blue, adjusted to pH 8 with NaOH; amino acids with 0.4% (w/v) ninhydrin in water-saturated butanol, and phosphorylated compounds by the spray and procedure of Hanes & Isherwood (1949).

RESULTS

Oxidation of tricarboxylic acid cycle intermediates

Washed suspensions of acetate-grown *Pseudomonas* KB1 oxidized acetate and all the intermediates of the tricarboxylic acid cycle (Fig. 1). The rates of oxygen uptake were highest with acetate, which had a Q_{O_2} value ($\mu\text{l. of O}_2/\text{mg. dry wt./hr.}$) of 120–140, and the α -oxo acids, α -oxo-glutarate and oxaloacetate. No lag was observed in the oxidation of malate. A lag of approx. 20 min. was noted with succinate and fumarate, and approx. 30 min. with isocitrate. Citrate was oxidized after a lag of 50–60 min. It is probable that these delays, noted also with *P. fluorescens* grown on acetate (Campbell & Stokes, 1951), fumarate (Barrett, Larson & Kallio, 1953) or succinate (Kogut & Podoski, 1953) as sole carbon sources, are associated with the elaboration of mechanisms for the transport of substrates into the cell (Davis, 1956).

Utilization of labelled acetate. When [2- ^{14}C]acetate was added to growing suspensions of *Pseudomonas* KB1, isotope was incorporated into both the ethanol-soluble and the protein fractions (Fig. 2). The incorporation of radioactive carbon into the soluble fraction was linear with time over the duration of the experiment (3.25 min.).

Distribution of ^{14}C amongst the labelled constituents of the soluble fractions. Two-dimensional chromatography and radioautography of the ethanol-soluble fractions obtained from each sample of the

Fig. 1. Oxidation of tricarboxylic acid cycle intermediates by *Pseudomonas* KB1. Manometer flasks contained washed bacteria (2 mg. dry wt.) in 78 mm-potassium phosphate, pH 7.1, in the main compartments, 20 μ moles of substrates (as indicated) in the side bulbs, and 400 μ moles of KOH in the centre well. Incubated in air at 30°; substrates were added at zero time. ●, Acetate; ○, oxaloacetate; △, α -oxoglutarate; ▼, malate; ×, succinate; ▲, fumarate; ▽, isocitrate; ○, citrate; ○, endogenous respiration.

Fig. 2. Incorporation of ^{14}C from [2- ^{14}C]acetate into the protein and ethanol-soluble fractions of *Pseudomonas* KB1 growing on acetate. Conditions were similar to those described in Table 1.

growing suspension showed that ^{14}C from acetate rapidly appeared in many of the intermediates of the tricarboxylic acid cycle and in amino acids, such as aspartate and glutamate, directly derived

therefrom (Table 1). Even after only 3 sec., isotope could be detected in malate, citrate, succinate and aspartate. Glutamate became detectably labelled after this time, and progressively increased in radioactivity until it formed the predominant fraction of the labelled compounds present. Phosphorylated compounds, such as phosphopyruvate and the phosphoglycerates, did not become labelled until 26 sec. after addition of the labelled acetate, by which time a high level of labelling had been reached in other compounds. This showed that carbon dioxide-fixing mechanisms, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955) organisms, were not of primary importance in the incorporation of acetate.

The procedure employed (rapid sampling with an automatic pipette delivering 1 ml. samples with a variation of ± 0.1 ml. between successive samples) yielded results which are quantitatively only approximately accurate. Thus no emphasis can be placed on the absolute values of the incorporated radioactivities; between 18 and 33 sec., for example, only the glutamate area apparently increased in activity, whereas after this time major increases were noted in all compounds (Table 1). This phenomenon is inevitably associated with the incomplete delivery of samples from the automatic pipette under conditions of very rapid operation. These considerations do not, however, render the results obtained useless. Within each sample, the distribution of radioactivity amongst the various labelled compounds fairly reflects the course of incorporation of acetate. When the proportion of the total radioactivity of each sample contributed by each labelled compound, expressed as the percentage of the total ^{14}C in the sample, was plotted against time, smooth curves could be drawn through the points obtained (Fig. 3). Initially, over 40% of the incorporated ^{14}C was present in malate and 30% in citrate. Succinate and fumarate contained a further 10% and aspartate approx. 11%. These proportions rapidly altered with time; both malate and citrate decreased sharply, whereas aspartate, after a rise to over 30%, decreased to a steady level at which it contributed some 18% to the total radioactivity of each sample. After 30 sec., a 'steady state' had been reached. At and after this time, the malate area contained approx. 10% of the total radioactivity, citrate approx. 15%, succinate plus fumarate 5% and glutamate approx. 40%. These steady levels may be taken to reflect also the 'pool sizes' of the various compounds, since, by this time, all these intermediates appear to be saturated with the isotope entering from the administered [^{14}C]acetate.

Since the fully-labelled malate 'pool' in the steady state was lower in radioactivity than the

Table 1. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of Pseudomonas KB1 incubated with potassium [2-¹⁴C]acetate*

A solution of $^{14}\text{CH}_3\cdot\text{CO}_2\text{K}$, containing 41 μmoles of acetate and 200 μC of ^{14}C , was added at zero time to 10 ml. of a suspension of bacteria, growing in an automatic pipette on 25 mM-ammonium acetate. Samples (approx. 1 ml.) were pipetted at known times into 3 ml. of hot absolute ethanol. The precipitates were each washed with 1 ml. of 20% (v/v) aqueous ethanol. Portions of the combined supernatant solutions were evaporated to dryness under N_2 , resuspended in 20% (v/v) aqueous ethanol and analysed by two-dimensional paper chromatography. The radioactive compounds were located by radioautography, and the ^{14}C content was determined by direct radioassay of the radioactive areas with a mica end-window β -counter tube.

Time (sec.)	Radioactivity (counts/min.) in							Total
	Malate	Citrate and isocitrate	Fumarate and succinate	Aspartate	Glutamate	Phosphorylated compounds	Other compounds	
3	21	15	5	6	0	0	3	50
8	42	28	14	39	13	0	4	140
12	49	40	15	68	73	0	7	252
18	133	142	48	209	390	0	18	940
26	133	164	62	195	443	20	34	1056
33	132	154	60	192	492	34	38	1102
48	244	273	116	312	760	78	67	1850
60	216	312	104	375	832	208	133	2180
79	272	296	122	464	964	360	198	2676

Fig. 3. Variation with time of the percentage distribution of ^{14}C from $[2\text{-}^{14}\text{C}]\text{acetate}$ incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas* KB1 growing on acetate. Conditions were similar to those described in Table 1.

citrate 'pool', the initially higher labelling of the malate area shows that, in the earliest stages of the incubation, isotope from acetate entered malate without passing through the stage of citrate. This therefore indicates that acetate entered the tri-carboxylic acid cycle at at least two sites, to form citrate at one, and a compound in ready isotopic equilibrium with, or identical with, malate at another. This conclusion is confirmed when the total radioactivity in the combined citrate plus glutamate fraction, and that in the C_4 compounds, is plotted against time (Fig. 4). The relative 'pool' sizes in the steady state, reached after 30 sec., show that the C_4 compounds contain approx. 35% less isotope than does the citrate plus glutamate 'pool'. Initially, however, this position is reversed:

at the earliest stages, the activity of the C_4 compounds is more than double that of the citrate plus glutamate area. This shows that isotope from acetate, appearing in the C_4 compounds, cannot exclusively have come via the stage of citrate, but must also have entered the tricarboxylic acid cycle by a route not involving a condensation of acetate and oxaloacetate to form citrate. The low initial radioactivity of phosphorylated compounds, shown in Fig. 4, again emphasizes that these substances do not play an essential role in the initial formation of C_4 compounds from acetate.

Experiments with Corynebacterium sp. Results substantially similar to those obtained with *Pseudomonas* KB1 were obtained with a Gram-positive organism, isolated from a vessel containing the 25 mM-ammonium acetate medium, which had been left exposed to the air for some days. (This organism was isolated in pure culture by Mr W. E. Patey, and experiments on it were performed in collaboration with him. It was tentatively identified as *Corynebacterium* sp. by Dr D. E. Hughes.)

This organism grew more slowly than did *Pseudomonas* KB1 (mean generation time 3.5 hr.). The percentage distributions of isotope from $[2\text{-}^{14}\text{C}]\text{acetate}$ (Fig. 5) were therefore inevitably different from those shown in Fig. 3. The citrate 'pool' contained relatively more isotope, and the increase in the label of the glutamate, derived from it, was less striking than was observed with *Pseudomonas* KB1. Similarly, the succinate and fumarate areas were relatively more radioactive. Yet, when the total radioactivities of the citrate plus glutamate, and the C_4 compounds, were plotted against time (Fig. 6), curves strikingly similar to those shown in Fig. 4 were obtained.

Fig. 4. Variation with time of the percentage distribution of ^{14}C from $[2-^{14}\text{C}]$ acetate incorporated into some components of the ethanol-soluble fraction of *Pseudomonas* KB1, growing on acetate. Conditions were similar to those described in Table 1.

Fig. 5. Variation with time of the percentage distribution of ^{14}C from $[2-^{14}\text{C}]$ acetate incorporated into the constituents of the ethanol-soluble fraction of *Corynebacterium* sp. growing on acetate. Conditions were similar to those described in Table 1.

Again the percentage labelling of the citrate plus glutamate area was higher than that of the C_4 compounds after establishment of the 'steady state', but much lower in the initial stages of the incubation. These results therefore show that in this organism, as in *Pseudomonas* KB1, isotope from acetate was initially introduced into C_4 compounds without passing through the stage of citrate.

DISCUSSION

When micro-organisms grow on acetate as sole source of carbon, intermediates are drained from the tricarboxylic acid cycle to provide the carbon skeletons of amino acids, and net syntheses of C_4 dicarboxylic acids must occur to provide the acceptor for C_2 units entering the cycle. These net

Fig. 6. Variation with time of the percentage distribution of the ^{14}C from $[2-^{14}\text{C}]$ acetate incorporated into some components of the ethanol-soluble fraction of *Corynebacterium* sp. growing on acetate. Conditions were similar to those described in Table 1.

syntheses can be explained in a number of ways. An oxidation of acetate to carbon dioxide, and subsequent fixation of carbon dioxide by the mechanism operating in photosynthetic (Calvin, 1955) and autotrophic (Santer & Vishniac, 1955; Trudinger, 1955) organisms, might occur. This is, however, rendered unlikely by the finding (Fig. 4) that phosphorylated compounds do not become labelled until other compounds contain relatively high levels of radioactivity. Similar objections apply to the suggestion that a fixation of carbon dioxide by acetate, to give phosphopyruvate (Abelson, Bolton, Britten, Cowie & Roberts, 1954) could supply the necessary intermediates. Studies of the incorporation of carbon dioxide by *Pseudomonas* KB1 growing on ammonium acetate showed that a compound in ready equilibrium with carbon dioxide does lie on the pathway of acetate (Kornberg, 1956), but that the total quantity of carbon fixed from carbon dioxide is only 15–17% of that incorporated from acetate (Kornberg, 1957b). These findings, and the results of degradative studies on the location within the molecules of synthesized amino acids of carbon derived from added carbon dioxide (Kornberg & Quayle, 1958), rule out carbon dioxide fixation as being of primary importance in the incorporation of acetate.

A direct condensation of two molecules of acetate to give succinate, first postulated by Thunberg (1920), would similarly supply necessary C_4 compounds. There is no evidence for the occurrence of this reaction. Although Seaman & Naschke (1955) have recently claimed a direct formation of succinate from acetyl-coenzyme A to occur in *Tetrahymena* and *Escherichia coli*, the present results show that this reaction plays no

major role in *Pseudomonas* KB1. After brief incubation of *Pseudomonas* KB1 with labelled acetate, only small amounts of ^{14}C were incorporated into succinate. A direct formation of succinate from the condensation of two molecules of acetyl-coenzyme A could not have given the results of Table 1. Similarly, no evidence for the direct formation of succinate from acetyl-coenzyme A was obtained with cell-free extracts (Kornberg & Madsen, 1957*a*, *b*).

The results given in this paper show that acetate enters the tricarboxylic acid cycle at at least two sites, to form citrate at one and a compound identical, or in close equilibrium, with malate at the other. Investigations of cell-free extracts of *Pseudomonas* KB1 (Kornberg & Madsen, 1957*a*, *b*) support this conclusion. Acetyl-coenzyme A has been shown to react both with oxaloacetate, to give citrate, and glyoxylate (derived from the aldol fission of isocitrate) to give malate. The net formation of malate and succinate from acetate and isocitrate (Kornberg & Madsen, 1957*a*, *b*) under the action of isocitritase (Smith & Gunsalus, 1954; Saz & Hillary, 1956) and malate synthetase (Wong & Ajl, 1956) would result in the net synthesis of C_4 dicarboxylic acids. The present results are in agreement with the operation of this mechanism *in vivo*, termed the 'glyoxylate cycle' (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958), which has been demonstrated *in vitro*. Such a mechanism, utilizing the intermediates of the tricarboxylic acid cycle, represents a modification of that cycle leading to a net synthesis of cell constituents from C_2 units and appears to be of importance not only in the growth of micro-organisms on acetate, but also in the metabolism of fungi (Kornberg & Krebs, 1957) and in conversions of fat into carbohydrate (Kornberg & Beevers, 1957; Callely, Dagley & Hodgson, 1957).

SUMMARY

1. Washed cells of *Pseudomonas fluorescens* KB1, grown on ammonium acetate as sole carbon source, rapidly oxidize acetate and all the intermediates of the tricarboxylic acid cycle, confirming that this cycle operates in this organism.

2. Cultures of *Pseudomonas* KB1 and of a Gram-positive organism tentatively identified as *Corynebacterium* sp., growing on ammonium acetate, were incubated for short periods (3 sec.–5 min.) with $[2-^{14}\text{C}]$ acetate. Isotope was incorporated into both the 75% aqueous ethanol-soluble, and into the protein, fractions.

3. The distribution of the incorporated isotope amongst the labelled compounds in the ethanol-soluble fraction showed that intermediates of the

tricarboxylic acid cycle, and amino acids directly derived therefrom, rapidly became labelled.

4. Although the proportion of the total radioactivity present in citrate and glutamate was higher than that present in the C_4 dicarboxylic acids after establishment of isotopic 'steady-state' conditions, the distribution of radioactivity during the earliest stages of the incubation was radically different. Initially, over 70% of the total isotope incorporated was present in C_4 compounds.

5. These results show that, initially, the label was introduced into malate without passing through citrate, and that therefore a second point of entry of acetate into the tricarboxylic acid cycle must exist in these organisms.

6. These findings are in accordance with the operation in intact cells of the 'glyoxylate by-pass' of the tricarboxylic acid cycle, demonstrated in cell extracts. This by-pass serves to replace, from acetate, C_4 dicarboxylic acids drained from the tricarboxylic acid cycle when micro-organisms grow on this single carbon source.

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The Metabolism of C₂ Compounds in Micro-organisms

2. THE EFFECT OF CARBON DIOXIDE ON THE INCORPORATION OF [14C]ACETATE BY ACETATE-GROWN *PSEUDOMONAS* KB1*

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When micro-organisms grow on acetate as sole source of carbon, intermediates are drained from the tricarboxylic acid cycle and are utilized to provide the carbon skeletons of amino acids. Under these circumstances, net syntheses of C₄ dicarboxylic acids must occur from acetate to replace the acceptor for C₂ units entering the cycle. Experiments on acetate-grown *Pseudomonas* KB1 (Kornberg, 1958) showed that acetate enters the cycle at two sites, to form citrate at one and a compound metabolically close to malate at the other. It is known that a reductive condensation of carbon dioxide with pyruvate is one of the routes of biosynthesis of malate (Wood & Werkman, 1938; Ochoa, Mehler & Kornberg, 1948; Davis, Cheldelin, Christensen & Wang, 1956), and it has been postulated that in acetate-grown *Escherichia coli* pyruvate or phosphopyruvate can arise from a condensation of carbon dioxide with acetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954). It is therefore feasible that the necessary net syntheses of C₄ dicarboxylic acids from acetate proceed via consecutive carboxylation reactions. Studies of the effect of unlabelled carbon dioxide on the incorporation of ¹⁴C by *Pseudomonas* KB1 growing on [¹⁴C]acetate were therefore undertaken on the assumption that the postulated reactions would be reflected in the quantity of carbon

from carbon dioxide appearing in proteins and in the labelling patterns of synthesized amino acids.

The results of such experiments rule out the occurrence of condensations of C₂ units with carbon dioxide as being of quantitative significance in the biosynthesis of cell constituents from acetate, and show that 15–17% of the total carbon incorporated into protein from acetate is exchangeable with carbon dioxide. The observed labelling patterns are in good agreement with the occurrence in the growing cells of the 'glyoxylate cycle' (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) demonstrated in bacterial extracts.

METHODS

Growth of organisms. *Pseudomonas fluorescens* KB1 (Kogut & Podoski, 1953) was grown in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland), each containing 400 ml. of a liquid phosphate-acetate-salts medium, as previously described (Kornberg, 1958). The bacterial suspensions were centrifuged at 10° when growth had reached the mid-point of the logarithmic phase, and were resuspended in fresh growth medium at concentrations of 2–3 mg. dry wt./ml. for short-term incubations, and at 0.1–0.2 mg. dry wt./ml. for long-term experiments.

Isotopic materials. [¹⁴C]Acetate, labelled in either the methyl or the carboxyl group, was obtained from the Radiochemical Centre, Amersham, Bucks., and was dissolved in water to contain 200 μC of ¹⁴C/ml. For the experiments with doubly-labelled acetate, equal volumes of each

* Part 1: Kornberg (1958).