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The Metabolism of C_2-Compounds in Micro-organisms

8. A DICARBOXYLIC ACID CYCLE AS A ROUTE FOR THE OXIDATION OF GLYCOLLATE BY *ESCHERICHIA COLI**

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(*Received* 25 *May* 1961)

Although the tricarboxylic acid cycle has been established as the major route for the oxidation of acetate, and of compounds catabolized to acetate, in a wide variety of organisms (for review, see Krebs & Lowenstein, 1960) little is known of the pathways of oxidation of C_2 compounds more highly oxidized than acetate, such as glycollate or glyoxylate. The main purpose of this paper is to show that, whereas the tricarboxylic acid cycle plays an essential role in the growth of *Escherichia coli* on glycollate as sole carbon source, this cycle is not necessary for glycollate oxidation. The results obtained with the mutant M22-64 of *E. coli*, strain *w*, which is devoid of the citrate-forming condensing enzyme (Gilvarg & Davis, 1957), and with its parent wild type, support the view that glyoxylate, derived from the oxidation of glycollate, can be oxidized completely via a dicarboxylic acid cycle in which glyoxylate condenses with acetyl-coenzyme A to form malate, which is oxidized via oxaloacetate and pyruvate to regenerate the acetyl-coenzyme A required for the initial condensation. An outline of this work has been published (Kornberg & Sadler, 1960).

MATERIALS AND METHODS

Maintenance and growth of micro-organisms. The micro-organisms used in this work were *E. coli*, strain *w*, and the mutant M22-64 isolated and characterized by Gilvarg & Davis (1957). The mutant differed from its parent wild strain in being unable to catalyse the condensation of acetyl-coenzyme A and oxaloacetate to form citrate, and hence required the addition of glutamate for growth. The original cultures of the mutant and of the parent wild type were gifts from Professor B. D. Davis (Harvard Medical School, Boston, Mass., U.S.A.).

Cultures of the mutant and of the parent wild type were grown and maintained on agar slopes, containing 50 mM-potassium phosphate buffer, pH 7·2, 50 mM-NH_4Cl, 50 mM-sodium glycollate, 2 mM-sodium glutamate and essential salts (4 mg. of $CaCl_2,6H_2O$; 0·2 mg. of $MnCl_2,4H_2O$; 4 mg. of $MgSO_4,7H_2O$ and 0·2 mg. of $FeSO_4,6H_2O$ per 100 ml. of medium) solidified with 1·5% (w/v) of agar agar (Hopkin 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 400 ml. of the above medium with the agar omitted (hereafter referred to as 'glycollate–glutamate' medium) in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland). The flasks were shaken for 30–35 hr. on a reciprocal shaker at 30°. Growth of the cells was determined by measurements in an EEL Unigalvo Type 20 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 shown to be adequate to sustain the growth of more than 1 mg. dry wt. of bacteria/ml., the organisms were harvested while in the phase of logarithmic growth, at densities of 0·2–0·5 mg. dry wt./ml. The contents of the Carrel culture flasks were centrifuged at 10° for 15 min. at 1500*g*.

Oxidation studies. The packed cells were suspended in 100–150 ml. of potassium phosphate, pH 7·2, centrifuged at 1500*g* for 10 min., and resuspended in a sufficient volume of buffer to give a cell density of 5 mg. dry wt./ml. Samples (2 ml.) of this washed cell suspension were pipetted into the main compartment of Warburg manometer cups.

* Part 7: Gotto & Kornberg (1961).

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The centre wells contained 0·2 ml. of 2N-KOH and a strip of Whatman no. 40 paper. Substrates and inhibitors were placed in the main compartments or in the side arms as required (see Tables). In those experiments in which evolved labelled CO_2 was quantitatively collected for radioassay, double-armed cups were used: in these cases, the second side arm contained 0·3 ml. of aqueous 50% (w/v) trichloroacetic acid, and the paper strips were omitted from the centre wells.

All manometric experiments were performed at 30°, with air as the gas phase.

Bacterial incorporation of [^{14}C]*glycollate and* [^{14}C]*acetate.* Batches of the wild type or mutant cells which had been grown on the glycollate–glutamate medium were centrifuged and resuspended without washing in a sufficient quantity of buffer and salts similar to those supplied in the growth medium, but lacking the carbon substrates, to give a cell density of approximately 5 mg. dry wt./ml. Portions (20–30 ml.) of this suspension were placed, in a constant temperature bath at 30°, in a wide tube and aerated vigorously by forcing a stream of air through a sintered disk fused into its lower end. After 5 min., sufficient quantities of 1M-sodium glycollate and 1M-sodium acetate were added to give final concentrations of 20 and 10mM respectively (the acetate being added only in those experiments where the labelled substrate was to be acetate). After a further 5 min., labelled substrate was added and samples were taken at measured times.

The sampling procedure consisted in drawing 1·0 ml. portions of the oxidizing bacterial suspension into 5 ml. hypodermic syringes containing air and 0·2 ml. of [1-^{14}C]-glycollate (50 μc/ml.; 26 mM), or of [2-^{14}C]glycollate (50 μc/ml.; 26 mM), or of [2-^{14}C]acetate (100 μc/ml.; 17 mM). The syringes containing the samples were shaken manually and, after a measured time interval, their contents were ejected into tubes containing 3 ml. of absolute ethanol. For incubations of over 30 sec. duration, the needles of the syringes were plunged into corks, and the syringes were shaken while immersed in the constant-temperature bath at 30°.

Analysis of samples obtained from incubation experiments. The aqueous ethanolic suspensions were analysed by two-dimensional chromatography and radioautography. The methods used, and the procedures for identification of labelled compounds, and for radioassay, were as described by Kornberg (1958).

Assay of $^{14}CO_2$. Labelled CO_2 absorbed in the centre-wells of Warburg vessels was converted into $Ba^{14}CO_3$ by precipitation with $BaCl_2$, and its radioactivity was assayed, as described by Kornberg & Gotto (1961).

Adaptation studies with Escherichia coli, *strain* w. Suspensions of the wild-type organism, grown on either the glycollate–glutamate medium or the standard acetate medium used in previous work (Kornberg, Phizackerley & Sadler, 1960) were centrifuged at 1500*g* for 15 min. The packed cells were suspended to a final cell concentration of 0·2 mg. dry wt./ml. in two Carrel flasks immersed in a constant-temperature bath at 30°, one containing 200 ml. of the glycollate–glutamate medium and the other 200 ml. of the acetate medium. Growth of the cultures was followed nephelometrically as described above, and sufficient substrate was provided to allow for approximately three doublings in cell density. Samples (20 ml.) were removed at zero time and after every hour. These were centrifuged at 18000*g* for 3 min. at 3°, resuspended in 5·5 ml. of ice-cold 0·9% (w/v) KCl and immediately frozen at −15°. The samples taken were later thawed and used for the preparation of extracts.

Preparation of cell extracts. Extracts of cells were prepared by exposing suspensions of cells (1–20 mg. dry wt./ml.) in 0·9% KCl to the output of a 600 w Mullard magnetostrictor oscillator, operating at 3·5A, 25 kcyc./sec., for 3 min. The extracts were centrifuged at 18000*g* at 0° for 15 min. and the supernatant solutions were used. The protein content of such solutions was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of enzymes in cell-free extracts. Glycollic acid oxidase was measured as the rate of increase in extinction at 550 mμ concomitant with the reduction of cytochrome *c* in the presence of glycollate, enzyme and catalytic quantities of phenazine methosulphate (Gotto, 1961). The citrate-forming 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 procedures of Dixon & Kornberg (1959). Malic dehydrogenase was estimated as described by Mehler, Kornberg, Grisolia & Ochoa (1948) and malic enzyme as described by Ochoa, Mehler & Kornberg (1948). Fumarase was measured by the method of Racker (1950). The pyruvic-oxidase system was assayed by the spectrophotometric procedure of Korkes (1955). The acyl thiol ester formed in this reaction was identified as acetyl-coenzyme A by its conversion into acetylhydroxamic acid, which was detected by chromatography (Stadtman & Barker, 1950). All spectrophotometric assays were carried out with a Cary model 14 recording spectrophotometer, at 23°.

Oxaloacetic-carboxylase activity (Utter & Kurahashi, 1954) was determined manometrically, by measuring the initial rate of CO_2 release from oxaloacetate under anaerobic conditions. Cups contained, in the main compartment, crude bacterial extract (containing 7·1 mg. of soluble protein), 100 μmoles of potassium phosphate buffer, pH 7·0, 10 μmoles of $CoSO_4$ and water to 2·6 ml. The side arm contained, in 0·5 ml., 40 μmoles of freshly dissolved, neutralized oxaloacetate, 10 μmoles of disodium adenosine triphosphate and 40 μmoles of potassium phosphate buffer, pH 7·0. After thermal equilibration at 30° for 10 min., the contents of the side arm were added to those of the main compartment. The manometers were read every 3 min. for 25 min.

Tests for reversion or contamination. Before use, all cultures of the mutant were tested to ensure the absence of the condensing enzyme. This was done either by spectrophotometric assay (Dixon & Kornberg, 1959) of sonic extracts, or by manometric tests for the ability of washed cell suspensions to oxidize acetate. Whereas the parent wild type readily oxidized acetate (Q_{O_2} = 60–70 μl. of O_2 absorbed/mg. dry wt./hr.), the mutant did not oxidize this substrate.

Materials. Purified sodium glyoxylate was a gift of Dr I. Zelitch (Connecticut Agricultural Experiment Station, New Haven, Conn., U.S.A.). Oxaloacetic acid and sodium pyruvate were gifts of Professor Sir Hans Krebs, F.R.S. Thiamine pyrophosphate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; adenosine triphosphate, pyridine nucleotides and coenzyme A from C. F. Boehringer und Soehne, Mannheim, Germany. Other

chemicals used were of the highest purity commercially available. Isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks.; they were purified before use as previously described (Kornberg, 1958; Kornberg & Gotto, 1961).

RESULTS

Growth on glycollate. The *E. coli* mutant M22-64, which required glutamate or α-oxoglutarate for growth, was isolated by Gilvarg & Davis (1957), who showed that its growth requirement was a consequence of the lack of the citrate-forming condensing enzyme.

The organism grew readily on a medium containing 50 mM-glycollate as main carbon source if this medium was supplemented with a small amount (2 mM) of glutamate, but did not grow in the absence of added glutamate. On this glycollate–glutamate medium, the mutant grew at approximately the same rate as did the parent wild type, *E. coli*, strain *w*: the mean generation times at 30° were, respectively, 229 ± 17 min. (eight experiments) and 190 ± 20 min. (four experiments). The growth yields of the two types of organisms were similarly not significantly different: under the conditions used, the medium supported an increase in dry weight of bacteria of $19{\cdot}3 \pm 0{\cdot}3$ μg./μmole of added glycollate (three experiments) for the mutant and $19{\cdot}4 \pm 0{\cdot}3$ μg./μmole of added glycollate (four experiments) for the wild type. These results, which confirm the findings of Gilvarg & Davis (1957) that *E. coli* cannot synthesize glutamate in the absence of the citrate-forming condensing enzyme, also indicate that, after relief of the enzyme deficiency of the mutant, both it and the wild-type organism utilized glycollate as a growth substrate by similar pathways.

Oxidation of glycollate and glyoxylate. Washed suspensions of the mutant, grown on the glycollate–glutamate medium, oxidized glycollate and glyoxylate at the same rate and to the same extent as did the parent wild type grown on this medium (Table 1). This indicates that the routes of oxidation of glycollate and glyoxylate are similar in the two types of organism, and shows that these routes are independent of a functioning tricarboxylic acid cycle.

Similar results were obtained with [^{14}C]glycollate. In both types of organism, the oxidation of [^{14}C]glycollate led to the evolution, as $^{14}CO_2$, of 93–98 % of the isotope in the carboxyl group, and 43–52 % of the isotope from the hydroxymethyl group.

2:4-Dinitrophenol (Clifton, 1946) acted in like manner on both mutant and wild-type organisms. Addition of this compound to 0·5–1 mM final concentration raised the quantity of oxygen absorbed by the wild type from 65 to 84 %, and by the mutant from 66 to 81 %, of that required for complete oxidation of glycollate, and also stimulated by 50 % the release of isotope as $^{14}CO_2$ from [2-^{14}C]glycollate.

The possibility that glycollate and glyoxylate oxidations proceeded via free formate was tested by the addition of 2·5 mM-hypophosphite, a specific inhibitor of formic dehydrogenase (Takamiya, 1953) to washed suspensions of mutant and wild-type organisms oxidizing glycollate, glyoxylate or formate. Formate oxidation, which was rapid and complete in the absence of this inhibitor, was abolished in its presence, but the oxidations of glycollate and glyoxylate were unaffected in rate or extent. This indicated that free formate was not an intermediate in their catabolism.

Incorporation of ^{14}C *from* [^{14}C]*glycollate.* When [1-^{14}C]- or [2-^{14}C]-glycollate was added to suspensions of the mutant or wild-type organisms oxidizing unlabelled glycollate, ^{14}C was rapidly incorporated into cellular materials soluble in aqueous ethanol. The rates of isotope incorporation were constant over the first 60–80 sec. of incubation, and corresponded to the utilization of [2-^{14}C]glycollate at the rate of 6 μmoles/hr./mg. dry wt. of mutant organism and of 7 μmoles/hr./mg.

Table 1. *Oxidation of glycollate and of glyoxylate by* Escherichia coli, *strain* w, *and its mutant M* 22-64

The main compartments of Warburg manometer flasks contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (5 mg. dry wt.). The centre wells contained 400 μmoles of KOH. Substrates as indicated were added from the side arms after thermal equilibration at 30°. Results are expressed as mean values ± standard deviation of the samples. The numbers of separate experiments are given in parentheses.

Quantity measured	Substrate	Organism used: *E. coli, w*	Organism used: M22–64
Extent of oxidation (% of theory for complete combustion)	Glycollate	61 ± 3 (3)	59 ± 7 (21)
	Glyoxylate	54 ± 7 (3)	57 ± 8 (8)
Q_{O_2} (μl. of O_2 absorbed/mg. dry wt./hr.)	Glycollate	111 ± 30 (3)	94 ± 23 (21)
	Glyoxylate	38 ± 7 (3)	45 ± 18 (6)
$Q_{substrate}$ (μmoles oxidized/mg. dry wt./hr.)	Glycollate	3·3 ± 0·9 (3)	2·8 ± 0·7 (21)
	Glyoxylate	1·7 ± 0·3 (3)	2·0 ± 0·8 (6)

dry wt. of wild-type cells. [1-^{14}C]Glycollate was incorporated at 3 μmoles/hr./mg. dry wt. by the mutant: this lower rate of incorporation would be expected from the rapid loss of isotope as $^{14}CO_2$ from the carboxyl group.

These rates are minimum ones, as neither volatile products such as carbon dioxide nor chromatographically fugitive materials such as oxaloacetate were assayed. They are, however, of the same order as those calculated from the observed rates of glycollate oxidation (Table 1) when these are corrected for assimilation.

Distribution of ^{14}C *amongst the labelled materials formed from* [^{14}C]*glycollate.* Two-dimensional chromatography and radioautography of the aqueous-ethanol fractions, obtained from each sample of the suspensions of mutant and wild-type organisms oxidizing glycollate, showed that isotope appeared rapidly in malate, fumarate, succinate, aspartate, alanine, carbohydrates and phosphorylated materials when the labelled substrate was either [1-^{14}C]- or [2-^{14}C]-glycollate. Labelled glycerate also appeared in all cases, but its contribution to the total amount of radioactivity varied and was greater for [2-^{14}C]- than for [1-^{14}C]-glycollate. Subsequent analysis of the area of phosphorylated material showed that, in the early samples at least, the major proportion of the isotope incorporated into this fraction was present in phosphoglyceric acid and phosphoenolpyruvate. No label was detected in citrate, α-oxoglutarate or glutamate in any sample from the mutant suspensions, though these compounds were heavily labelled in samples taken from the wild type. The general pattern of isotope distribution found was, with the exception of these compounds, identical in the experiments with either mutant or wild-type organisms: for simplicity, only the former are shown in Figs. 1 and 2.

When the proportions of the total radioactivity contributed by each compound, expressed as the percentage of the total isotope incorporated in each sample, were plotted against the times at which such samples were taken, smooth curves could be drawn through the points obtained (Figs. 1, 2). After approx. 150 sec., steady-state conditions had apparently been reached, as indicated by the

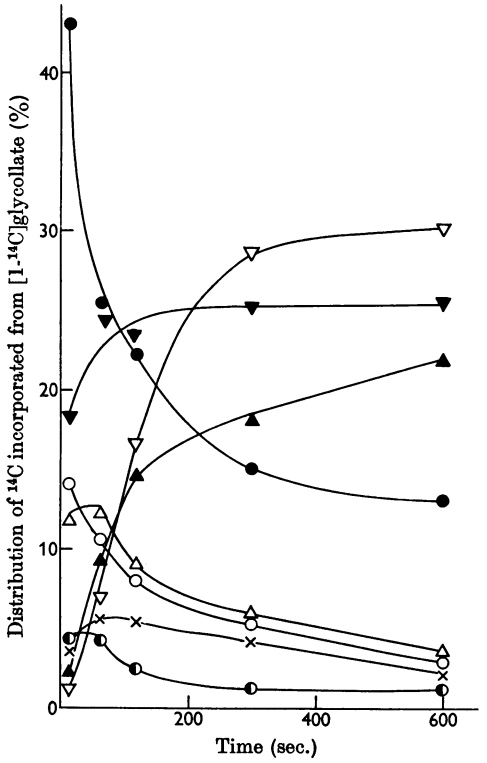


Fig. 1. Variation with time in the distribution of isotope from [1-^{14}C]glycollate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were polysaccharide (▽), phosphorylated materials (▼), glucose (▲), succinate (△), fumarate (○), alanine (×), aspartate (◐) and (predominating at early times) malate (●).

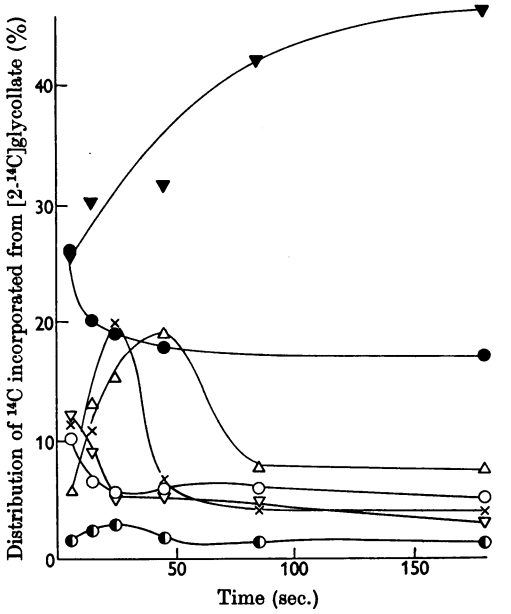


Fig. 2. Variation with time in the distribution of isotope from [2-^{14}C]glycollate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were phosphorylated compounds (▼), succinate (△), fumarate (○), alanine (×), glycerate (▽), aspartate (◐) and (predominating at the earliest times) malate (●).

relative constancy of isotope distribution amongst the components of the various samples. At and after this time, most of the incorporated isotope was found in carbohydrates and phosphorylated compounds, and 14–18% in malate. At earlier times, however, this distribution was reversed: malate contained the highest proportion of ^{14}C incorporated and, whereas the relative amounts of most other labelled materials initially rose with time, that of malate showed a continuous decrease. These results indicated that isotope from [^{14}C]glycollate was initially incorporated into malate by organisms oxidizing this labelled substrate; the initially low and rising proportions of isotope in succinate suggested that this compound had acquired label from malate and not vice versa. The negative slope of the glycerate curve indicated that isotope from glycollate also entered this material before passing on to other intermediates: this could be explained by the operation of a pathway for net synthesis of cell materials from glycollate similar to, or identical with, that found by Kornberg & Gotto (1959, 1961) with *Pseudomonas* spp.

Similar results were obtained with the wild-type organism oxidizing [1-^{14}C]- and [2-^{14}C]-glycollate, except that citrate and glutamate also became labelled. The relative proportion of the isotope incorporated into glutamate rose to a high level with time whilst that of citrate fell. Since neither of these materials was formed by the mutant, these results indicate that the tricarboxylic acid cycle functions in the wild-type organisms as a means of providing precursors of cell materials from glycollate rather than as a stage in glycollate oxidation.

Analysis of 2:4-dinitrophenylhydrazones prepared from all samples further showed that [^{14}C]-glycollate oxidation by the mutant resulted in rapid and heavy labelling of glyoxylate, oxaloacetate and pyruvate. At the earliest times, the bulk of the radioactivity was contained in glyoxylate, with oxaloacetate and pyruvate acquiring ^{14}C thereafter.

Effect of acetate on growth. Although the wild-type organism grew readily on acetate, the mutant did not grow on this carbon source even when the medium was supplemented with glutamate. This finding was expected as the growth of *E. coli* on acetate, which has been shown to be effected via the tricarboxylic acid and glyoxylate cycles (Kornberg, Phizackerley & Sadler, 1959, 1960; Reeves & Ajl, 1960), is not possible in the absence of a means of forming citrate. However, the addition of acetate (25 mM) to the glycollate–glutamate growth medium consistently stimulated the rate of growth of the mutant and reduced the mean generation time, by about 20%, to 183 min. (five experiments). Similarly, the addition to the glycollate–glutamate medium of acetate to a final concentration of 50 mM increased the growth yield of the mutant by 40%, to 29 μg. dry wt./μmole of added glycollate. Cells thus grown still failed to oxidize acetate alone and did not contain the citrate-forming condensing enzyme. This suggested that acetate, which, in the absence of glycollate, was not utilized by the organism, could enter metabolic pathways in its presence; it therefore indicated a point of entry into such routes other than via the condensing enzyme.

Incorporation of ^{14}C from [2-^{14}C]acetate. When [2-^{14}C]acetate was added to washed suspensions of either the mutant or wild-type organisms oxidizing unlabelled glycollate, ^{14}C was incorporated into cellular materials soluble in aqueous ethanol. The rates of isotope incorporation were constant over the first 90–100 sec. of incubation and corresponded to the uptake by respectively mutant and wild-type cells of 0·05 and 0·8 μmole of acetate/hr./mg. dry wt. The comparatively low rate of uptake of isotope from acetate is, in part, due to the small quantities of acetic thiokinase in glycollate-grown cells: the activation of [2-^{14}C]acetate to [2-^{14}C]-acetyl-coenzyme A would thus be expected to impose a rate-limiting step. The difference in the rates observed with the mutant and wild-type organisms indicates that, under these conditions, the major route for the utilization of exogenous acetate is via the tricarboxylic acid cycle.

Distribution of ^{14}C amongst the labelled materials formed from [2-^{14}C]acetate. With the exception of glutamate and citrate, which formed the bulk of labelled materials in the wild-type organisms but were absent from the mutant, the relative distribution of labelled products and their changes with time were similar with mutant and wild-type cells: again, for simplicity, only the results obtained with the mutant are shown in Fig. 3. As had been observed with [^{14}C]glycollate, one of the earliest labelled materials formed from [^{14}C]acetate was malate, which contained approx. 20% of the total isotope incorporated after 5 sec. incubation and the contribution of which continuously declined over the next 55 sec. to a steady-state level, at which it contained 8·4% of the total isotope incorporated. A similar high proportion of isotope was incorporated into aspartate, the contribution of which compound rose from an initial value of 19% at 5 sec. to a maximum of 21% at 23 sec., and declined continuously thereafter to a steady-state value at which it contributed 6% to the total radioactivity of the samples. Analysis of 2:4-dinitrophenylhydrazones prepared from each sample further showed that isotope from [^{14}C]-acetate rapidly appeared in oxaloacetate and pyruvate, but not in glyoxylate. It is therefore probable that, since oxaloacetate is fugitive on

two-dimensional chromatograms, the time course of labelling of aspartate is a reflexion of the rapid formation of labelled oxaloacetate from labelled malate.

These results show that the mutant, which, in the absence of glycollate cannot utilize acetate, can in its presence incorporate isotope from [^{14}C]-acetate into cellular materials, and that at the earliest times malate and oxaloacetate thus acquire label and in that order.

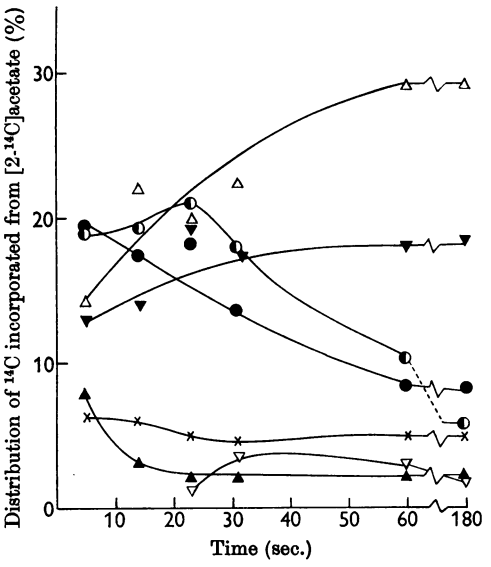


Fig. 3. Variation with time in the distribution of isotope from [2-^{14}C]acetate incorporated by *E. coli*, mutant M22–64, oxidizing unlabelled glycollate. For details, see Materials and Methods. The labelled products were succinate (△), phosphorylated compounds (▼), aspartate (◐), alanine (×), glycine (▲), glycerate (▽) and (predominating at the earliest times) malate (●).

Effect of acetate on the oxidation of glycollate or glyoxylate. Although acetate, added as sole substrate, was not oxidized by washed suspensions of the mutant, the total quantities of oxygen absorbed during the oxidation of glyoxylate were raised consistently by 20% when acetate was also added to various final proportions (Table 2). Moreover, the maximum rates of oxygen uptake observed showed a small but reproducible increase in the presence of acetate.

Effect of glycollate or glyoxylate on the oxidation of acetate. When washed suspensions of the mutant were incubated with [1-^{14}C]- or [2-^{14}C]-acetate, only traces of isotope (0·5 and 0·3% respectively of that added) were evolved as labelled carbon dioxide. However, this small amount of acetate oxidation was stimulated 70–80-fold when unlabelled glyoxylate or glycollate was also added to suspensions of the mutant incubated with [1-^{14}C]-acetate: of the isotope added, 44% was recovered as labelled carbon dioxide in the presence of glyoxylate, and 36% in the presence of glycollate. Pyruvate was ineffective in promoting this oxidation of [1-^{14}C] acetate (Table 3).

Similar results were obtained with [2-^{14}C]-acetate (Table 4). Addition of unlabelled glycollate or glyoxylate to suspensions of the mutant incubated with [2-^{14}C]acetate raised the proportion of added isotope recovered as labelled carbon dioxide from 0·3 to 12%. This stimulation was abolished by addition of arsenite, and was also not observed when pyruvate or L-malate was added instead of glycollate or glyoxylate.

Table 2. *Stimulation by acetate of the oxidation of glyoxylate by the mutant M 22-64*

The main compartments of Warburg manometer flasks contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (3–5 mg. dry wt.). The centre wells contained 400 μmoles of KOH. Substrates as indicated were added from the side arms after thermal equilibration at 30°.

Substrate	Quantity added (μmoles)	O_2 uptake observed (μl.)	Increase in O_2 uptake in the presence of acetate (μl.)	(%)
Acetate	20	0	—	—
Glyoxylate	10	116	—	—
Glyoxylate + Acetate	10 + 20	139	23	20
Glyoxylate	5	69	—	—
Glyoxylate + Acetate	5 + 10	83	14	20
Glyoxylate + Acetate	5 + 20	82	13	19
Glyoxylate	4	62	—	—
Glyoxylate + Acetate	4 + 40	75	13	21

Oxidation of L-*malate, oxaloacetate and pyruvate.* Washed suspensions of the wild-type organisms readily oxidized L-malate, oxaloacetate and pyruvate, the quantities of oxygen absorbed being respectively 70, 68 and 66% of those required for complete oxidation. In contrast, washed suspensions of the mutant oxidized these substrates to a much smaller extent. Of the quantities of oxygen required for their complete oxidation, only 21% was absorbed when L-malate or oxaloacetate were the substrates, and only 26% when pyruvate was used. These quantities are respectively 62, 63 and 80% of those required for the oxidation of these compounds to acetate.

Table 3. *Effect of added materials on* [1-^{14}C]*acetate oxidation by the mutant M* 22-64

The main compartments of double-armed Warburg manometer cups contained, in 2·5 ml., 100 μmoles of potassium phosphate, pH 7·2, and washed cells (10 mg. dry wt.). Sodium [1-^{14}C]acetate (6·8 μmoles; giving $1·3 \times 10^6$ counts/min. under the conditions of radioassay used) and unlabelled substrates (20 μmoles) as indicated were placed in one side arm, 0·3 ml. of 50% (w/v) trichloroacetic acid in the second. The centre wells contained 400 μmoles of KOH. After thermal equilibration at 30° for 10 min., the contents of the first side arm were mixed with those of the main compartment. The incubation was continued until oxygen uptake ceased, after which time the contents of the second side arm were added. After a further 15 min., the cups were detached and the contents of the centre wells were precipitated as $BaCO_3$ for radioassay as described by Kornberg & Gotto (1961).

Unlabelled substrate added	$10^{-3} \times$ Radioactivity of $BaCO_3$ (counts/min.)	Fraction of added isotope recovered as $BaCO_3$ (%)
None	7	0·5
Pyruvate	4	0·3
Glycollate	466	36
Glyoxylate	575	44

Table 4. *Effect of added substrates on* [2-^{14}C]*acetate oxidation by the mutant M* 22-64

For conditions, see Table 3. Sodium arsenite was added to the contents of the main compartments to a final concentration of 10 mM where indicated. The sodium [2-^{14}C]-acetate added, 5·8 μmoles/cup, gave $7·8 \times 10^5$ counts/min. under the conditions of radioassay used.

Unlabelled materials added	$10^{-3} \times$ Radioactivity of $BaCO_3$ (counts/min.)	Fraction of added isotope recovered as $BaCO_3$ (%)
None	2·5	0·32
Arsenite	2·1	0·26
Glycollate	97	12·4
Glycollate + arsenite	2·7	0·34
Glyoxylate	91	11·7
Glyoxylate + arsenite	2·6	0·33
L-Malate	2·3	0·30
Pyruvate	2·4	0·31

Similar results were found with labelled malate. After incubation of [1-^{14}C]malate with washed suspensions of the mutant, 35% of the added isotope was recovered as $^{14}CO_2$ and 10% as oxaloacetate and pyruvate. Addition of unlabelled pyruvate or acetate did not significantly alter these quantities (Table 5). With [2:3-$^{14}C_2$]malate or [3-^{14}C]pyruvate, only 1·5 and 1·6% respectively of the added isotope was evolved as $^{14}CO_2$.

However, as the results of the isotope-incorporation experiments had indicated labelled malate and oxaloacetate to be formed readily from labelled glycollate or labelled acetate, and as glycollate was oxidized (presumably via malate and oxaloacetate) to an extent much greater than suggested by the above results, it was probable that the presence of glycollate or glyoxylate promoted the oxidation of malate, oxaloacetate and pyruvate, as they promoted the oxidation of acetate. This is confirmed by the results shown in Table 5. Addition of unlabelled glycollate to suspensions of the mutant oxidizing [1-^{14}C]malate resulted in a 44% increase in the quantities of $^{14}CO_2$ evolved, the total amounts of isotope released being raised from 35 to 50·5% of those added. A like effect was noted after addition of unlabelled glyoxylate: this material stimulated the release of $^{14}CO_2$, derived from the oxidation of [1-^{14}C]malate, from 35 to 51%; of [2:3-^{14}C]malate, from 1·5 to 8·5%; and of [3-^{14}C]-pyruvate, from 1·6 to 10% of the isotope added. With [1-^{14}C]malate, the addition of unlabelled glycollate or unlabelled glyoxylate also caused a decrease in the quantities of labelled oxaloacetate and pyruvate formed, which indicated that the unlabelled materials had been in part converted into these α-oxo acids in the course of these oxidations.

Malate synthetase and condensing enzyme in Escherichia coli. The finding that labelled malate was formed as the earliest labelled product by cells oxidizing either [^{14}C]glycollate or [^{14}C]acetate in the presence of unlabelled glycollate or glyoxylate, indicated that the malate was probably formed by the action of malate synthetase (Wong & Ajl, 1956), which catalyses, reaction (1):

$$\text{Acetyl-coenzyme A} + \text{glyoxylate} + H_2O \rightarrow \text{malate} + \text{coenzyme A} \quad (1)$$

Assay of cell-free extracts of the mutant and the wild-type organism, grown on a variety of substrates, confirmed that this enzyme was present in glycollate-grown cells in concentrations three to four times higher than in cells grown on acetate, on which substrate the enzyme is known to participate in the biosynthesis of cell materials (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957, 1958; Kornberg, Collins & Bigley, 1960; Kornberg, Phizackerley & Sadler, 1960). Moreover, glycollate-grown cells contained the citrate-forming con-

densing enzyme in less than one-third the quantity observed in similar cells grown on acetate. This suggested that, when *E. coli* grows on glycollate, malate synthetase may function as an enzyme of a respiratory pathway in addition to its involvement in a biosynthetic route.

This is supported by studies of the changes of the intracellular concentration of malate synthetase and the citrate-forming condensing enzyme during the adaptation of wild-type organisms to growth on acetate or glycollate (Fig. 4). Continued growth of acetate-grown cells on acetate was accompanied by a maintenance of the ratio of activities of malate synthetase to condensing enzyme at 0·43 throughout an increase of cell density from 0·2 to 1·6 mg. dry wt./ml. (Fig. 4, curve *C*). However, when acetate-grown cells continued to grow on glycollate (Fig. 4, curve *A*) this ratio changed from an initial value of 0·3 to a final value of 2·1. Similarly, when glycollate-grown cells continued to grow on acetate (Fig. 4, curve *B*) the ratio of activities of malate synthetase to condensing enzyme fell from an initial value of 1·7 to a final figure of 0·5.

Enzymes oxidizing malate in glycollate-grown Escherichia coli. The rapid labelling of oxaloacetate,

Table 5. *Effect of added substrates on oxidation of* [^{14}C]*malate and* [^{14}C]*pyruvate by the mutant M 22-64*

The conditions used were similar to those of Table 3, but each cup received 2–5 mg. dry wt. of washed cells. The labelled substrates were mixed with unlabelled carriers to a final quantity of 5 μmoles/cup.

Labelled substrate	10^{-3} × Radioactivity added (counts/min.)	Unlabelled compound added	Quantity of unlabelled compound added (μmoles)	10^{-3} × Radioactivity of $BaCO_3$ (%)	Fraction of added isotope recovered as $BaCO_3$ (%)	10^{-3} × Radioactivity of oxaloacetate and pyruvate	Fraction of added isotope recovered as oxaloacetate and pyruvate (%)
[1-^{14}C]Malate	22·8	None	0	7·98	35	2·22	9·7
[1-^{14}C]Malate	22·8	Acetate	10	7·80	34	2·28	10
[1-^{14}C]Malate	22·8	Pyruvate	10	8·07	35	1·98	8·7
[1-^{14}C]Malate	22·8	Glycollate	10	11·8	51	1·08	4·7
[1-^{14}C]Malate	22·8	Glyoxylate	10	10·8	47	1·14	5·0
[2:3-^{14}C$_2$]Malate	36·6	None	0	0·57	1·5	—	—
[2:3-^{14}C$_2$]Malate	36·6	Glyoxylate	20	3·04	8·5	—	—
[3-^{14}C]Pyruvate	130	None	0	2·13	1·6	—	—
[3-^{14}C]Pyruvate	130	Glyoxylate	20	13·0	10	—	—

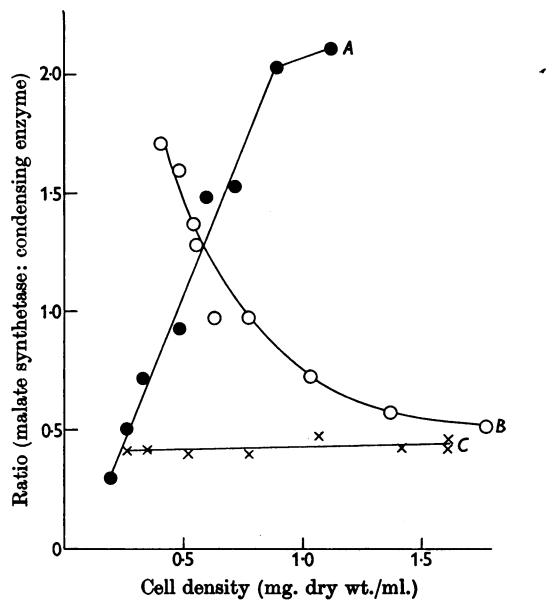


Fig. 4. Effect of change of growth substrate on the ratio of the activities of malate synthetase to condensing enzyme in extracts of *E. coli*, strain *w*. For details, see Materials and Methods. The ratio increased when acetate-grown cells continued to grow on glycollate (●, *A*), decreased when glycollate-grown cells continued to grow on acetate (○, *B*) and remained constant when acetate-grown cells continued to grow on acetate (×, *C*).

Table 6. *Enzymic activities in extracts of* Escherichia coli *strain* w *and M* 22-64 *grown on the glycollate–glutamate medium*

The extracts were prepared and the enzymes were assayed as described under Materials and Methods. Specific activities are expressed as μmoles of substrate transformed/mg. of soluble protein/hr.

Enzyme	Specific activity in *E. coli, w*	Specific activity in M22–64
Glycollic acid oxidase	6	6
Malate synthetase	25	21
Malate dehydrogenase	70	70
Oxaloacetic carboxylase	15	13
Pyruvate oxidase	10*	6*
Fumarase	52	32

* Minimum values, as the DPNH-oxidase activity of *E. coli* extracts was too high for precise quantitative assay.

phosphopyruvate and pyruvate noted during the oxidation of labelled glycollate, or labelled malate, or labelled acetate in the presence of unlabelled glycollate or glyoxylate, suggested that the malate formed from acetyl-coenzyme A and glyoxylate, by reaction (1), was oxidized via oxaloacetate, phosphopyruvate and pyruvate to re-form acetyl-coenzyme A. Enzymes capable of catalysing these oxidations were present in high activity in extracts of both mutant and wild-type cells grown on the glycollate–glutamate medium (Table 6). The enzymic activities observed were 3–25 times greater than required to account for the observed rates of glycollate or glyoxylate oxidation by intact cells.

DISCUSSION

Little is known of the metabolic routes whereby micro-organisms oxidize glycollate or glyoxylate to carbon dioxide and water. A possible pathway might involve a stepwise degradation of glycollate via glyoxylate and formate (reactions 2–4).

$$\text{Glycollate} + \tfrac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O \quad (2)$$

$$\text{Glyoxylate} + \tfrac{1}{2}O_2 \rightarrow \text{formate} + CO_2 \quad (3)$$

$$\text{Formate} + \tfrac{1}{2}O_2 \rightarrow CO_2 + H_2O \quad (4)$$

The occurrence in glycollate-grown micro-organisms of the first enzyme of this sequence, glycollic acid oxidase (reaction 2), has been established (Kornberg & Gotto, 1959, 1961; Table 6), and the enzyme has been shown to be formed only in organisms grown on glycollate (Gotto, 1961). Evidence on the mechanism of the further oxidation of glyoxylate is sparse and confusing. Although the glycollic acid-oxidase system of plants seemed also to catalyse the oxidation of glyoxylate to formate and carbon dioxide (Claggett, Tolbert & Burris, 1949), this oxidation was shown to be a non-enzymic decomposition of glyoxylate by hydrogen peroxide produced in the oxidation of glycollate (Kenten & Mann, 1952; Kolesnikov, 1952; Zelitch & Ochoa, 1953). The formation of [^{14}C]formate and unlabelled carbon dioxide from [1-^{14}C]glyoxylate was observed by Campbell (1954, 1955) when extracts of a strain of *Pseudomonas* were shaken in air for 6 hr. in the presence of sodium hypophosphite, which was added to inhibit the oxidation of formate. It is difficult to envisage the mechanism of this reaction, which was stimulated by Mg^{2+} ions and thiamine pyrophosphate and which effected the transformation of the carboxyl group of glyoxylate to formic acid with concomitant oxidation of the aldehyde group to carbon dioxide: it is conceivable that this is not a one-step reaction but that it might involve a process analogous to the conversion of $^{13}CH_2(OH)\cdot{}^{14}CH_2(NH_2)$ into $^{13}CH_2(NH_2)\cdot{}^{14}CO_2H$ observed by Weissbach & Sprinson (1953) in rats. The oxidation of glyoxylate to formate and carbon dioxide has also been reported as an intermediate step in the oxidation of glyceric acid by a soil diphtheroid (Taylor & Juni, 1959).

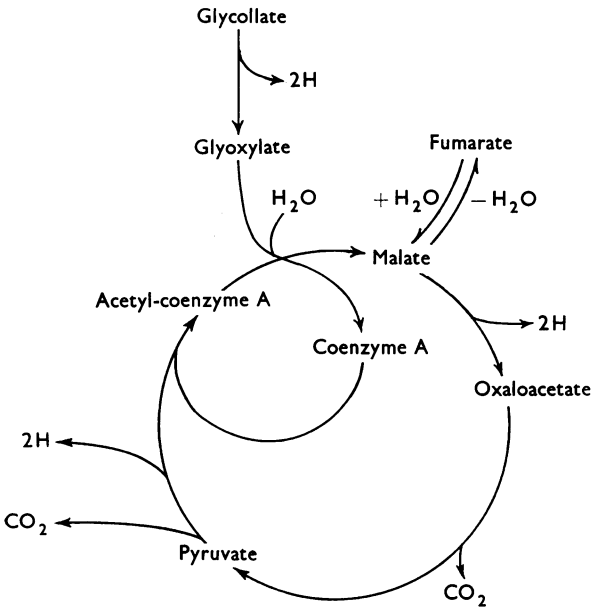
In contrast with these observations, Bachrach (1957) and Kornberg & Gotto (1959, 1961) failed to detect formate as a product of glyoxylate oxidation by several strains of *Pseudomonas*. Similarly, although sodium hypophosphite abolished the oxidation of formate by *E. coli, w* or M22-64, this compound did not affect the oxidation of glycollate or of glyoxylate.

The inhibition of glycollate oxidation by fluoroacetate, observed by Kalnitsky & Barron (1947), and the accumulation of citrate as a consequence of this inhibition (Jayasuriya, 1955, 1956), are strong indications that the tricarboxylic acid cycle is involved in the microbial metabolism of glycollate. However, it is unlikely that this cycle functions as the main respiratory pathway under these conditions. Jayasuriya (1955, 1956), in an extensive investigation of the effect of fluoroacetate on the metabolism of *Pseudomonas* OD1, noted that, although isotope from [2-^{14}C]glycollate and (to a lesser extent) from [1-^{14}C]glycollate was incorporated into the citrate accumulated as a consequence of fluoroacetate inhibition, the carbon dioxide evolved from the oxidation of the labelled glycollate contained isotope at a specific activity much higher than that contained in the carboxyl groups of citrate, which are the sources of the carbon dioxide evolved in the tricarboxylic acid cycle.

Similar conclusions may be drawn from the experiments reported in this paper. The rapid incorporation of isotope from [^{14}C]glycollate or from [^{14}C]acetate into citrate and glutamate by *E. coli*, strain *w*, simultaneously oxidizing unlabelled glycollate indicates that the tricarboxylic acid cycle operates under these conditions. The failure

of the mutant M22-64, which lacks the citrate-forming condensing enzyme, to grow on glycollate unless the medium is supplemented with glutamate further shows that the tricarboxylic acid cycle is as essential for growth on glycollate as for growth on other carbon sources (Gilvarg & Davis, 1957). But the ability of washed suspensions of the mutant to oxidize glycollate and glyoxylate at the same rate and to the same extent as washed suspensions of the wild type rules out this cycle as a necessary route for the oxidation of glycollate.

Instead, it is postulated that the complete oxidation of glycollate and of glyoxylate may be effected via a dicarboxylic acid cycle (Scheme 1).



Scheme 1. Postulated dicarboxylic acid cycle for the oxidation of glycollate and of glyoxylate. Each turn of the cycle results in the oxidation of one molecule of glyoxylate:

$$CHO \cdot CO_2H + O_2 \longrightarrow 2CO_2 + H_2O$$

The evidence for this cycle rests on five main considerations:

(1) Isotope from labelled glycollate is incorporated into glyoxylate and into the intermediates of the cycle in the expected sequence.

(2) Although acetate alone cannot be utilized by the mutant, acetate stimulates both growth on, and the oxidation of, glycollate.

(3) In the presence of unlabelled glycollate, labelled acetate is incorporated into the intermediates of the cycle but not into glyoxylate.

(4) Extracts of glycollate-grown *E. coli* contain the enzymes of the cycle in activity greater than required to account for the rates of oxidation of glycollate by whole cells.

(5) Labelled acetate alone is not oxidized by the mutant, but is oxidized in the presence of glycollate or glyoxylate.

Moreover, the relative activities of malate synthetase and condensing enzyme in glycollate-grown *E. coli*, strain *w*, and their variations with change of growth substrate (Fig. 4), suggest that the dicarboxylic acid cycle effects the oxidation of glycollate whereas the tricarboxylic acid cycle supplies the carbon skeletons of cell materials synthesized during growth on glycollate. Many of the enzymic steps of the dicarboxylic acid cycle are also those of the tricarboxylic acid cycle, but an inverse relationship emerges between oxidations effected by these two cycles. Whereas in each turn of the tricarboxylic acid cycle one molecule of acetate (entering the cycle as acetyl-coenzyme A) is totally oxidized with the obligatory participation of an α-oxo acid (which is regenerated), in each turn of the dicarboxylic acid cycle, one molecule of α-oxo acid is totally oxidized with the obligatory participation of acetyl-coenzyme A (which is regenerated).

Although this dicarboxylic acid cycle has been postulated to participate in the oxidation of glycollate and of glyoxylate by several strains of *Pseudomonas* (Hullin & Hassall, 1960; Kornberg & Gotto, 1961) and has been shown to be a quantitatively important route for glycollate oxidation by *E. coli*, strain *w*, and its mutant M22-64, it is likely that it is not the only route for the microbial oxidation of these C_2 compounds. For example, an organism isolated from soil has been found to grow readily on glycollate, and to be rich in the enzymes which effect the biosynthesis of cell materials from glycollate (Kornberg & Gotto, 1961), but to contain relatively small amounts of malate synthetase (C. T. Gray & H. L. Kornberg, unpublished experiments). The nature of such alternative routes is under investigation.

SUMMARY

1. *Escherichia coli*, strain *w*, grew readily in a medium containing 50 mM-glycollate as carbon source. The mutant M22-64 (Gilvarg & Davis, 1957), devoid of the citrate-forming condensing enzyme, failed to grow in this medium unless small quantities (2 mM) of glutamate were also added.

2. The mutant and the wild-type organisms, grown on 50 mM-glycollate plus 2 mM-glutamate, oxidized glycollate or glyoxylate at the same rate and to the same extent. These oxidations were not affected by 2·5 mM-hypophosphite, which abolished formate oxidation.

3. Isotope from [1-^{14}C]- or [2-^{14}C]-glycollate was incorporated at early times, by organisms oxidizing unlabelled glycollate, into glyoxylate, malate,

oxaloacetate and pyruvate. Under these conditions, isotope from [2-^{14}C]acetate appeared in malate, oxaloacetate and pyruvate.

4. Although the mutant did not oxidize acetate, acetate stimulated the rate and extent of oxygen uptake by cells oxidizing glyoxylate.

5. Washed suspensions of the mutant evolved, as $^{14}CO_2$, 0·5% of the isotope from [1-^{14}C]acetate. This was increased 70–80-fold when unlabelled glycollate or glyoxylate was also added. Pyruvate was ineffective in promoting this oxidation of [1-^{14}C]acetate. Similar results were obtained with [2-^{14}C]acetate.

6. Addition of unlabelled glycollate or glyoxylate stimulated the release of isotope as $^{14}CO_2$ from [1-^{14}C]malate, [2:3-$^{14}C_2$]malate and [3-^{14}C]pyruvate.

7. These results suggest the operation of a dicarboxylic acid cycle, in which glyoxylate is totally oxidized with the catalytic participation of malate, oxaloacetate, pyruvate and acetyl-coenzyme A.

8. Measurement of the absolute levels of enzymes in glycollate-grown cells, and alterations in their relative proportions when cells adapt to grow on glycollate, support the occurrence of this postulated cycle.

We thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement. This work was done during the tenure by J. R. S. of a Rhodes Scholarship and of a Pre-doctoral Fellowship of the National Science Foundation (Washington, D.C., U.S.A.). It was supported by the Rockefeller Foundation, the U.S. Public Health Service, and by the Office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under contract no. AF61(052)-180.

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