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The Metabolism of C₂ Compounds in Micro-organisms

3. SYNTHESIS OF MALATE FROM ACETATE VIA THE GLYOXYLATE CYCLE*

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When micro-organisms, such as *Pseudomonas*, grow on acetate as sole source of carbon, acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and a compound metabolically close to or identical with malate at the other (Kornberg, 1958; Kornberg & Quayle, 1958). The experiments described in this paper were undertaken to elucidate the intermediate steps of these reactions. The results show that cell-free extracts of *Pseudomonas* grown on acetate, which catalyse the formation of acetyl-coenzyme A from acetate, can promote the condensation of acetyl-coenzyme A

both with oxaloacetate to form citrate (Ochoa, Stern & Schneider, 1951) and with glyoxylate to form malate (Wong & Ajl, 1956). Glyoxylate, which serves as substrate for the latter condensation, can be derived from the aldol fission of isocitrate to succinate and glyoxylate (Smith & Gunsalus, 1954; Olson, 1954; Wong & Ajl, 1955; Saz & Hillary, 1956).

In conjunction with the known reactions leading from citrate to isocitrate, and from malate to oxaloacetate, these reactions represent a variant of the tricarboxylic acid cycle, leading to the net synthesis of one molecule of C₄ dicarboxylic acid from two molecules of acetate. The two reactions which involve glyoxylate have been referred to as

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the 'glyoxylate by-pass' (Kornberg & Madsen, 1957b), and the entire cycle has been termed the 'glyoxylate cycle' (Kornberg & Krebs, 1957). This cycle is an essential link in the synthesis of cell constituents by micro-organisms growing on acetate as sole carbon source.

This work has been presented in part to the Biochemical Society (Kornberg & Madsen, 1957a).

METHODS

Growth of organisms. *Pseudomonas fluorescens* KB1 (Kogut & Podoski, 1953) was grown in 4 l. batches of the medium (containing salts, 50 mM-phosphate buffer, pH 7.2, and 50 mM-ammonium acetate) previously described (Kornberg, 1958), at 30° with aeration at the rate of approx. 3 l. of air/min. When the cells had reached a density of 0.5–0.6 mg. dry wt./ml., they were harvested by centrifuging at 1500 g at 0°, washed with 0.9% KCl and stored at –12°.

Escherichia coli, mutant M 22–64 (Gilvarg & Davis, 1956), was maintained on slopes containing 0.5% of glucose, 0.2% of L-glutamate, 0.7% of K_2HPO_4 , 0.8% of KH_2PO_4 , 1% of $(NH_4)_2SO_4$, 0.1% of $MgSO_4$ and 1.5% of agar agar (all concentrations are %, w/v).

For experimental use, cells were grown in 400 ml. of liquid medium similar to that used for agar slopes, but with the agar omitted, in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) at 30° with shaking. The harvest and subsequent treatment of cells were similar to those used for *Pseudomonas* KB1.

Preparation of extracts. Batches of frozen cells were crushed at –25° in a Hughes press (Hughes, 1951). The crushed material was homogenized with 2 vol. of 0.1M-potassium phosphate, pH 7.5, in a glass homogenizer, and the suspension was centrifuged at 25 000 g for 30 min. at 0°. The clear supernatant fraction was used in all experiments except when otherwise specified.

Isotopic solutions used. Solutions of [2- ^{14}C]acetate, prepared as previously described (Kornberg, 1958), contained 200 μ C of ^{14}C and 36 μ moles of acetate/ml., and gave 1.4×10^7 counts/min./ml. under the conditions of radioassay used. A solution of $KH^{14}CO_3$, prepared from $Ba^{14}CO_3$ (Radiochemical Centre, Amersham, Bucks) as previously described (Kornberg, Davies & Wood, 1952), contained 200 μ C of ^{14}C and 250 μ moles of $KHCO_3$ /ml., and gave 8×10^6 counts/min. under the conditions of radioassay used.

Chemicals. Sodium pyruvate, sodium oxaloacetate and barium isocitrate, prepared according to the method of Fittig & Miller (1889) (see Deutsch & Phillips, 1957), were gifts from Professor H. A. Krebs, F.R.S. The isocitrate was converted into the potassium salt by removal of the barium with K_2SO_4 . Samples of sodium glyoxylate were gifts from Dr D. B. Sprinson (Columbia University Medical School, New York) and from Dr I. Zelitch (Connecticut Agricultural Experimental Station, New Haven, Conn., U.S.A.). Reduced glutathione was a gift from The Distillers Co. (Biochemicals) Ltd., Speke, Lincs. Pyridine nucleotides were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Other reagents were of analytical grade.

Chemical and enzyme determinations. Glyoxylate and pyruvate were measured as 2:4-dinitrophenylhydrazones by

the procedure of Friedemann & Haugen (1943). The 2:4-dinitrophenylhydrazones were chromatographically identified by the procedure of El Hawary & Thompson (1953). *iso*Citrate was determined spectrophotometrically by the production of reduced triphosphopyridine nucleotide (TPNH) from triphosphopyridine nucleotide (TPN) catalysed by isocitric dehydrogenase. Malate was measured manometrically with the malic decarboxylase of *Lactobacillus arabinosus* (Nossal, 1951); succinate with succinic oxidase.

Acetate-activating enzyme (acetic-thiokinase) was determined as described by Jones & Lipmann (1955), and isocitrate by a modification of the procedure of Saz & Hillary (1956).

Incubation and subsequent treatment of extracts. For experiments on the incorporation of ^{14}C from [2- ^{14}C]acetate, the incubation mixture used contained 100 μ moles of potassium phosphate, pH 7.5, 10 μ moles of reduced glutathione, 10 μ moles of $MgCl_2$, 0.08 μ mole of coenzyme A (Sigma Chemical Co., St Louis, Mo., U.S.A.), 0.1 ml. of bacterial extract, 1.8 μ moles of [2- ^{14}C]acetate (containing 10 μ C of ^{14}C and giving 6.8×10^6 counts/min.), 5 or 10 μ moles of unlabelled potassium acetate, 10 μ moles of other unlabelled substrates as indicated and water to 0.9 ml. The mixture was incubated at 30° for 2–3 min., after which time were added 10 μ moles of adenosine triphosphate (ATP), tetrasodium salt (Zellstoff-Fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany) or disodium salt (Sigma Chemical Co., St Louis, Mo., U.S.A.). Incubations were continued for various lengths of time, the reactions being stopped by the addition of 3 ml. of hot absolute ethanol. The 75% (v/v) aqueous ethanolic suspensions were centrifuged, the precipitates washed with 1 ml. of 20% (v/v) ethanol and the combined supernatant solutions, evaporated to dryness at 60° under a stream of H_2SO_4 -washed N_2 , were analysed by chromatography and radioautography as previously described (Kornberg, 1958).

The experiments on the anaerobic net synthesis of malate from acetate with unlabelled materials (Table 3, expt. II) and on the formation of malate from pyruvate, TPNH and $KH^{14}CO_3$ (Table 5) were carried out manometrically, Warburg double side-armed flasks, gassed with N_2 , being used.

RESULTS

Reactions of acetate

Suspensions of crushed *Pseudomonas* did not consume oxygen and did not incorporate ^{14}C from labelled acetate when this material was added to them, either alone or in conjunction with members of the tricarboxylic acid cycle. Enzymic assay by the method of Jones & Lipmann (1955) showed, however, that such suspensions were still capable of activating acetate, in the presence of added coenzyme A, glutathione and ATP, to form acetyl-coenzyme A, which was detected in the assay by the formation of hydroxamic acid (cf. Fig. 1). The rate of formation of hydroxamic acid was linear with time, and in the extract illustrated corresponded with the activation of approx. 1.6 μ moles of acetate/hr./extract from 6 mg. dry wt. of crushed bacteria. Higher rates (up to 8 μ moles/hr./

Table 1. Reactions of acetyl-coenzyme A with tricarboxylic acid-cycle intermediates

The complete system contained 100 μ moles of potassium phosphate, pH 7.5, 10 μ moles of glutathione, 0.08 μ mole of coenzyme A, 0.1 ml. of *Pseudomonas* extract (supernatant fraction), 10 μ moles of acetate (containing 16 μ C of 14 C), 10 μ -moles of unlabelled intermediates as indicated, 10 μ moles of ATP and water to 1 ml. Incubation was at 30° for 30 min.

Tube	Compound added	Radioactivity (counts/min.) in			
		Malate	Fumarate	Acetyl-coenzyme A	Citrate or isocitrate
a	None	119	258	1385	10
b	Glyoxylate	15 660	4030	62	6
c	isoCitrate	43 880	1511	91	4 283
d	Succinate	84	170	1420	0
e	α -Oxoglutarate	90	210	1396	25
f	Citrate	2 420	175	1047	616
g	Oxaloacetate	810	677	120	11 806
h	Malate	208	400	980	9 700

Fig. 1. Rates of acetate activation (x) and of malate formation (O) from acetate and glyoxylate. For measurement of acetate activation, the incubation system contained 10 μ moles of potassium acetate, 100 μ moles of potassium phosphate, pH 7.5, 10 μ moles of ATP, 0.08 μ mole of coenzyme A, 10 μ moles of reduced glutathione, 10 μ moles of $MgCl_2$, 50 μ moles of KF, 200 μ moles of freshly neutralized hydroxylamine and water to 0.9 ml. *Pseudomonas* extract, supernatant fraction (0.1 ml., containing extract from 6 mg. dry wt. of crushed bacteria), was added at zero time. The quantities of hydroxamic acid formed were determined by measurement of the optical density at 540 $m\mu$ after addition of 1.5 ml. of the $FeCl_3$ reagent of Jones & Lipmann (1955). The formation of [^{14}C]malate from [$2-^{14}C$]acetate and glyoxylate was measured by radioassay of the labelled malate on paper-partition chromatograms (Kornberg, 1958). The incubation system used was similar to that described above but contained [$2-^{14}C$]acetate (0.05 ml., containing 1.8 μ moles of acetate and giving 6.8×10^6 counts/min.), 5 μ moles of unlabelled acetate and 10 μ moles of sodium glyoxylate. Fluoride and hydroxylamine were omitted.

mg. dry wt. of bacteria) were obtained with subsequent extracts. No differences were observed between suspensions of crushed bacteria and the supernatant fractions obtained therefrom; for convenience, the latter preparations were therefore used.

When [$2-^{14}C$]acetate was added to such supernatant fractions in the presence of added coenzyme A, glutathione and ATP, ^{14}C was incorporated chiefly into acetyl-coenzyme A, with traces in malate, fumarate, and citrate or isocitrate (Table 1, tube a). No succinate or succinyl-coenzyme A was detected, indicating that the reactions described by Seaman & Naschke (1955) in *Tetrahymena* were not present in these preparations. The presence of condensing enzyme (Ochoa *et al.* 1951) was shown by the heavy incorporation of ^{14}C into citrate on addition of oxaloacetate (Table 1, tube g). Considerable amounts of ^{14}C (seven times that observed in the absence of added oxaloacetate) appeared also in malate, accompanied by a trace of labelled succinate. Since it was known that *Pseudomonas* KB1 contained isocitritase (Saz & Hillary, 1956), this finding suggested that malate might be formed in these supernatant fractions via the actions of aconitase, isocitritase and the malate synthetase of Wong & Ajl (1956). That malate synthetase was present in the extracts is shown by the heavy incorporation of ^{14}C from acetate into malate in the presence of glyoxylate (Table 1, tube b). The combined action of isocitritase (1) and malate synthetase (2) is demonstrated by the similar formation of labelled malate from labelled acetate and isocitrate (Table 1, tube c):

The quantity of ^{14}C incorporated into malate was nearly three times higher in the presence of isocitrate than in the presence of glyoxylate. A similar effect has been observed in extracts of castor beans (Kornberg & Beevers, 1957). In such extracts, the addition of glyoxylate from a motor-driven Agla syringe at 2 μ moles/min., more than doubled the amount of ^{14}C incorporated into

malate than when, as in the present instance, the 10 μ moles of glyoxylate were added at the start of the incubation. These findings are consistent with the view that the presence of large amounts of glyoxylate inhibit metabolic reactions (cf. D'Abramo, Romano & Ruffo, 1957). Greater incorporation would be expected from the gradual addition of glyoxylate, as is the case when glyoxylate is produced from the enzymic fission of *isocitrate*.

Amongst the substrates tested, only glyoxylate and its precursor, *isocitrate*, led to a major incorporation of ^{14}C into malate. Succinate and α -oxoglutarate were without effect (Table 1, tubes *d* and *e*). The weak effect of citrate (Table 1, tube *f*) suggested that aconitase was present only in low concentration. This suggestion was confirmed spectrophotometrically (see later) and explains the relatively low incorporation of [^{14}C]acetate into malate on addition of oxaloacetate (Table 1, tube *g*); had aconitase been present in higher activity, more labelled malate would have been expected to be formed from the synthesized labelled citrate, and the amount of labelled succinate formed would also have been greater.

In the presence of unlabelled malate, ^{14}C from acetate was incorporated chiefly into citrate or *isocitrate* (Table 1, tube *h*). This finding could be explained by the formation of oxaloacetate from malate, catalysed by the action of malic dehydrogenase, which, by condensation with labelled acetate, would lead to the formation of labelled citrate. The formation of oxaloacetate from malate in this sequence of reactions would require a concomitant reduction of another substrate. Although the nature of this substrate is not known, it is established that the overall reaction (3)

can take place in biological systems and has been used in the assay of condensing enzyme (Ochoa, 1955).

In all cases, the formation of labelled malate was accompanied by the formation of labelled fumarate, showing the presence of fumarase in the extracts.

Malate synthetase

The experiments reported in Table 1 show that the extracts contain both malate synthetase, discovered in *Escherichia coli* by Wong & Ajl (1956), and the condensing enzyme of Ochoa *et al.* (1951). Both enzymes catalyse the reaction of the active methyl group of acetyl-coenzyme A with the carbonyl group of an α -oxo acid. The question arises whether these interactions are catalysed by one enzyme or by separate enzymes.

This point was tested with extracts of crushed *E. coli*, mutant M 22-64, which was known to be deficient in the condensing enzyme (Gilvarg & Davis, 1956). Such extracts rapidly catalysed the formation of labelled acetyl-coenzyme A, and its interaction with glyoxylate, to form labelled malate (Table 2), showing that malate synthetase was present. Addition of oxaloacetate instead of glyoxylate did not lead to any incorporation of ^{14}C ; in particular, the citrate or *isocitrate* area of the chromatograms was devoid of radioactivity. This confirms the absence of condensing enzyme from the mutant, and shows this enzyme to be different from malate synthetase.

The formation of labelled malate from labelled acetate and glyoxylate, catalysed by the malate synthetase of *Pseudomonas* KB1, proceeded linearly with time (Fig. 1). The extract used in this experiment, derived from 6 mg. dry wt. of bacteria, catalysed the formation of 1.2 μ moles of malate/hr. This is too low to account for the observed growth of *Pseudomonas* KB1 on acetate. However, the activity of acetic-thiokinase (Jones & Lipmann, 1955) in this extract was low; comparison of the rate of formation of labelled malate with that of formation of hydroxamic acid indicates the activation of acetate to be the rate-limiting step in this reaction.

Table 2. Incorporation of radioactive carbon from [$2\text{-}^{14}\text{C}$]acetate, catalysed by extracts of *Escherichia coli* mutant M 22-64

The conditions of incubation were similar to those used in Table 1, but only half the quantity of [$2\text{-}^{14}\text{C}$]acetate was used. Incubation was for 20 min. at 30°.

Tube no.	Substrate added	Total radioactivity (counts/min.) in			
		Acetyl-coenzyme A	Malate	Fumarate	Citrate or <i>isocitrate</i>
1	None	816	112	12	0
2	Oxaloacetate	747	81	9	0
3	Glyoxylate	76	9720	580	0
4	Glyoxylate; no coenzyme A	0	5526	540	0
5	Glyoxylate; no ATP	0	0	0	0
6	<i>iso</i> Citrate	354	700	202	0

Table 3. *Synthesis of malate from acetate*

The flasks used in Expts. I and II contained, respectively, 300 and 300 μ moles of potassium phosphate, pH 7.6, 30 and 10 μ moles of $MgCl_2$, 30 and 5 μ moles of glutathione, 0.6 and 0.16 μ mole of coenzyme A, 30 and 40 μ moles of ATP, and other substrates as indicated. All flasks contained 0.5 ml. of bacterial extract and water to 3 ml. Expt. I was carried out in air, the glyoxylate being added in ten portions of 2.5 μ moles each. Expt. II was performed with Warburg manometers, gassed with N_2 . All flasks were incubated at 30° for 1 hr.

Expt. no.	Flask no.	Reactants (μ moles)					Products (μ moles)			
		Potassium acetate	Sodium glyoxylate	Potassium (+)-isocitrate			Succinate	Malate	Glyoxylate*	Sum of malate + glyoxylate
				Initial	Final	Decrease				
I	1	30	—	—	—	—	—	—	—	—
	2	30	25	—	—	—	—	9.9	—	—
II	3	300	—	15.8	2.7	13.1	11.8	8.2	0.9	9.1
	4	—	—	31.6	16.8	14.8	13.4	1.1	9.5	10.6

* Identified by chromatography of its 2:4-dinitrophenylhydrazone, by the method of El Hawary & Thompson (1953).

Fig. 2. *iso*Citric dehydrogenase and the reversibility of *isocitritase*. Beckman quartz cells, 1 cm. light path, contained 100 μ moles of potassium phosphate, pH 7.6, 10 μ moles of $MgCl_2$, 10 μ moles of glutathione, 0.27 μ -mole of TPN, 0.1 ml. of *Pseudomonas* supernatant fraction and water to 3 ml. Optical density was measured at 340 m μ . Curve A, 5 μ moles of *isocitrate* were added at 2 min.; curve B, 10 μ moles of succinate were added at 2 min. 15 sec., and 20 μ moles of glyoxylate at 3 min. 45 sec.; curve C, no succinate: 20 μ moles of glyoxylate were added at 6 min.

Enzymes acting on *isocitrate*

The bacterial extracts used contained two enzymes capable of acting on *isocitrate*. The cleavage of *isocitrate* (1), catalysed by *isocitritase* (Smith & Gunsalus, 1954; Wong & Ajl, 1955; Saz & Hillary, 1956), was demonstrated both by the

formation of succinate, estimated manometrically with succinic oxidase, and by the simultaneous appearance of equal quantities of an α -oxo compound, the 2:4-dinitrophenylhydrazone of which had the spectrographic and chromatographic characteristics of glyoxylate 2:4-dinitrophenylhydrazone (Table 3).

Besides *isocitritase*, the extracts also contained an active *isocitric dehydrogenase* (Fig. 2, curve A) catalysing the oxidation of *isocitrate* to α -oxoglutarate, with concomitant reduction of TPN:

This reaction was utilized to estimate *isocitrate* and also to demonstrate the reversibility of *isocitritase*. The addition of succinate (Fig. 2, curve B) or glyoxylate alone (Fig. 2, curve C) to the bacterial extract, to which TPN had been added, caused no change in optical density at 340 m μ and hence no reduction of the nucleotide. The addition of glyoxylate to an extract already containing succinate and TPN resulted in an immediate reduction of the nucleotide. This was due to a combination of reaction (1), acting from right to left, and reaction (4), and confirmed the reversibility of *isocitritase* (Smith & Gunsalus, 1954; Saz & Hillary, 1956).

Formation of malate from acetate and *isocitrate*

That the glyoxylate produced from the action of *isocitritase* on *isocitrate* (1) served as substrate for the action of malate synthetase (2) was shown by the incorporation of ^{14}C from acetate into malate (Table 1, tube c).

Since it was thought that the activity of acetylthiokinase in the extracts was the rate-limiting step, the rate of formation of labelled malate was measured after pre-incubation of half the normal quantity of extract with $[2\text{-}^{14}\text{C}]\text{acetate}$, glutathione, ATP and coenzyme A in five times higher concentration than normally used. *iso*Citrate was added

Fig. 3. Formation of [^{14}C]malate from [$2\text{-}^{14}\text{C}$]acetate and isocitrate. The incubation mixture (0.95 ml.) contained 100 μmoles of potassium phosphate, pH 7.6, 10 μmoles of glutathione, 10 μmoles of MgCl_2 , 0.4 μmole of coenzyme A, 10 μmoles of ATP, 10 μmoles of potassium [$2\text{-}^{14}\text{C}$]acetate (giving 1.4×10^6 counts/min.) and 0.05 ml. of *Pseudomonas* supernatant fraction. After pre-incubation for 15 min. at 30° , 15 μmoles of (+)-isocitrate were added at zero time. Samples (0.2 ml.) were removed and analysed at the times indicated.

after 15 min. Over the next 8 min. the incorporation of ^{14}C into malate proceeded at a linear rate (Fig. 3) corresponding to the formation of 2.5 μmoles of malate/hr./extract from 1 mg. dry wt. of bacteria. Rates of 3 $\mu\text{moles/hr./extract}$ from 1 mg. dry wt. of bacteria have been obtained. These rates are sufficiently high to account for synthesis of cell constituents by *Pseudomonas* KB1 growing on ammonium acetate as sole carbon source.

The appearance of ^{14}C from [$2\text{-}^{14}\text{C}$]acetate in malate in the presence of either glyoxylate or isocitrate could conceivably be the result of an isotope-exchange reaction, and might not represent a net synthesis. That this is not the case is shown by the net formation of malate from acetate and isocitrate (Table 3). Incubation of the extract with acetate, ATP, glutathione and coenzyme A (flask 1) did not result in the appearance of malate. However, when glyoxylate was added to this system, 9.9 μmoles of malate were formed (flask 2). When isocitrate replaced glyoxylate in this system, 0.9 μmole of glyoxylate and 8.2 μmoles of malate were formed, accompanied by the appearance of 11.8 μmoles of succinate and the disappearance of 13.1 μmoles of isocitrate (flask 3). This result confirms the net synthesis of stoichiometric amounts of malate and succinate from acetate and isocitrate. In the absence of added acetate (flask 4), the disappearance of 14.8 μmoles of isocitrate was accompanied by the appearance of 13.4 μmoles of succinate and 9.5 μmoles of glyoxylate. The formation of 1.1 μmoles of malate must be ascribed to

Fig. 4. Malic dehydrogenase activity of *Pseudomonas* extract. Beckman cells, 1 cm. light path, contained 60 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.2, 3 μmoles of MnCl_2 , 0.3 μmole of DPNH, and water to 2.9 ml. One cell ($\circ$) received 0.05 ml. of the supernatant solution obtained from boiled *Pseudomonas* extract, the other ($\bullet$) received 0.05 ml. of fresh bacterial extract. Oxaloacetate (5 μmoles) was added at 2 min. Optical density was measured at 340 $\text{m}\mu$.

the presence of residual acetate in the extract prepared from these acetate-grown organisms.

Reactions of malate

The bacterial extracts contained both malic dehydrogenase (Fig. 4), assayed by the oxidation of reduced DPN caused by addition of oxaloacetate, and malic enzyme (Ochoa, Mehler & Kornberg, 1948). This latter enzyme catalyses the reversible conversion of malate into pyruvate and carbon dioxide in the presence of TPN:

The stoichiometry of this reaction was demonstrated by incubation of bacterial extract with malate and TPN. The increase in the optical density at 340 $\text{m}\mu$ observed (Table 4), which did not occur with DPN, corresponded to the reduction of 0.15 μmole of TPN in 10 min. When, at this time, the reaction was stopped by addition of trichloroacetic acid, determination of the α -oxo compounds present by the procedure of Friedemann & Haugen (1943), and chromatographic analysis of their 2:4-dinitrophenylhydrazones, showed the 0.13 μmole of pyruvate formed to be the only α -oxo compound present. The occurrence of the reverse reaction was

Table 4. *Formation of pyruvate from malate by Pseudomonas extract*

Two Beckman quartz cells, 1 cm. light path, each contained 100 μ moles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.6, 3 μ moles of MnCl_2 , 0.1 ml. of *Pseudomonas* supernatant fraction, and water to 2.9 ml. (cell A) or 2.7 ml. (cell B). TPN (1 μ mole in 0.2 ml. of water) was added to cell B. The optical density at 340 $m\mu$. was measured at 30-sec. intervals for 2 min., by which time no further changes were observed. Sodium L-malate (10 μ moles in 0.1 ml. of water) was added to both cells. The reaction was stopped after 5 min. by adding 0.3 ml. of 100% (w/v) trichloroacetic acid. The cell contents were centrifuged and analysed for keto compounds. The spectrum of the 2:4-dinitrophenylhydrazone was that of pyruvate. The optical density at 445 $m\mu$ of 0.1 μ mole of the 2:4-dinitrophenylhydrazone of authentic pyruvate was 0.60.

Cell	Change in optical density at 340 $m\mu$	TPN reduced (μ mole)	$E_{445\text{ }m\mu}$ for 2:4-dinitrophenylhydrazone	Pyruvate formed (μ mole)	Pyruvate formed, corrected for blank (μ mole)
A	+0.008	—	0.037	0.006	—
B	+0.324	0.15	0.82	0.135	0.13

Table 5. *Formation of malate from pyruvate and potassium [^{14}C]bicarbonate in the presence of reduced triphosphopyridine nucleotide*

The main compartments of three Warburg manometer flasks contained 60 μ moles of tris buffer, pH 7.6, 3 μ moles of MnCl_2 and 10 μ moles of unlabelled and 0.05 ml. of labelled KHCO_3 (giving 4×10^6 counts/min.). Flasks 1 and 3 also contained 20 μ moles of sodium pyruvate, and flasks 2 and 3 1 μ mole of TPNH. The side bulbs contained 0.1 ml. of *Pseudomonas* supernatant fraction. The manometers were gassed with N_2 . The contents of the side bulbs and main compartments were mixed and incubated for 30 min. at 30°. After this time, the flasks were detached and the contents pipetted into 9 ml. of ethanol at 70°. The suspensions were centrifuged and the supernatant solutions, evaporated to dryness, were analysed by two-dimensional chromatography and radioautography.

Flask no.	Contents	Radioactivity (counts/min.) in		
		Citrate	Malate	Fumarate
1	No TPNH	90	186	38
2	No pyruvate	48	106	25
3	Complete system	42	2740	520

demonstrated by anaerobic incubation of bacterial extract with TPNH, pyruvate and [^{14}C]bicarbonate (Table 5). Malate and fumarate were the only labelled compounds formed, the amount of incorporation of ^{14}C into these compounds being over 20 times that observed in the absence of pyruvate or in the absence of TPNH. No ^{14}C was incorporated by the complete system containing boiled extract.

DISCUSSION

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 simple substrates, such as acetate or ethanol as the sole sources of carbon, processes must operate to replenish from these substrates the intermediates

drained from the cycle. Studies on *Pseudomonas* KB1 growing on ammonium acetate (Kornberg, 1958; Kornberg & Quayle, 1958) showed that acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and a compound identical with or metabolically close to malate at the other. These findings suggest the occurrence of reactions ancillary to the tricarboxylic acid cycle leading to the net formation of C_4 dicarboxylic acids from acetate. The number and complexity of processes occurring in whole cells make it difficult to investigate individual steps in overall reactions. Cell-free extracts were therefore used, as they neither consume oxygen nor remove malate and citrate (except under special circumstances) and allow the study of reactions leading to the net accumulation of intermediates.

The experiments reported in this paper provide evidence that acetyl-coenzyme A can react both with oxaloacetate, to form citrate, and with glyoxylate, to form malate. The aldol fission of *isocitrate* provides the glyoxylate necessary for this latter condensation. Since the extracts also contain the enzymes capable of catalysing the conversion of citrate into *isocitrate* and of malate into oxaloacetate, it is possible to construct a modification of the tricarboxylic acid cycle (scheme I) leading to the net synthesis of C_4 dicarboxylic acids from acetate (scheme II). Kornberg & Krebs (1957), in their review of this work, proposed that this synthesizing tricarboxylic acid cycle be termed the 'glyoxylate cycle'.

Whereas the net effect of the tricarboxylic acid cycle is the combustion of one molecule of acetate to two molecules of carbon dioxide, the operation of the glyoxylate cycle, which contains only one oxidative step (that leading from malate to oxaloacetate), leads to no such loss of carbon atoms. The succinate formed is envisaged to enter the metabolic reactions of the tricarboxylic acid cycle; the glyoxylate cycle thus serves as a means

of replenishing the supply of intermediates removed in the synthesis of cell constituents. The significance of this cycle in the metabolism of bacteria, fungi and fat-containing seeds has been previously discussed (Kornberg & Madsen, 1957*b*; Kornberg & Krebs, 1957; Kornberg & Beevers, 1957).

In studies of the role of carbon dioxide fixation in growing cells (Kornberg & Quayle, 1958), no evidence was found for the existence of reactions leading to the synthesis of C_3 units from a combination of acetate with carbon dioxide, but isotope-exchange reactions were present relating C_4 compounds with C_3 units and carbon dioxide. These findings are consistent with the demonstration of 'malic enzyme' in the bacterial extracts.

SUMMARY

1. Reactions of acetate were studied in cell-free extracts of acetate-grown *Pseudomonas* KB1. Such extracts are capable of catalysing the formation of acetyl-coenzyme A from acetate.

2. Acetyl-coenzyme A thus formed reacts both with oxaloacetate, to form citrate, and with glyoxylate to form malate.

3. By the use of extracts from mutant M 22-64 of *Escherichia coli*, it has been shown that these condensations are catalysed by different enzymes.

4. The aldol fission of isocitrate, to succinate and glyoxylate, can provide the glyoxylate necessary for the formation of malate.

5. The cell-free extracts catalyse the net anaerobic formation of malate and succinate from acetate and isocitrate.

6. These reactions, in conjunction with the reactions leading from acetate to citrate, from citrate to isocitrate and from malate to oxaloacetate, shown to occur in the bacterial extracts, represent a synthesizing variant of the tricarboxylic acid cycle leading to the formation of one molecule of C_4 dicarboxylic acid from two molecules of acetate.

7. The rate of formation of malate from acetate and isocitrate is at least $3 \mu\text{moles/hr.}/\text{extract}$ from

1 mg. dry wt. of bacteria. Since malate is known to be a precursor of most cell constituents, this rate is sufficiently high to account for the observed rates of growth of *Pseudomonas* KB1 on ammonium acetate.

8. The bacterial extracts also catalyse an inter-conversion of malate with pyruvate and carbon dioxide that is linked with triphosphopyridine nucleotide.

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Effects of Cations on Amino Acid Decarboxylases

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Metzler, Ikawa & Snell (1954) have proposed a general mechanism for reactions catalysed by pyridoxal in which there is a requirement for a multivalent metal ion; a metal chelate of the Schiff base formed from pyridoxal phosphate and the amino acid is postulated as an intermediate. Most of their evidence in support of this theory was obtained from non-enzymic reactions of amino acids catalysed by pyridoxal which required one of the metal ions Cu^{2+} , Fe^{3+} or Al^{3+} for activity. Guirard & Snell (1954) found that the histidine decarboxylase of *Lactobacillus* 30a required pyridoxal phosphate and Fe^{3+} or Al^{3+} ions for activity, other cations being relatively ineffective or inhibitory. A requirement for Fe^{3+} ions was also found by McCullough, Pilgion & Daniel (1957) for an enzyme in *Escherichia coli* O111:B4, which decarboxylated aminobenzoates in the presence of pyridoxal phosphate. Copper ions are known to inhibit many bacterial amino acid decarboxylases (Taylor & Gale, 1945).

It was therefore thought to be of interest to test whether an activation by multivalent cations is a characteristic property of other amino acid decarboxylases. The experiments described in this paper show that many bacterial decarboxylases are activated by bivalent cations.

EXPERIMENTAL

The strains of *Escherichia coli* and *Clostridium welchii*, their growth, collection and washing and the preparation of cell-free extracts were as previously described (Eggleson, 1957). Dialysis of cell-free extracts was carried out against water at 2° for 18–24 hr.

Amino acid decarboxylase activities of bacterial preparations were examined in a Warburg apparatus at 25°. In most experiments the cups contained 0.2 ml. of m-sodium acetate buffer, pH 4.5, 20 µg. of pyridoxal phosphate, 0.6–10 mg. dry wt. of enzyme; metal salt solution (where added) was in the main compartment, and 0.2 ml. of 0.25 M-L-amino acid was in the side bulb. The final volume in each cup was 2 ml. The histidine decarboxylase of *E. coli* F was found to have a higher pH optimum than the other bacterial enzymes; these experiments were therefore carried out at pH 5.5.

Extracts were made of vegetable marrow or orange fruit, by macerating the tissue in a Waring Blender and straining the pulp through muslin, followed by dialysis against ice-water overnight. Experiments with the glutamic acid decarboxylase in citrus fruit (Axelrod, Jang & Lawrence, 1955) and in marrow were carried out in a total volume of 4 ml., at least half of which consisted of enzyme solution. The cups also contained 0.4 ml. of m-acetate buffer, pH 5.5, 20 µg. of pyridoxal phosphate and 0.2 ml. of 0.25 M-L-glutamate.

Brains from rats were ground up in a mortar with sand and 2 vol. of water and then dialysed for about 5 hr. against ice-water. Each Warburg cup contained 3 ml. of enzyme, 0.2 ml. of m-sodium phosphate buffer, pH 6.8, 20 µg. of pyridoxal phosphate and 0.2 ml. of 0.25 M-L-glutamate in a total volume of 4 ml. The gas space was filled with N_2 , a freshly scraped stick of yellow phosphorus placed in the centre well and the incubation carried out at 35°.

The cations used were in the form of solutions of: KCl , Li_2SO_4 , NH_4Cl , AgNO_3 , MgCl_2 , MnCl_2 , CoCl_2 , CaCl_2 , BaCl_2 , NiSO_4 , FeSO_4 , ZnCl_2 , CuSO_4 , SnCl_2 , HgCl_2 , FeCl_3 , AlCl_3 (SnCl_2 and FeCl_3 were dissolved in 0.01 N-HCl; FeSO_4 was dissolved in water immediately before use). Unless otherwise stated, the final concentration of the salt added to the Warburg cups was mM. Each cation effect was examined at least twice with each of the enzymes tested.