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The Metabolism of C_2 Compounds in Micro-organisms

5. BIOSYNTHESIS OF CELL MATERIALS FROM ACETATE IN *ESCHERICHIA COLI**

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The tricarboxylic acid cycle (Krebs & Johnson, 1937) has been shown to be the major route for the oxidation of acetate by *Escherichia coli* (Swim & Krampitz, 1954; Gilvarg & Davis, 1956) and for the provision of the carbon skeletons of cell constituents (Roberts, Abelson, Cowie, Bolton & Britten, 1955; Gilvarg & Davis, 1956). When the organisms grow on acetate as sole carbon source and intermediates are removed from the cycle to supply cell materials, reactions must occur to replenish from acetate the supply of such intermediates. It has been postulated that this may be effected by condensation of acetate with carbon dioxide, to yield a C_3 compound which, by further carboxylation, yields malate or oxaloacetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954; Roberts *et al.* 1955), or by the direct formation of succinate (Thunberg, 1920) from the oxidative condensation of 2 molecules of acetate (Glasky & Rafelson, 1957, 1959; Glasky, Eichholz & Rafelson, 1958). A third possible route is the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The purpose of this paper is to show that isotope from [1-^{14}C]acetate is incorporated into cellular materials by *E. coli* strain *w*, growing on acetate, in a manner consistent with the simultaneous operation of the tricarboxylic acid and glyoxylate cycles, and that extracts of such cells contain the requisite enzymes in high activity. No evidence for the postulated successive carboxylation of acetate or for the direct formation of succinate was obtained. Since this latter finding might have been due to species differences, these experiments were repeated with *E. coli*, Crooks strain, as used by Glasky & Rafelson (1957, 1959) and Glasky *et al.* (1958). The results obtained again exclude the direct formation of succinate from acetate. They support the view that acetate is catabolized via the tricarboxylic acid cycle, and that the glyoxylate cycle operates as a means of supplying intermediates to that cycle. This work has been presented in part to The Biochemical Society (Kornberg, Phizackerley & Sadler, 1959).

* Part 4: Collins & Kornberg (1960).

METHODS

Maintenance and growth of organisms. A culture of *E. coli* strain *w* was the gift of Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). *E. coli*, Crooks strain, was obtained from The National Collection of Industrial Bacteria, Teddington, Middx. (catalogue no. 8545).

The organisms were maintained on agar slopes consisting of 50 mM-potassium phosphate buffer, pH 7·2, 50 mM-ammonium chloride, 50 mM-sodium acetate, essential salts (4 mg. of $CaCl_2,6H_2O$; 8 mg. of $MgSO_4,7H_2O$; 0·4 mg. of $MnSO_4,4H_2O$; 0·4 mg. of $FeSO_4,7H_2O$/100 ml. of medium), solidified with 2% (w/v) of agar agar (Hopkins and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism 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 the above-mentioned medium but with the agar agar omitted. The flasks were shaken at 30° for 16–24 hr. on a reciprocal shaker. Growth of the cells 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 readings 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 while still in the phase of logarithmic growth, at densities of 0·2–0·4 mg. dry wt./ml. The contents of the Carrel flasks were centrifuged at 10° for 15 min. at 1500 *g*.

Bacterial incorporation of [1-^{14}C]*acetate.* (*a*) Experiments with *E. coli*, strain *w*. Bacteria that had been centrifuged after growth in Carrel culture flasks were suspended without washing in fresh 50 mM-acetate growth medium, and the vigorously aerated cell suspension was placed in a water bath at 30°. Growth was followed for about an hour (mean generation time, 130 min.).

In those experiments in which the cells were to be incubated with the isotope for 15 sec. or less, 1·0 ml. samples of a suspension of growing cells, at 3·1 mg. dry wt./ml., were drawn into a hypodermic syringe containing 0·4 ml. of [1-^{14}C]acetate (120 μc/ml.; 20 mM) and 0·6 ml. of air; they were shaken manually and then ejected after a measured time interval into tubes containing 3·0 ml. of absolute ethanol.

In those experiments in which the cells were incubated with isotope for 15 sec. or longer, the procedure of Kornberg (1958) was followed, except that the incubation was terminated by transfer of the samples of bacterial suspension to centrifuge tubes chilled in crushed ice. The samples were quickly centrifuged in the high-speed head of an International refrigerated centrifuge (25 000 *g*) at 0°, the supernatant solutions were discarded and the packed cells were immediately mixed with 3·0 ml. of absolute ethanol. Although this procedure introduces some uncertainty into the measurements of the times of sampling, and is thus unsuitable for very brief incubations, it has the advantage that most of the unassimilated isotope as well as the salts in the suspending medium are removed, and the resulting chromatograms are of better quality.

(*b*) Experiments with *E. coli*, Crooks strain. In order to reproduce as closely as possible the conditions used by Glasky & Rafelson (1959), but with shorter periods of incubation, the technique used was as described above, but the experiments were performed with thicker cell suspensions (about 8 mg. dry wt./ml.) at a lower incubation temperature (20°).

Analysis of samples obtained from incubation experiments. The treatment of the aqueous ethanolic suspension, the techniques of chromatographic analysis, of radioautography and radioassay, and of the methods of identification of labelled compounds, were as previously described (Kornberg, 1958).

Preparation of cell extracts. Extracts of cells were prepared in two ways: (*a*) by disintegration of washed packed cells in the Hughes (1951) press, and (*b*) by ultrasonic oscillation of suspensions of washed cells. Material obtained after crushing of cells was homogenized in 20 mM-potassium phosphate, pH 7·2, to which a single crystal of ribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks) had been added, and the resulting suspension was centrifuged at 25 000 *g* at 0°. The supernatant solution, which contained 5–10 mg. of soluble protein/ml., was used for subsequent experiments.

Extracts of cells disrupted by ultrasonic oscillation were prepared by exposing suspensions of cells (10–30 mg. dry wt./ml.) in either 20 mM-potassium phosphate or 20 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·2, to the output of a 600 w Mullard magnetostrictor oscillator, operating at 3·5 A, 25 kcyc./sec., for 2 min. The extracts were centrifuged at 25 000 *g* at 0° for 10 min. and the supernatant solutions were used.

Reactions of [1-^{14}C]*acetate in cell extracts.* Reactions of [1-^{14}C]acetate in extracts of *E. coli* were studied by the procedures previously described (Kornberg & Madsen, 1958; Collins & Kornberg, 1960).

Assays of L-iso*citrate, glyoxylate and malate.* L-*iso*Citrate was assayed by means of the triphosphopyridine nucleotide (TPN)-linked *iso*citric dehydrogenase obtained from extracts of glucose-grown *E. coli* (which contained negligible *iso*citratase). Glyoxylate was assayed as the phenylhydrazone, by measurement of light-absorption at 324 mμ (Dixon & Kornberg, 1959). Malate was assayed by the fluorescence method of Hummel (1949).

Assays of enzymes. Condensing enzyme (Ochoa, Stern & Schneider, 1951), malate synthetase (Wong & Ajl, 1956) and *iso*citratase (Smith & Gunsalus, 1954, 1957; Olson, 1954, 1959) were assayed by the spectrophotometric procedure of Dixon & Kornberg (1959).

Partial purification of iso*citratase and malate synthetase.* In order better to demonstrate the net formation of malate from acetyl-coenzyme A and *iso*citrate, extracts of *E. coli*, strain *w*, were partially purified to remove interfering enzymes (e.g. aconitase, *iso*citric dehydrogenase, phosphotransacetylase). Packed cells (35 g. wet wt.) were suspended in 3 vol. of 50 mM-potassium phosphate, pH 7·2, and disrupted by sonic oscillation. The resulting 120 ml. of solution was centrifuged at 25 000 *g* in an International refrigerated centrifuge at 0° for 15 min. The supernatant solution was treated with 10 ml. of 2% (w/v) protamine sulphate to remove nucleic acids, and was fractionated with ammonium sulphate. The fractions precipitated between 40 and 55% saturation (rich in *iso*citratase), and between 55 and 70% saturation (rich in malate synthetase), were dissolved in 20 ml. of 20 mM-potassium phosphate, pH 7·2, and dialysed overnight at 3° in 10 l. of the same buffer containing in addition 0·1 g. of cysteine–HCl. The clear solutions after dialysis were used experimentally.

Materials. Potassium dihydrogen L$_s$-*iso*citrate and highly purified sodium glyoxylate were gifts from Dr H. B. Vickery and Dr I. Zelitch respectively (Connecticut Agricultural Experimental Station, New Haven, Conn., U.S.A.). Pyridine nucleotides and coenzyme A were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals used were of A.R. grade. Isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks.; they were purified before use as described by Kornberg (1958).

RESULTS

Utilization of [1-^{14}C]*acetate.* When [1-^{14}C]acetate was added to suspensions of *E. coli* growing on acetate, isotope was rapidly incorporated into cellular materials soluble in aqueous ethanol. The rates of incorporation of ^{14}C were constant over the first minute and corresponded to the uptake respectively of 12·6 μmoles of acetate/mg. dry wt. of cells/hr. for strain *w* at 30° (Fig. 1, *A*) and 7·6 μmoles/mg. dry wt. of cells/hr. for the Crooks strain at 20° (Fig. 1, *B*). These values are minimum ones,

since neither volatile products (such as carbon dioxide) nor chromatographically fugitive materials (such as oxaloacetate or pyruvate) were assayed, but the rates of entry of acetate are of the order required (9 μmoles/hr./mg. dry wt. of cells) to account for the observed rates of growth of *E. coli* on acetate at 30° (mean generation time: 130 min.).

Distribution of ^{14}C *amongst the labelled constituents of the soluble fraction.* Two-dimensional chromatography and radioautography of the fractions soluble in aqueous ethanol, obtained from each sample of the growing suspensions, showed that ^{14}C from acetate rapidly appeared in many intermediates of the tricarboxylic acid cycle and in amino acids directly derived therefrom. Even at the shortest times of incubation (3–5 sec.), isotope was detected in citrate, glutamate, malate and aspartate, with traces also in succinate and fumarate. These last-named compounds were only partially separated by the chromatographic procedures used. Phosphorylated compounds became appreciably radioactive only after longer times of incubation (more than 1 min.), which indicated that carbon dioxide-fixing reactions, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955, 1956) organisms, were not of quantitative importance in the growth of *E. coli* on acetate.

When the proportion of the total radioactivity contributed by each labelled compound, expressed as the percentage of the total ^{14}C in the sample, was

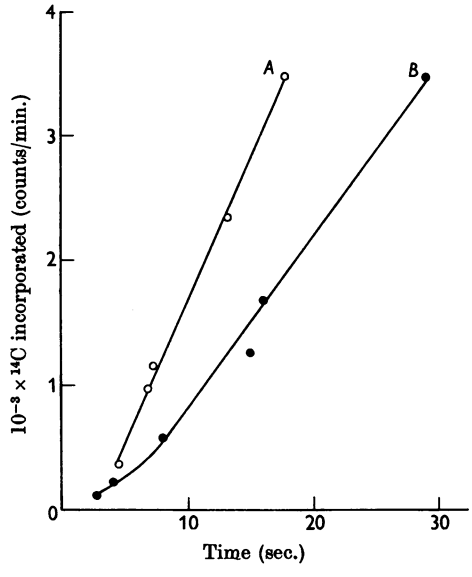

Fig. 1. Incorporation of ^{14}C from [1-^{14}C]acetate into the fractions soluble in aqueous ethanol of *E. coli* strain *w* (○) and Crooks (●) growing on acetate. For conditions, see Methods section.

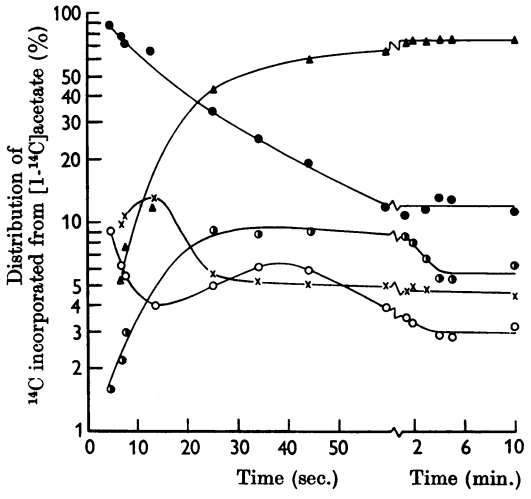

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from [1-^{14}C]acetate into the constituents of fractions soluble in aqueous ethanol of *E. coli*, strain *w*, growing on acetate. ●, Citrate; ○, malate; ▲, glutamate; ×, aspartate; ◑, (succinate + fumarate).

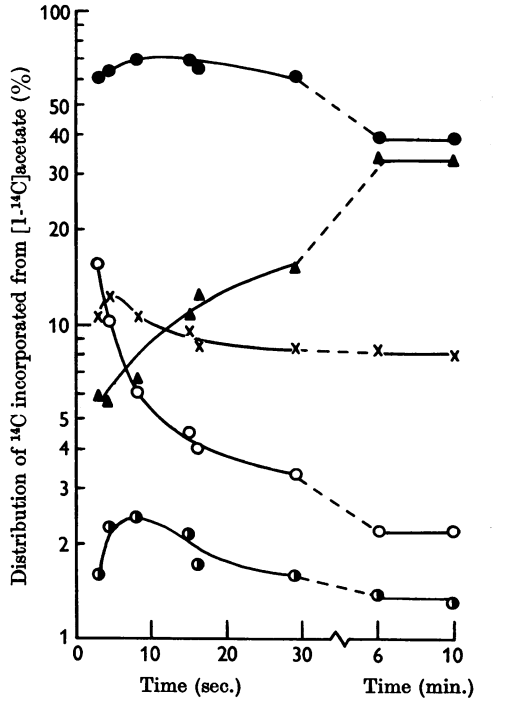

Fig. 3. Variation with time of the percentage distribution of ^{14}C incorporated from [1-^{14}C]acetate into the constituents of fractions soluble in aqueous ethanol of *E. coli*, Crooks strain, growing on acetate. ●, Citrate; ○, malate; ▲, glutamate; ×, aspartate; ◑, (succinate + fumarate).

plotted against time of incubation, smooth curves could be drawn through the points obtained (Figs. 2, 3). The general pattern of distribution of isotope amongst the labelled materials was similar for the two strains of organism tested. After an incubation of approx. 1 min., a 'steady state' of isotopic distribution had been reached, in which the proportion contributed by each labelled compound to the total radioactivity of the samples changed little with time. At and after this time, malate contained 3 % and 2·2 % respectively of the total ^{14}C incorporated into *E. coli*, strain *w*, and the Crooks strain. (Succinate plus fumarate) contained respectively 6 % and 1·5 % and citrate contributed 12 % and 39 % to the total radioactivity. The ratio of the isotope present in the malate to that present in (succinate plus fumarate) also remained constant after the 'steady state' had been reached: ratios of 1:2 were observed with strain *w* and 2:1 with the Crooks strain (Fig. 4).

At the earliest times of incubation, these isotope-distribution patterns were markedly different. Whereas the proportion of label contributed by (succinate + fumarate) rose with time, that of malate fell rapidly in both strains of *E. coli* (Figs. 2, 3). Whereas malate contained approximately half the radioactivity of (succinate + fumarate) after the achievement of the 'steady state' in strain *w*, at the earliest times malate contained approximately six times as much label as did (succinate + fumarate) (Figs. 2 and 4). Similarly, the earliest samples taken from the Crooks strain contained over nine times as much labelled malate as they did labelled (succinate + fumarate) (Figs. 3, 4). In both strains, the greatest proportion of isotope incorporated at the earliest times was found in citrate, as expected from the operation of the tricarboxylic acid cycle.

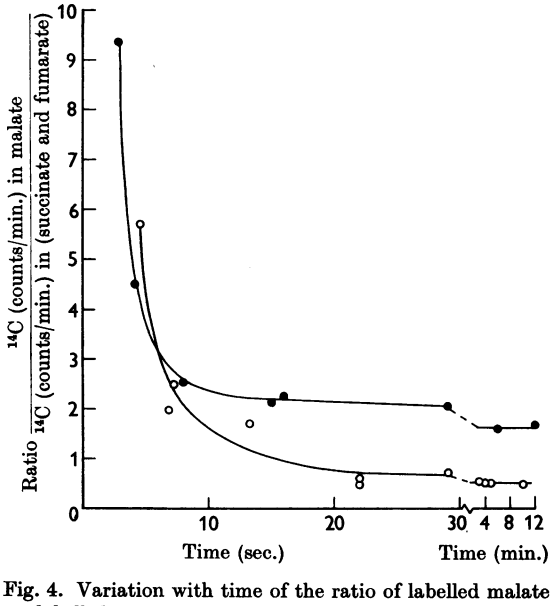

Fig. 4. Variation with time of the ratio of labelled malate to labelled (succinate + fumarate) formed from [1-^{14}C]-acetate by *E. coli* strains *w* (○) and Crooks (●) growing on acetate.

These results show that the direct formation of succinate from acetate, postulated by Glasky & Rafelson (1957, 1959) and Glasky *et al.* (1958), plays no quantitatively important role in the growth on acetate of either *E. coli* strain tested: the initially low and rising radioactivity of succinate precludes its primary formation from labelled acetate. The high proportion of isotope present in malate, and its rapid fall with time, shows also that succinate could not have been the major isotopic precursor of labelled malate but that labelled malate must have been formed from labelled acetate without passing through the stage of succinate.

It was conceivable that the high initial labelling of malate was due to fixation of labelled carbon dioxide (produced from the oxidation of [1-^{14}C]-acetate) by a C_3 compound also derived from acetate (Abelson *et al.* 1954; Roberts *et al.* 1955). This was tested by allowing 10 ml. of a suspension of *E. coli* strain *w* to grow on acetate in the presence of 1 ml. of 40 mM-sodium [^{14}C]bicarbonate (containing 200 μc of ^{14}C). Analysis of the samples as for the similar experiments with [1-^{14}C]acetate showed that the rate of incorporation of isotope was less than 2 % of that observed with a similar suspension of cells growing on [1-^{14}C]acetate with the same mean generation time; moreover, only a small proportion of the isotope incorporated from sodium [^{14}C]bicarbonate appeared in malate. These findings render it unlikely that *E. coli* growing on acetate derives cellular materials from successive carboxylations of acetate to C_3 and C_4 compounds or from the direct formation of succinate from acetate, but offer presumptive evidence for the operation of the glyoxylate cycle in the two strains tested.

Reactions of [1-^{14}C]*acetate in* Escherichia coli *extracts.* Evidence in support of the operation of the glyoxylate cycle was obtained when extracts of acetate-grown *E. coli* strain *w* [prepared in the Hughes (1951) press] were incubated with sodium [1-^{14}C]acetate, adenosine triphosphate, magnesium chloride, glutathione, coenzyme A and added unlabelled substrates. In the absence of such unlabelled substrates, only traces of non-volatile radioactive materials were formed (Table 1). Addition of oxaloacetate resulted in the formation of labelled citrate and a small quantity of labelled succinate. Addition of glyoxylate resulted in the formation of labelled malate and fumarate, which

Table 1. *Incorporation of isotope from* [1-^{14}C]*acetate into extracts of* Escherichia coli *strain* w

Incubation mixtures contained: 120 μmoles of potassium phosphate, pH 7·2; 10 μmoles of $MgCl_2$; 10 μmoles of adenosine triphosphate; 0·08 μmole of coenzyme A; 10 μmoles of glutathione; 4·5 μmoles of sodium [1-^{14}C]-acetate (containing 10 μc of ^{14}C and giving $6·5 \times 10^5$ counts/min. under the conditions of radioassay used); 0·10 ml. of an extract of crushed acetate-grown *E. coli* strain *w* (containing 0·9 mg. dry wt. of crushed cells); additional substrates as indicated and water to 1·0 ml. The mixtures were incubated for 30 min. at 30°. The reaction was terminated by addition of 3 ml. of ethanol and the labelled materials were analysed as previously described (Kornberg, 1958).

Additional substrates	10^{-3} × Radioactivity (counts/min.)			
	Citrate	Malate	Succinate	Fumarate
None	0·98	0·25	0·00	0·18
Oxaloacetate (10 μmoles)	13·4	0·18	0·76	0·35
Glyoxylate (10 μmoles)	0·51	12·0	0·20	3·13
*iso*Citrate (10 μmoles)	2·5	15·3	0·43	1·36
Diphosphopyridine nucleotide (0·4 mg.)	0·34	0·59	0·18	0·04

was also achieved when *iso*citrate was used instead of glyoxylate. These findings can be explained by the action of the key enzymes of the glyoxylate cycle. The citrate-forming condensing enzyme (Ochoa *et al.* 1951) catalyses the reaction (1)

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \text{oxaloacetate} + \text{water} \rightarrow [1\text{-}^{14}C]\text{citrate} + \text{coenzyme A} \quad (1)$$

If the extract used also contained aconitase, part of the labelled citrate would isomerize to *iso*-citrate; subsequent cleavage of this material by *iso*citratase (2) (Smith & Gunsalus, 1954, 1957; Saz & Hillary, 1956; Olson, 1954, 1959) would explain the formation of the small amount of labelled succinate observed:

$$\textit{iso}\text{Citrate} \rightleftharpoons \text{succinate} + \text{glyoxylate} \quad (2)$$

The formation of labelled malate in the presence of unlabelled glyoxylate is due to the action of malate synthetase (3), discovered in extracts of *E. coli* by Wong & Ajl (1956):

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \text{glyoxylate} + \text{water} \rightarrow [^{14}C]\text{malate} + \text{coenzyme A} \quad (3)$$

Enzymic removal of water, catalysed by fumarase, from the [^{14}C]malate thus formed would account for the observed formation of [^{14}C]fumarate from [^{14}C]acetate. The combined action of *iso*citratase (2) and malate synthetase (3) accounts for the observed formation of labelled malate when *iso*-citrate was added as unlabelled substrate (4).

$$[1\text{-}^{14}C]\text{Acetyl-coenzyme A} + \textit{iso}\text{citrate} + \text{water} \rightarrow [^{14}C]\text{malate} + \text{succinate} + \text{coenzyme A} \quad (4)$$

The addition of diphosphopyridine nucleotide did not stimulate the incorporation of isotope from [1-^{14}C]acetate: hence the extract did not catalyse the oxidative condensation of acetate to succinate (Seaman & Naschke, 1955).

Enzymes of the glyoxylate cycle in extracts of Escherichia coli. The presence of the enzymes which catalyse reactions (1)–(3) in extracts of both *E. coli* strain *w* and the Crooks strain was demonstrated with the spectrophotometric assays of Dixon & Kornberg (1959). In these, *iso*citratase activity was measured as the rate of increase of extinction at 324 mμ concomitant with the formation of glyoxylate phenylhydrazone from phenylhydrazine and glyoxylate produced by the aldol cleavage of *iso*citrate; the activities of malate synthetase and condensing enzyme were determined as the rate of decrease of extinction at 232 mμ consequent upon the cleavage of the acyl thio-ester bond of acetyl-coenzyme A in the presence of either glyoxylate or oxaloacetate. It was found (Table 2) that extracts of both strains of acetate-grown *E. coli* were rich in the three enzymes assayed. The specific activities (μmoles of substrate transformed/hr./mg. of soluble protein) of condensing enzyme averaged 23 for strain *w* and 14 for the Crooks strain; of malate synthetase 7 and 5·5 respectively; and of *iso*citratase 5·3 and 6·3 respectively. The activities of these enzymes, acting in conjunction with the other enzymes of the tricarboxylic acid cycle, are therefore sufficient to catalyse the net formation from acetate of at least 6 μmoles of C_4 compounds/hr./mg. of soluble protein. This value is a minimum one, as neither *iso*-citratase nor malate synthetase is at its optimum temperature or pH under the conditions of assay (Smith & Gunsalus, 1957; Dixon, Kornberg & Lund, 1960), but the value is of the order required to account for the observed rates of formation of cell material during growth on acetate.

Net formation of malate from acetate by extracts of acetate-grown Escherichia coli. That extracts of acetate-grown *E. coli* can catalyse the net formation of malate from acetyl-coenzyme A and *iso*citrate, via reaction (4), was demonstrated with partially purified *iso*citratase and malate synthetase obtained from *E. coli* strain *w* (Table 3). The quanti-

Table 2. *Average specific activities of key enzymes of the glyoxylate cycle in extracts of* Escherichia coli *strains* w *and Crooks*

Extracts were prepared by disintegration of washed cells, grown on the substrates indicated, for 2 min. in a 600 w Mullard magnetostrictor oscillator operating at 3·5 A, 25 kcyc./sec. The suspensions obtained were centrifuged at 0°, 25 000 *g* for 10 min.; enzymic assays were performed on the clear supernatant solutions by the procedures of Dixon & Kornberg (1959). Specific activities are expressed as μmoles of substrate transformed/mg. of soluble protein/hr.

Carbon source for growth	*E. coli* strain used	Average specific activity		
		*iso*Citratase	Malate synthetase	Condensing enzyme
Acetate	*w*	5·3	7·0	23
Acetate	Crooks	6·3	5·5	14
Succinate	*w*	0·47	0·55	1·4
Succinate	Crooks	0·70	—	—
Malate	*w*	0·64	0·39	1·3
Lactate	*w*	0·22	0·40	7·0

Table 3. *Formation of malate from acetyl-coenzyme A and* isocitrate

The complete system contained, in a silica cell (vol. 0·45 ml.), 70 μmoles of tris buffer, pH 7·2; 1 μmole of $MgCl_2$; 0·05 μmole of acetyl-coenzyme A (purified by electrophoresis and paper chromatography: Dixon, Kornberg & Lund, 1960); 0·02 μmole of cysteine–HCl; 1 μmole of L-*iso*citrate; 0·02 ml. of partially purified *E. coli* extract (containing 20 units of *iso*citratase and 1 unit of malate synthetase) and water to 0·41 ml. A unit of enzyme is that quantity catalysing the transformation of 1 μmole of substrate/hr. under the conditions of assay described by Dixon & Kornberg (1959). The amounts of acetyl-coenzyme A utilized were calculated from the observed decrease in extinction at 232 mμ (Stadtman, 1957); the amounts of malate formed were determined by the fluorimetric procedure of Hummel (1949).

Expt. no.	Conditions	Time of incubation (min.)	Acetyl-coenzyme A utilized (μm-moles)	Malate formed (μm-moles)
1	*iso*Citrate omitted	10	0	0
1	Complete system	10	22	22
2	*iso*Citrate omitted	24	0	0
2	Enzyme omitted	24	0	0
2	Acetyl-coenzyme A omitted	24	—	0
2	Complete system	24	38	37

ties of acetyl-coenzyme A utilized by the extract incubated with excess of *iso*citrate were calculated from the measured decrease in extinction at 232 mμ (Stadtman, 1957); the amounts of malate formed were determined fluorimetrically (Hummel, 1949). It was found that for each μmole of acetyl-coenzyme A split an equivalent amount of malate was formed.

Influence of growth substrates on enzymes of the glyoxylate cycle. Extracts prepared by ultrasonic disintegration of both strains of *E. coli* grown on carbon sources other than acetate contained only low activities of malate synthetase and *iso*citratase (Table 2), the specific activities of these enzymes being less than 10% of those observed in extracts of the organisms which had been grown on acetate. The specific activity of condensing enzyme was also markedly decreased in proportion. These results, which differ from those reported for *Pseudomonas* (Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959), *Micrococcus denitrificans* (Kornberg, Collins & Bigley, 1960) and *Aspergillus niger* (Collins & Kornberg, 1960), indicate that all the key enzymes of the glyoxylate cycle are under adaptive control in *E. coli* (cf. Wong & Ajl, 1957). The high activities of these enzymes in extracts of *E. coli* grown on acetate therefore provide further evidence for their involvement in the growth of the organisms on acetate.

DISCUSSION

The growth of micro-organisms on acetate as sole carbon source necessitates the occurrence of reactions, ancillary to the tricarboxylic acid cycle, which effect the net formation of intermediates of that cycle from acetate. Three possible routes whereby this might be achieved have been postulated for *E. coli*: (*a*) a direct formation of succinate from the oxidative condensation of 2 molecules of acetate; (*b*) successive carboxylations of acetate, to yield first a C_3 and then a C_4 compound; (*c*) the glyoxylate cycle.

'*Thunberg condensation*'. The formation of succinate by direct oxidative condensation of acetate was first postulated by Thunberg (1920), and is often referred to as the 'Thunberg condensa-

tion'. Although this reaction has been assumed to play a role in the metabolism of many micro-organisms (for review, see Ajl, 1951, 1958; Foster, 1949, 1958; Kornberg, 1959), it has not been possible to demonstrate convincingly its occurrence in cell-free extracts. There are several lines of evidence against its occurrence in *E. coli*.

Gilvarg & Davis (1956) isolated a mutant (M 22–64) from *E. coli* strain *w* which lacked the citrate-forming condensing enzyme. This mutant was unable to oxidize acetate, although it readily oxidized succinate: no direct formation of succinate from acetate could therefore occur in this organism.

Swim & Krampitz (1954) incubated suspensions of *E. coli* anaerobically in the presence of fumarate and [2-^{13}C]acetate. Under these conditions succinate accumulates by the overall reaction (5):

$$\text{Acetate} + 4\ \text{fumarate} + 2\ \text{water} \rightarrow 4\ \text{succinate} + 2\ \text{carbon dioxide}, \quad (5)$$

as first established by Krebs (1937). The evolved carbon dioxide was not enriched with ^{13}C, and ethylene, obtained from the decarboxylation of the succinate formed, was found to be a mixture of molecular species with masses 28 and 29. Had any succinate been formed via the 'Thunberg condensation' this succinate would have been labelled in both methylene groups and on chemical decarboxylation would have produced ethylene of configuration $^{13}CH_2$:$^{13}CH_2$ and mass 30. No such ethylene was detected, and hence the succinate arose from acetate via the reactions of the tricarboxylic acid cycle and not via the 'Thunberg condensation'. A similar explanation applies to the earlier work of Kalnitsky, Wood & Werkman (1943).

The results presented in the present paper confirm these findings. Both *E. coli* strain *w* and the Crooks strain incorporated isotope from [1-^{14}C]-acetate initially into citrate and malate. The primary formation of labelled citrate is expected from the operation of the tricarboxylic acid cycle. The heavy labelling of malate at times when succinate was only slightly radioactive (Fig. 4) indicates that succinate was not an isotopic precursor of malate at these times, and also excludes the 'Thunberg condensation' as being of quantitative importance in these strains of *E. coli*. It is possible that the results obtained by Glasky & Rafelson (1959), which are also not consistent with the operation of the tricarboxylic acid cycle, were vitiated by technical difficulties.

Carboxylation of acetate. The formation by *E. coli* of pyruvate or phosphopyruvate by carboxylation of acetate was postulated by Abelson *et al.* (1954) and Roberts *et al.* (1955). Although it is known that extracts of *E. coli* (Strecker & Ochoa, 1954) can catalyse the exchange of the carboxyl group of pyruvate with carbon dioxide, no evidence exists that such extracts catalyse the net formation of pyruvate from acetate and carbon dioxide. The results obtained by Bagatell, Wright & Sable (1958), in which isotope from [1-^{14}C]acetate was found to be incorporated significantly only into carbon atoms 3 and 4 of glucose synthesized by *E. coli* from acetate, further exclude such a carboxylation as being of quantitative importance. This is confirmed by the present work: isotope from added sodium [^{14}C]bicarbonate was incorporated into *E. coli* growing on acetate at less than 2% of the rate at which isotope from [1-^{14}C]acetate was incorporated.

Glyoxylate cycle. The overall reaction of the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) is identical with that of the 'Thunberg condensation', but the mechanism is entirely different: acetate enters this cycle at two sites, to form citrate at one and malate at the other. The results presented in this paper are consistent with the operation of this scheme in both strains of *E. coli* studied. In particular, isotope from acetate was incorporated initially into citrate and malate, and the ratio of labelled malate to labelled succinate formed at early times precluded the formation of malate from labelled citrate via succinate. Cell-free extracts of the acetate-grown bacteria, supplemented with necessary cofactors, readily catalysed the formation of labelled citrate from labelled acetate and oxaloacetate, and of labelled malate from labelled acetate and either glyoxylate or *iso*citrate. Moreover, the cell-free extract catalysed the net formation of malate from acetyl-coenzyme A and *iso*citrate. Direct enzymic assay showed that the key enzymes of the glyoxylate cycle were present in such extracts in amounts adequate to account for the observed rates of growth of the organisms on acetate, and that growth on substrates other than acetate was accompanied by a marked decrease in the quantities of these enzymes formed. These findings are consistent with the view that the biosynthesis of cell materials from acetate by *E. coli* involves the necessary operation of both the tricarboxylic acid and glyoxylate cycles.

SUMMARY

1. In brief incubation periods, suspensions of two strains of *Escherichia coli* (*w* and Crooks) growing on acetate as the sole carbon source incorporated isotope from [1-^{14}C]acetate only into intermediates of the tricarboxylic acid cycle and into amino acids derived therefrom.

2. The distribution of isotope amongst the labelled products was not consistent with the

operation of the tricarboxylic acid cycle as the sole route of acetate metabolism, but showed that acetate entered the cycle at two sites, to form citrate at one and malate at the other. Succinate was not labelled at the earliest times. These results indicate that the glyoxylate cycle also operated in these cells, and exclude the primary formation of succinate by oxidative condensation of acetate.

3. Isotope from sodium [^{14}C]bicarbonate was incorporated by *E. coli* growing on acetate into cellular materials at less than 2% of the rate at which [1-^{14}C]acetate was utilized.

4. Cell-free extracts of acetate-grown cells, fortified with necessary cofactors, catalysed the incorporation of isotope from [1-^{14}C]acetate into citrate in the presence of oxaloacetate, and into malate in the presence of either glyoxylate or *iso*-citrate. They also catalysed the net formation of malate from acetyl-coenzyme A and *iso*citrate.

5. Direct enzymic assay showed that such extracts contained high activities of key enzymes of the glyoxylate cycle. The specific activities (μmoles of substrate transformed/mg. of soluble protein/hr.) of condensing enzyme averaged 23 for strain *w* and 14 for the Crooks strain, of malate synthetase 7·0 and 5·5, and of *iso*citratase 5·3 and 6·3 respectively. The activities of these enzymes, acting in conjunction with other enzymes of the tricarboxylic acid cycle, are adequate to account for the observed rates of growth of the organisms on acetate.

6. Cell-free extracts of *E. coli* grown on carbon sources other than acetate contained malate synthetase and *iso*citratase at less than one-tenth of the specific activities observed with acetate-grown cells. This supports the essential role of the glyoxylate cycle in the growth of *E. coli* on acetate.

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