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The Role and Control of the Glyoxylate Cycle in *Escherichia coli*

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It is now generally accepted (for reviews see Ajl, 1958; Kornberg, 1959) that, in *Escherichia coli* as in other aerobic organisms capable of effecting the complete combustion of food materials, the tricarboxylic acid cycle (Krebs & Johnson, 1937) is the only pathway for the complete oxidation of acetate. In each turn of this cycle, acetate (as acetyl-coenzyme A) condenses with oxaloacetate to form citrate; subsequent reactions effect the stepwise oxidation of citrate to regenerate the oxaloacetate, with concomitant evolution of 2 molecular units of carbon dioxide for each molecular unit of acetate entering the cycle.

In addition to its role as terminal pathway of respiration, the tricarboxylic acid cycle serves another function in most micro-organisms. Many of the intermediates of the cycle are obligatory precursors of the macromolecular constituents of the cell (Roberts, Abelson, Cowie, Bolton & Britten, 1955; Gilvarg & Davis, 1956). To give just one example: oxaloacetate supplies not only aspartate itself, but also, from aspartate, the methionine, lysine, threonine and isoleucine components of proteins, and the pyrimidines of nucleic acids. The growth of micro-organisms on any carbon source is thus necessarily accompanied by a continuous removal of oxaloacetate (as well as of other intermediates) from the tricarboxylic acid cycle. If this cycle is to continue to function, then oxaloacetate or precursors thereof have either to be supplied in the growth medium or, when growth occurs on any single compound the catabolism of which does not directly yield intermediates of the cycle, this necessary replenishment must be achieved by some reactions ancillary to the cycle.

The necessity for the occurrence of such a pathway of replenishment—a type of metabolic route generically described as an anaplerotic sequence (Kornberg, 1965*a*,*b*)—is immediately apparent when acetate serves as sole carbon source for growth, and it is the nature of the pathway that replenishes the tricarboxylic acid cycle under these conditions which forms the subject of this lecture.

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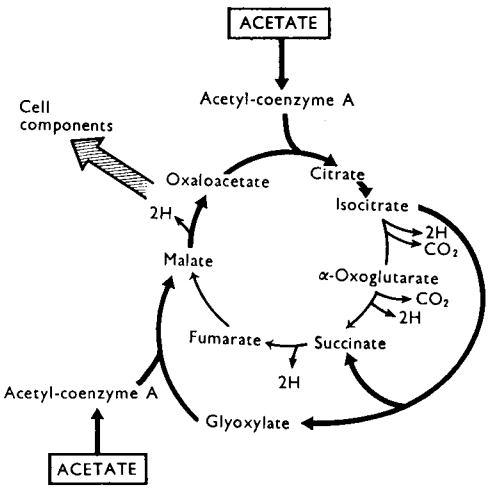
The nature and role of the glyoxylate cycle

The glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957) consists of six of the eight reactions of the tricarboxylic acid cycle (Scheme 1) but differs from it in by-passing the two oxidative steps in which carbon dioxide is evolved. Instead, isocitrate initially undergoes an aldol cleavage to succinate (an intermediate of the tricarboxylic acid cycle) and glyoxylate (reaction 1):

$$HO_2C\cdot CH_2\cdot CH(CO_2H)\cdot CH(OH)\cdot CO_2H \rightleftharpoons HO_2C\cdot CH_2\cdot CH_2\cdot CO_2H + O{:}CH\cdot CO_2H \quad (1)$$

This reaction is catalysed by isocitrate lyase (EC 4.1.3.1) and was first described by Campbell, Smith & Eagles (1953), who observed that ultrasonic extracts of *Pseudomonas aeruginosa* catalysed the formation of glyoxylate and succinate if either citrate or *cis*-aconitate was added to them, and



Scheme 1. Routes for the provision of energy and of cell components during microbial growth on acetate. The catabolic route (tricarboxylic acid cycle) is shown as light arrows, the anaplerotic pathway (glyoxylate cycle) as heavy arrows.

that this cleavage of the C_6 acids to a C_2 compound and a C_4 compound was reversible. This initial observation was speedily confirmed by Olson (1954), by Saz (1954) and by Smith & Gunsalus (1954), working with fungi and with several species of bacteria, who also established that the true substrate of the reaction was D$_s$(+)-isocitrate rather than either of the two C_6 acids in equilibrium with it.

The second component enzyme of the by-pass, malate synthase (EC 4.1.3.2), was discovered by Wong & Ajl (1956) in extracts of *E. coli*. This enzyme catalyses the condensation of acetyl-coenzyme A with glyoxylate, to yield malate—a second intermediate of the tricarboxylic acid cycle (reaction 2):

$$CH_3 \cdot CO \cdot S \cdot CoA + O:CH \cdot CO_2H + H_2O \rightarrow HO_2C \cdot CH_2 \cdot CH(OH) \cdot CO_2H + CoA \cdot SH \quad (2)$$

Together with the other enzymes of the tricarboxylic acid cycle, the concerted action of isocitrate lyase and malate synthase would thus effect the required net formation of C_4 acids from acetate (Scheme 1, heavy arrows).

That this concerted action indeed occurs was first shown with acetate-grown pseudomonads (Kornberg, 1958; Kornberg & Quayle, 1958; Kornberg & Madsen, 1958); subsequent experiments with intact *E. coli* and extracts thereof (Kornberg, Phizackerley & Sadler, 1960) gave virtually identical results. It was established: (*a*) that the addition of labelled acetate to the growing cells resulted, at the earliest times, in the appearance of isotope in malate before its appearance in succinate, though this ratio was reversed after more prolonged exposure to the labelled acetate; (*b*) that the distribution of isotope, derived from labelled acetate, within the carbon skeletons of a number of amino acids was intermediate between the distribution expected from the sole operation of the tricarboxylic acid cycle and from that of the glyoxylate cycle; (*c*) that acetate-grown cells contained the component enzymes of the glyoxylate cycle in activities more than sufficient to account for the observed rates of growth of the intact organisms; (*d*) that extracts of cells which had been grown on substances other than acetate (such as glucose or C_4 acids) contained the anaplerotic enzymes of the glyoxylate cycle in amounts considerably lower than did similar extracts of acetate-grown cells; moreover, when lactate-grown cells were transferred to acetate growth medium, isocitrate lyase and malate synthase were preferentially synthesized before growth recommenced (Fig. 1).

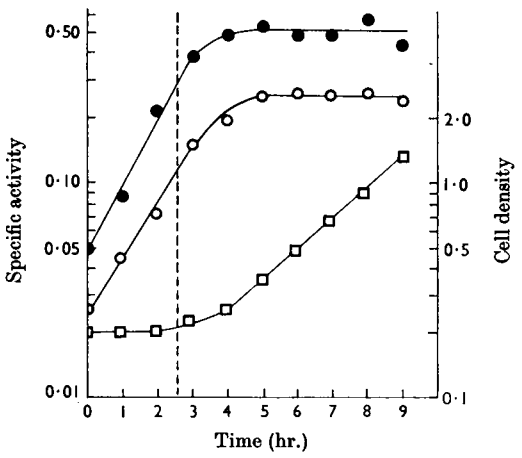
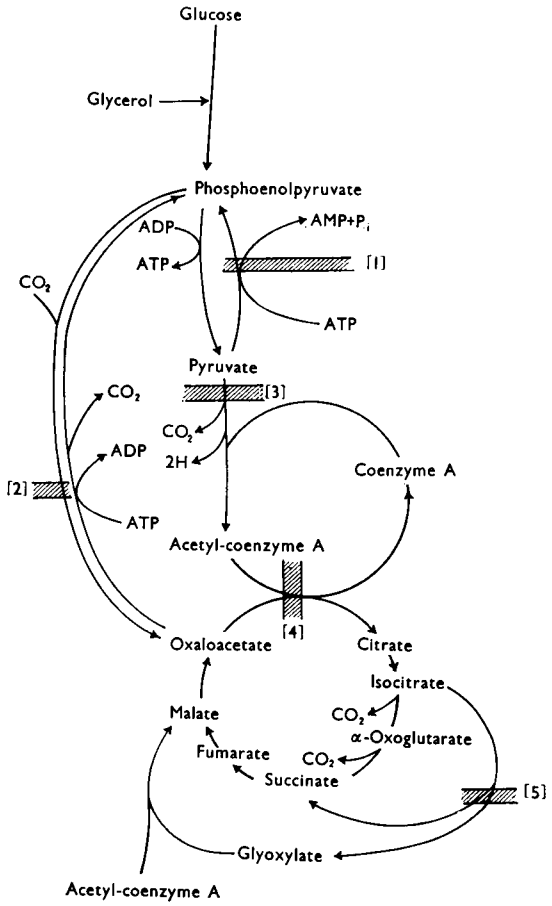


Fig. 1. Adaptive changes in the specific activities of isocitrate lyase and malate synthase after transfer of lactate-grown *E. coli* W into a medium containing acetate as sole carbon source. Isocitrate lyase (○) was measured as described by Kornberg (1963), malate synthase (●) as described by Dixon & Kornberg (1959). Specific activities are expressed as μmoles of substrate transformed/mg. of protein/min. Growth (□) was measured as $\Delta E_{680m\mu}$ and is expressed as mg. dry wt. of bacteria/ml.

But, although such observations provide evidence for the operation of the glyoxylate cycle, they do not show that the cycle is necessary as well as sufficient for its anaplerotic role. Such evidence was, however, obtained with auxotrophic mutants devoid of isocitrate lyase (Ashworth & Kornberg, 1964). Mutants of this type (Scheme 2, [5]) do not grow on acetate, though they readily oxidize acetate and though they grow on glucose or utilizable intermediates of the tricarboxylic acid cycle with the same facility as do their wild-type parents. If such mutants are now smeared thickly on agar plates containing acetate as sole carbon source, occasional colonies appear: these are largely the result of further and relatively rare mutations, which are selected with great sensitivity by this procedure. Examination of such further mutants showed that, in each colony of organisms which had regained the ability to grow on acetate, isocitrate-lyase activity had also been restored—albeit, in many instances, the enzyme now present had physical properties markedly different from that normally found in the wild-type organism. However, these observations show that the enzymic activity of isocitrate lyase and the ability to grow on acetate are causally, and not casually, related.

Control of the glyoxylate cycle

Role of acetate in isocitrate-lyase formation. The rapid appearance of isocitrate lyase and malate synthase in cells transferred to a medium containing acetate as sole carbon source (Fig. 1) might suggest that acetate (or acetyl-coenzyme A) acts as a direct inducer of the synthesis of these anaplerotic



Scheme 2. Enzymic dysfunctions in mutants of *E. coli* used in this work. The relationship of the metabolic lesions to the central pathways of metabolism is indicated by hatched bars. [1] Phosphoenolpyruvate synthase; [2] phosphoenolpyruvate carboxylase; [3], pyruvate-dehydrogenase complex of enzymes; [4], citrate synthase; [5], isocitrate lyase.

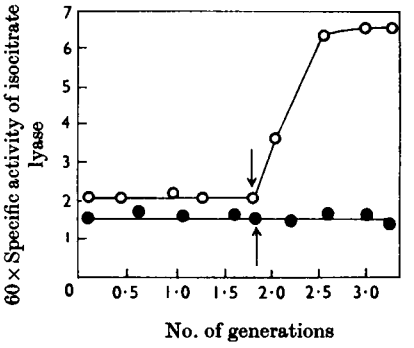


Fig. 2. Effect of 20mM-acetate on the specific activity of isocitrate lyase in *E. coli* W (○) and its mutant *E. coli* M22–64 (●) (which lacks citrate synthase) during growth on 25mM-proline at 30°. Acetate was added at the points indicated by arrows. For ease of representation, the specific activity has been multiplied by 60.

enzymes. There are several lines of evidence against this view (Kornberg, 1963).

When acetate was added, in equimolar concentration, to cultures of *E. coli* growing on glucose, pyruvate, or utilizable intermediates of the tricarboxylic acid cycle, the continued growth of the organisms occurred without significant increase in the specific activities of these enzymes. Only when substances such as proline, glutamate or γ-aminobutyrate served as carbon and/or nitrogen sources was the rate of isocitrate-lyase synthesis stimulated markedly by the addition of acetate (Fig. 2). However, this effect is not due to a direct induction by acetate of isocitrate-lyase formation, as it does not occur with organisms devoid of citrate-synthase activity (EC 4.1.3.7; Scheme 2, [4]). Such mutants require glutamate for growth, as they are unable to synthesize this amino acid in consequence of their inability to form citrate (Gilvarg & Davis, 1956), but grow on glutamate as sole carbon source at rates equal to and to extents similar to their wild-type parents; however, they are still capable of activating acetate to acetyl-coenzyme A (Gilvarg & Davis, 1956; Kornberg & Sadler, 1961). The failure of acetate to stimulate isocitrate-lyase synthesis in these mutants, under conditions identical with those in which acetate apparently induced the formation of this enzyme in wild-type *E. coli*, thus argues against acetate or acetyl-coenzyme A as a direct inducer. Rather do these observations suggest either that citrate or some other product derived from citrate is the true inducer, or that acetate exerts an 'inducer-like' effect (Rosenberger, 1962), by removing a repressor metabolically close to oxaloacetate: in the wild-type organism, acetate might lower the intracellular concentration of such a repressor by combining with oxaloacetate to form citrate but, in the mutant, would be unable to act in this manner.

Since *E. coli* does not grow on citrate, it is difficult to test the former of these alternatives. However, pseudomonads and another member of the Enterobacteriaceae, *Salmonella typhimurium*, do, and produce very little isocitrate lyase while doing so: this suggests that neither citrate nor a product of citrate catabolism is an inducer of the enzyme.

A second observation in favour of the view that the inducer-like action of acetate is a consequence of its combination with a repressor metabolite or with a precursor of a repressor metabolite arises from

experiments in which the mutant devoid of citrate-synthase activity grew on media containing either glycollate plus glutamate, or this mixture plus acetate (Fig. 3). In the absence of acetate, isocitrate lyase was produced at a specific activity of 0·037 [μmole of glyoxylate formed from isocitrate/min./mg. of protein, in the assay procedure of Kornberg (1963)], which remained constant over nearly 2 generations. The addition of acetate after this time not only failed to induce isocitrate-lyase formation but caused a sharp decrease in the specific activity of the enzyme without causing any decrease in the growth rate. Similarly, when glycollate/glutamate-grown cells were transferred to a medium containing this mixture plus acetate, the further growth of the mutant was accompanied by a decrease in isocitrate lyase to a new level of activity, which was less than 60% of that present in cells growing in the absence of acetate; this lower level was maintained throughout the subsequent growth. Since it is known (Kornberg & Sadler, 1961) that acetate can enter the metabolic pathways of this mutant during growth on glycollate, by combining with glyoxylate to form malate (reaction 2), these observations suggest the formation of a repressor from malate.

Influence of carbon sources on isocitrate-lyase formation. Since significant amounts of isocitrate lyase were observed in extracts of mutants devoid of citrate-synthase activity—and hence, presumably, also devoid of isocitrate—this enzyme must have been formed gratuitously under these conditions (Fig. 2 and 3). Similarly, since isocitrate-lyase-less mutants of *E. coli* (Scheme 2, [5]) grow readily on substrates other than acetate, isocitrate lyase cannot play any necessary role in the utilization of such substrates. Consequently, any variation in the activity of isocitrate lyase present in wild-type cells during growth on these different carbon sources is likely to indicate, not that such substrates are utilized via the glyoxylate cycle to some extent, but that different carbon sources produce different concentrations of repressors in the cell. This is borne out by the observation (Fig. 4) that, although only relatively low levels of isocitrate lyase are found in cells growing on compounds other than acetate, the magnitude of the specific activities achieved appears to vary inversely with the ease with which those carbon sources enter the main metabolic routes of the cell. Thus the highest specific activities of isocitrate lyase (up to 30% of that found in extracts of acetate-grown *E. coli* W) were observed during growth on proline, glutamate, γ-aminobutyrate and glycollate, which require the occurrence of one or more reactions before they can enter the Embden–Meyerhof pathway or the tricarboxylic acid cycle: growth on these substances was slower than on the intermediates of the central pathways to which they are transformed.

Identity of the repressor of isocitrate-lyase synthesis. As might be expected from the cyclic nature of the reaction sequences through which

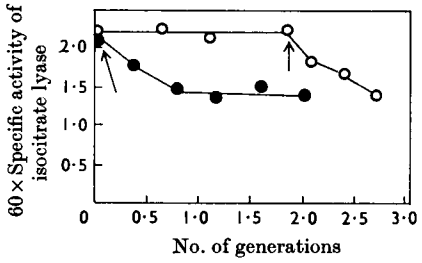


Fig. 3. Effect of acetate on the specific activity of isocitrate lyase in *E. coli* M22–64 (which lacks citrate synthase) during growth at 30° on 20mM-glycollate plus 2mM-glutamate (○) or on this medium plus 50mM-acetate (●). The experiment was begun with cells grown on glycollate plus glutamate. Acetate was added at the points indicated by arrows. For ease of representation, the specific activities have been multiplied by 60.

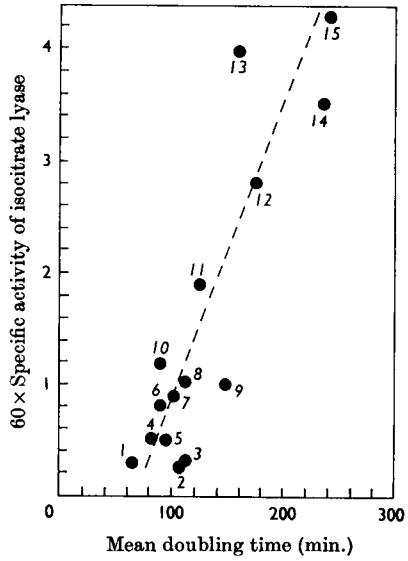


Fig. 4. Relationship between mean doubling time at 30° and specific activity of isocitrate lyase in *E. coli* W grown on the following substances as sole carbon sources: *1*, glucose; *2*, pyruvate; *3*, DL-glycerate; *4*, lactate; *5*, fructose; *6*, L-malate; *7*, fumarate; *8*, succinate; *9*, L-α-alanine; *10*, glycerol; *11*, L-aspartate; *12*, glycollate; *13*, L-glutamate; *14*, γ-aminobutyrate; *15*, L-proline. For ease of representation, the specific activities have been multiplied by 60.

Table 1. *Wild-type* E. coli, *and mutants derived therefrom, mentioned in the text*

Organism	Parentage of mutant	Enzymic dysfunction	Position on Scheme 2	Special growth characteristics	Source
W	—	None	—	None	Gift from Professor B. D. Davis (Harvard)
M22–64	W	Lacks citrate synthase	[4]	Requires glutamate	Gilvarg & Davis (1956); gift from Professor B. D. Davis (Harvard)
M191–6	W	Lacks first enzyme of pyruvate-dehydrogenase complex	[3]	Requires acetate	Gounaris & Hager (1961); gift from Professor B. D. Davis (Harvard)
M1	W	Lacks isocitrate lyase	[5]	Does not grow on acetate	Ashworth & Kornberg (1964)
UH–Ac2	K12	Lacks first enzyme of pyruvate-dehydrogenase complex	[3]	Requires acetate	Henning, Herz & Szolyvay (1964); gift from Dr U. Henning (Cologne)
B	—	None	—	None	Gift from Professor L. Gorini (Harvard)
Bm	B	Lacks phosphoenolpyruvate carboxylase	[2]	Does not grow on glucose unless medium supplemented with tricarboxylic acid-cycle intermediates; grows on acetate	Gift from Professor L. Gorini (Harvard)
PN–1	B	Lacks phosphoenolpyruvate synthase	[1]	Does not grow on pyruvate; grows on glucose or acetate	Cooper & Kornberg (1965)
PN–1/1	PN–1	Lacks phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase	[1] and [2]	Does not grow on pyruvate or glucose unless media supplemented with tricarboxylic acid-cycle intermediates; grows on acetate	Isolated by R. A. Cooper
PN–1/1R	PN–1/1	As PN–1/1	[1] and [2]	Grows slowly on pyruvate, rapidly on acetate plus pyruvate; does not grow on glucose	Isolated by J. Feldman
PN–1PD$^-$	PN–1	Lacks phosphoenolpyruvate synthase and first enzyme of pyruvate-dehydrogenase complex	[1] and [3]	Requires acetate	Isolated by R. A. Cooper & J. Smith
ILc–1	K12	Lacks phosphoenolpyruvate synthase and is constitutive for isocitrate lyase	[1]	Grows on pyruvate	Isolated by H. L. Kornberg

energy and biosynthetic precursors are obtained, attempts to pin-point the metabolite(s) responsible for the repression of isocitrate-lyase synthesis are beset by great difficulties: in the wild-type organisms any intermediate of the central pathways of metabolism can be derived from, and converted into, any other. In an effort to overcome these problems, a variety of auxotrophic mutants have been selected and used: these are listed in Table 1, and the relationship of the enzymic dysfunction to the overall metabolism of the organisms is diagrammatically represented in Scheme 2.

The first clue as to the possible identity of the metabolite(s) responsible for isocitrate-lyase repression came from studies with mutants which lacked a component enzyme of the pyruvate-dehydrogenase complex (EC 1.2.4.1) (Scheme 2, [3]). When such mutants were transferred from a medium containing acetate as sole carbon source to one also containing glyoxylate, the continued growth of the organism was accompanied by a virtual cessation of isocitrate-lyase synthesis: the specific activity of the enzyme decreased by 50% for each generation of growth (Fig. 5). A culture continuing to grow on acetate, on the other hand, maintained unchanged the high concentration of isocitrate lyase present in the inoculum. Change-over of the acetate-grown cells, to medium containing glyoxylate plus acetate, led to a similar cessation of isocitrate-lyase synthesis, whereas transfer of the cells, previously grown on glyoxylate plus acetate, to acetate growth medium resulted in rapid resumption of isocitrate-lyase synthesis.

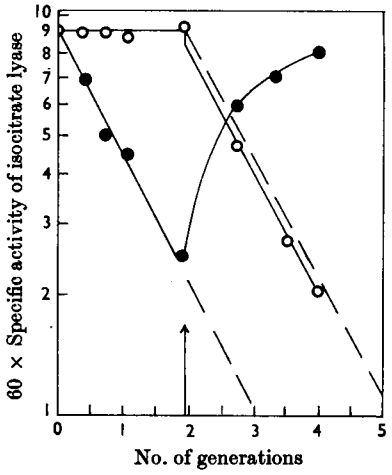


Fig. 5. Effect of glyoxylate on the specific activity of isocitrate lyase during the growth on acetate of *E. coli* M191–6 (which lacks the first enzyme of the pyruvate-dehydrogenase complex). The acetate-grown inoculum was suspended in growth medium, containing either 25 mM-acetate (○) or 25 mM-acetate plus 5 mM-glyoxylate (●), to a cell density of approx. 0·2 mg. dry wt./ml. When the cells had grown at 30° to 0·3 mg. dry wt./ml., samples were withdrawn for analysis and fresh medium was added to restore the cell density to 0·2 mg. dry wt./ml. After approx. 2 generations of growth, the cells were harvested (arrow); the acetate-grown cells were now resuspended in medium containing acetate plus glyoxylate, whereas the cells previously grown on acetate plus glyoxylate were suspended in acetate medium. The experiment was continued for a further 2 generations. For ease of representation, the specific activities have been multiplied by 60.

These findings contrast sharply with those reported in Fig. 3: in both cases, the organism grew on media containing glyoxylate (either supplied as such, or derived from glycollate) plus acetate, yet, in the absence of pyruvate-oxidase activity, isocitrate-lyase synthesis was virtually abolished, whereas, in a mutant containing this enzyme complex, it continued at a significant rate. This suggested that the agent responsible for the drastic repression of isocitrate-lyase synthesis was either the metabolite which could not be oxidized, namely pyruvate, or some precursor of pyruvate which could also not be oxidized under these conditions. Indeed, analysis of the media in which the organisms were growing (Fig. 5) showed that pyruvate accumulated during the growth of the mutant on glyoxylate plus acetate, but not during growth on acetate alone.

The hypothesis that the repressor metabolite of isocitrate-lyase synthesis was a C_3 compound rather than an intermediate of the tricarboxylic acid cycle was supported by experiments with a similar mutant of *E. coli*, but derived from strain K12 rather than W, and also lacking the first enzyme of the pyruvate-dehydrogenase complex (Henning, Herz & Szolyvay, 1964). During growth in a medium containing acetate as carbon source and aspartate as nitrogen source, isocitrate lyase was synthesized at a rate only slightly lower than that observed during growth on acetate alone: no keto compounds accumulated under these conditions, and the aspartate must therefore have been also utilized for growth (Fig. 6) but without causing significant repression of isocitrate-lyase formation. However, the addition of pyruvate to such cultures resulted again in virtual cessation of the synthesis of this enzyme, without impairing the continued growth of the mutant.

Though this experiment suggested that C_4 acids were not the metabolites responsible for repression, it was possible that the rate at which aspartate was utilized as nitrogen source, coupled with the rapid removal by acetate of the oxaloacetate produced from it, did not sufficiently raise the concentration of C_4 compounds to produce a significant repression. Direct evidence on this point was provided by studies with mutants of *E. coli* B (Ashworth &

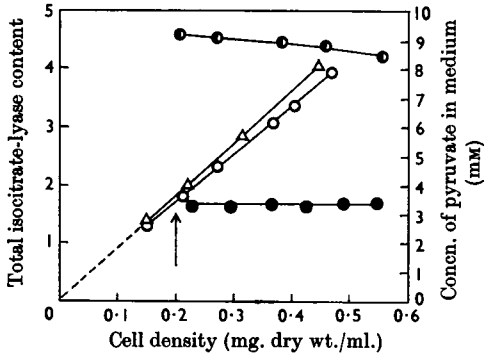


Fig. 6. Effect of 10mM-pyruvate on the synthesis of isocitrate lyase by *E. coli* UH Ac–2 (a mutant of *E. coli* K12 which lacks an enzyme of the pyruvate-dehydrogenase complex). Acetate-grown cells were inoculated into media containing 50mM-acetate plus 50mM-NH_4Cl (△) or 50mM-acetate plus 5mM-aspartate as nitrogen source (○). To a flask containing the latter medium, 10mM-pyruvate was added shortly after growth commenced (●), at the point indicated by an arrow. The pyruvate content of the medium was determined at intervals (◐) by the procedure of Friedemann & Haugen (1943). The isocitrate-lyase content of the culture is expressed as 60 × specific activity × cell density.

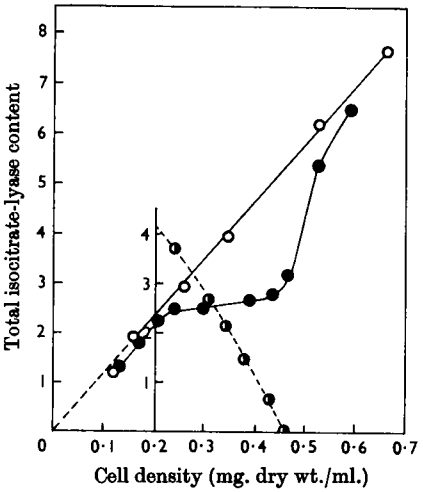


Fig. 7. Effect of pyruvate on isocitrate-lyase formation by *E. coli* Bm (which lacks phosphoenolpyruvate carboxylase) during growth on 50mM-acetate plus 5mM-glutamate, in the absence of other nitrogen sources. Pyruvate (final concn. 4mM) was added to one culture (●); the decrease in the pyruvate content of the medium is indicated by (◑). The other culture (○) received no pyruvate. The isocitrate-lyase content of the culture is expressed as 60 × specific activity × cell density.

Kornberg, 1963) or K12 (Ashworth, Kornberg & Ward, 1965) which lacked phosphoenolpyruvate-carboxylase activity (EC 4.1.1.31). Such mutants (Scheme 2, [2]) are unable to effect the net formation of C_4 acids from phosphoenolpyruvate or pyruvate, and hence do not grow on glucose or pyruvate unless utilizable intermediates of the tricarboxylic acid cycle are also supplied, but grow readily on acetate, since the glyoxylate cycle does not involve the carboxylation of C_3 acids.

The addition of pyruvate to cultures of such mutants, growing on acetate as carbon source and aspartate or glutamate as nitrogen source, did not decrease the rate of growth, but caused a sharp decrease in the high rate of isocitrate-lyase synthesis (Fig. 7). Again, the amino acids must have been utilized for growth, since no keto compounds accumulated in the growth media before the addition of pyruvate, but without impairing the rate of isocitrate-lyase formation: such impairment was, however, produced by pyruvate although in these mutants this C_3 compound could not give rise to the net formation of C_4 acids or other intermediates of the tricarboxylic acid cycle.

These and other experiments (Kornberg, 1963) indicated that, if any single metabolite may be regarded as a specific repressor of isocitrate-lyase synthesis, that metabolite cannot be a C_4 acid, an intermediate of the tricarboxylic acid cycle, or an oxidation product of pyruvate. It was thus likely to be either pyruvate itself, or some substance (such as phosphoenolpyruvate) which can readily give rise to pyruvate.

Two considerations stood in the way of acceptance of pyruvate itself as the repressor metabolite. In the first place, *Achromobacter* spp., like *E. coli*, form isocitrate lyase in only low amounts during growth on C_4 acids but, unlike *E. coli*, form considerable quantities of this enzyme during growth on lactate, alanine or pyruvate (Kornberg, Dennis & Wilson, 1964). Pyruvate itself is thus unlikely to be the metabolite responsible for isocitrate-lyase repression in these organisms and, on the assumption that the regulatory mechanisms governing the synthesis of anaplerotic enzymes in one micro-organism are likely to be similar to those operative in most others, these observations also raise doubts of the repressor role of pyruvate in *E. coli*.

In the second place, it was clear that experiments which indicated pyruvate as the repressor metabolite did not rule out substances catabolized to pyruvate. The observation that mutants unable to carboxylate phosphoenolpyruvate (Ashworth & Kornberg, 1963; Vanderwinkel, Liard, Ramos & Wiame, 1963; Theodore & Englesberg, 1964; Ashworth *et al.* 1965; Scheme 2, [2]) also could not grow on pyruvate suggested that some enzymic system must be present which effects the net formation of phosphoenolpyruvate from pyruvate.

Studies with *E. coli* B (Cooper & Kornberg, 1965) and K 12 revealed the nature of this enzyme system. Like pyruvate kinase (EC 2.7.1.40), it catalyses the formation of phosphoenolpyruvate from pyruvate and ATP, but, unlike pyruvate kinase, the other products of the reaction are AMP and inorganic phosphate, and not ADP. Although the mechanism of this phosphoenolpyruvate-synthase reaction (reaction 3)

$$\text{Pyruvate} + \text{ATP} \rightarrow \text{phosphoenolpyruvate} + \text{AMP} + P_i \quad (3)$$

is not yet fully elucidated, it has been shown (R. A. Cooper, personal communication) that the inorganic phosphate is derived from the γ-phosphate group of ATP, whereas the β-phosphate of ATP is the source to the phosphate group of phosphoenolpyruvate. It is thus probable that a pyrophosphorylpyruvate or a pyrophosphoryl-enzyme is an intermediate in this process. More relevantly to the present theme, mutants have been isolated which grow readily on glucose or acetate, but do not grow on pyruvate: such mutants lack phosphoenolpyruvate-synthase activity (Scheme 2, [1]; Table 1) but contain pyruvate kinase in quantities similar to those found in wild-type *E. coli*.

When a mutant of this type, which lacked both phosphoenolpyruvate-synthase and phosphoenolpyruvate-carboxylase activities (Scheme 2, [1] and [2]), was grown on acetate, with either ammonium chloride or aspartate as nitrogen source, isocitrate lyase was synthesized at the customary high rate. However, the addition of pyruvate to such a culture virtually abolished isocitrate-lyase synthesis when aspartate was the nitrogen source: if the C_4 acid was omitted, pyruvate failed to produce this striking repression, and isocitrate-lyase production continued at a somewhat diminished but still significant rate (Fig. 8). It was thus apparent that the absence of phosphoenolpyruvate synthase was associated also with an amelioration of the repressor-like action of pyruvate. But an amelioration is not an abolition, and this interpretation of the results could still be open to doubt.

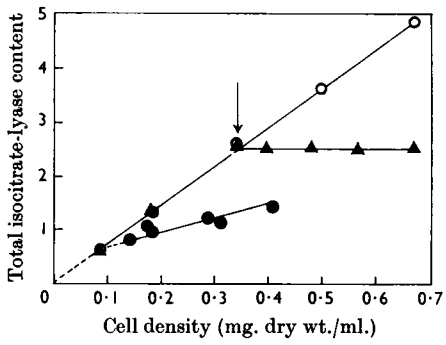


Fig. 8. Effect of pyruvate on the formation of isocitrate lyase by *E. coli* PN-1/1 (which lacks both phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase). Acetate-grown cells were used as inoculum for further growth, either on media containing 50 mM-NH_4Cl and 50 mM-acetate (○), or 50 mM-acetate and 50 mM-NH_4Cl plus 5 mM-pyruvate (●), or 50 mM-acetate plus 5 mM-aspartate in the absence of other nitrogen sources (▲). Pyruvate (final concn. 5 mM) was added to this last culture at the point indicated by an arrow. The isocitrate-lyase content is expressed as 60 × specific activity × cell density.

Fortunately, a means of resolving this doubt came to hand when it was observed (Fig. 10) that such mutants, which lack both phosphoenolpyruvate-synthase and phosphoenolpyruvate-carboxylase activities (Scheme 2, [1] and [2]), grow rather slowly on acetate if pyruvate is also present: this phenomenon will be discussed later. By selecting further mutants which grow rapidly in the presence of pyruvate, it could be shown that the addition of pyruvate to a culture of such pyruvate-resistant organisms, growing on acetate, now produced but a negligible repression of isocitrate-lyase synthesis (Fig. 9). On the other hand, the addition of glucose speedily caused the organisms to stop growing: growth was resumed when glyoxylate was now also supplied. But, whereas growth on acetate plus pyruvate did not result in significant repression of isocitrate-lyase synthesis, growth in the presence of glucose and the C_4 acids presumably derived from the interaction of acetate and glyoxylate resulted in total cessation of the formation of this enzyme—indeed, the enzyme appeared to be destroyed during further growth. It is thus clear that in *E. coli*, as in *Achromobacter* d–15, pyruvate itself is not the repressor of isocitrate-lyase synthesis, and that this repressor metabolite must be either phosphoenolpyruvate or some metabolite readily derived therefrom.

'Fine control' of the glyoxylate cycle. Although the variations described, in the differential rate of synthesis of isocitrate lyase, represent one type of control of the glyoxylate cycle, such a regulatory process must of necessity be a relatively 'coarse' control mechanism. In addition, a 'fine' control operates which governs the rate of anaplerosis achieved through the cycle by regulating the activity of isocitrate lyase.

An indication of the mechanism of this control was provided by the observation (Fig. 10) that mutants devoid of phosphoenolpyruvate-carboxylase activity (Scheme 2, [2]), which grow readily on acetate, do not grow when pyruvate is also present: it may be recalled (Fig. 7), however, that growth is not impaired if C_4 acids are also added. A similar arrest of growth on acetate, but not on acetate plus

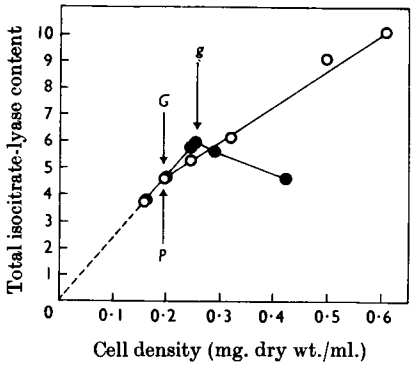


Fig. 9. Effect of pyruvate, or glucose and subsequently glyoxylate, on isocitrate-lyase formation by acetate-grown *E. coli* PN-1/1R. The mutant used was selected from *E. coli* PN-1/1 for its ability to grow rapidly on acetate plus pyruvate (Table 1); like *E. coli* PN-1/1, it lacks both phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase. Acetate-grown cells were used as inoculum for growth on 25mM-acetate. To one flask (○), pyruvate (final concn. 10mM) was added at the point marked *P*. To the second flask (●), glucose (final concn. 10mM) was added at *G* and glyoxylate (final concn. 5mM) at *g*. (The growth of these cultures is shown in Fig. 14.). The isocitrate-lyase content is expressed as 60 × specific activity × cell density.

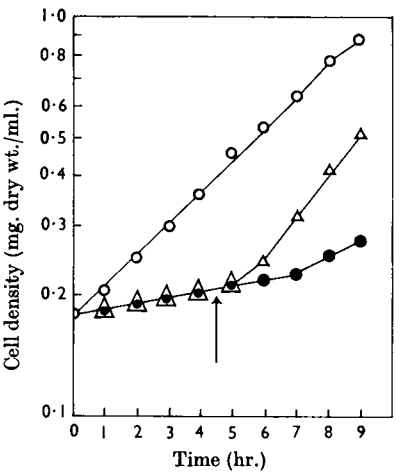


Fig. 11. Effect of pyruvate and glyoxylate on the growth on acetate of *E. coli* PN-1PD⁻ (which lacks phosphoenolpyruvate synthase and the first enzyme of the pyruvate-dehydrogenase complex). Acetate-grown cells were inoculated into media containing 50mM-acetate (○), or 50mM-acetate plus 1mM-pyruvate (●), or 50mM-acetate plus 10mM-pyruvate (△). Glyoxylate (final concn. 5mM) was added to this last culture after 4·5hr. (arrow).

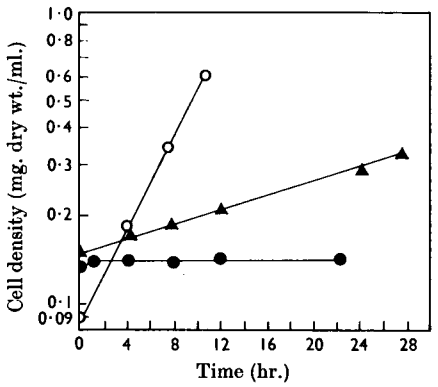


Fig. 10. Effect of 5mM-pyruvate on the growth, on 25mM-acetate, of *E. coli* B (○), *E. coli* Bm (●) (which lacks phosphoenolpyruvate carboxylase) and *E. coli* PN-1/1 (▲) (which lacks both phosphoenolpyruvate carboxylase and phosphoenolpyruvate synthase). The pyruvate was added 5min. after the beginning of the experiment and was maintained at 3–5mM throughout.

utilizable intermediates of the tricarboxylic acid cycle, results from the addition of glucose (Vanderwinkel *et al.* 1963) or glycerol (Ashworth, 1965) to cultures of such mutants. This suggests that pyruvate or a product of pyruvate metabolism inhibits the supply of C_4 acids from acetate, and hence (since pyruvate does not inhibit acetate oxidation by washed suspensions of such mutants) a component reaction of the glyoxylate cycle (Ashworth & Kornberg, 1963).

The demonstration that pyruvate is readily convertible into phosphoenolpyruvate via the action of phosphoenolpyruvate synthase (Cooper & Kornberg, 1965) also suggests that this arrest of growth on acetate is a consequence of the formation of elevated concentrations of phosphoenolpyruvate, which has been shown (Ashworth & Kornberg, 1963) to be a powerful non-competitive inhibitor of isocitrate-lyase activity. Two other observations lend credence to this view.

In the first place, mutants devoid of phosphoenolpyruvate-synthase activity (Scheme 2, [1]) are not arrested by pyruvate in their growth on acetate (Fig. 10), although, if phosphoenolpyruvate carboxylase is also lacking (Scheme 2, [1] and [2]), growth is still abolished by the addition of glucose: phosphoenolpyruvate would be expected to be formed from glucose but not from pyruvate. However, it will be apparent from Fig. 10 that, although pyruvate does not arrest the growth of phosphoenolpyruvate-synthase-less mutants, it does slow it down. This slowing down appears to be a direct effect of pyruvate, as it occurs in mutants which lack both phosphoenolpyruvate-synthase and

pyruvate-oxidase activities (Scheme 2, [1] and [3]; Fig. 11); it can be overcome by the addition of glyoxylate—the product of isocitrate-lyase activity. It is known (Ashworth & Kornberg, 1963) that phosphoenolpyruvate is a much more powerful inhibitor of isocitrate lyase than is pyruvate (Fig. 12). However, in mutants which cannot form phosphoenolpyruvate from pyruvate, high concentrations of this keto acid may also affect isocitrate-lyase activity. Indeed, even mutants constitutive for isocitrate lyase but lacking phosphoenolpyruvate-synthase activity (Table 1), which must derive their necessary biosynthetic intermediates from pyruvate entirely through the glyoxylate cycle, grow less rapidly on pyruvate than they do on acetate (Fig. 13), which shows that isocitrate lyase may be partially inhibited by pyruvate even though in such mutants this enzyme is present in amounts fivefold greater than are present in the wild-type organism during growth on acetate. But it must be stressed that, whereas the inhibition of isocitrate lyase through phosphoenolpyruvate is achieved at concentrations of this inhibitor, and in a manner, quite consistent with its postulated role as a physiological agent of 'fine' control, the effect of pyruvate on isocitrate-lyase activity is likely to be of very minor significance, if any, in the normal organism.

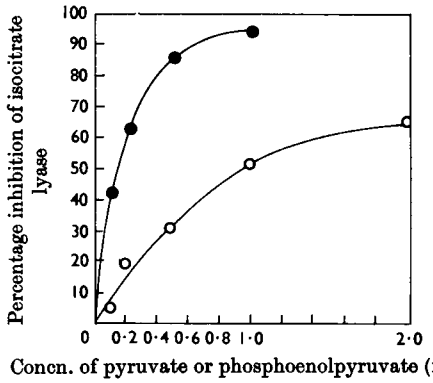


Fig. 12. Effect of pyruvate (○) or phosphoenolpyruvate (●) on the formation of glyoxylate from the enzymic cleavage of 0·5 mM-isocitrate by ultrasonic extracts of acetate-grown *E. coli* PN–1 PD⁻. The cells were disrupted by ultrasonic oscillation for 2 min. in 10 mM-tris buffer (pH 8·0)–10 mM-$MgCl_2$–1 mM-EDTA and centrifuged at 20000*g* for 5 min.; the supernatant solution was incubated for 10 min. at 30° in this buffer with 0·5 mM-isocitrate and either water, or pyruvate, or phosphoenolpyruvate. The glyoxylate formed was determined colorimetrically by the method of Kromer, Klein & Baselice (1959).

A second piece of evidence which directly supports this hypothesis has already been briefly referred to (Fig. 9). It may be recalled that mutants devoid of phosphoenolpyruvate-synthase and phosphoenolpyruvate-carboxylase activities (Scheme 2, [1] and [2]), which grow but slowly on acetate in the presence of pyruvate (Fig. 10), give rise to further mutants which now grow rapidly in the presence of pyruvate: such further mutants are also not repressed by pyruvate. However, if glucose is added to a culture of such organisms growing on acetate, growth is soon arrested (Fig. 14). That this effect is due to the inhibition of isocitrate-lyase activity is shown by the resumption of growth when glyoxylate —the product of isocitrate-lyase action—is now

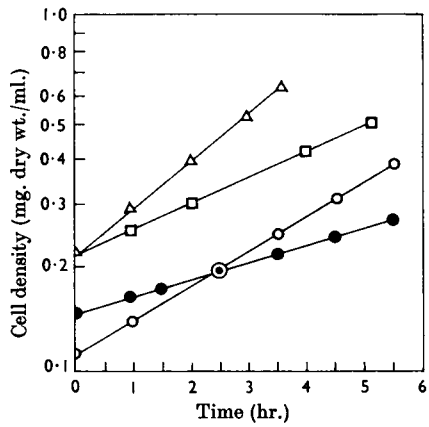


Fig. 13. Rates of growth of *E. coli* K12 (wild type) *E. coli* IL^c–1 (mutant of *E. coli* K12 which lacks phosphoenolpyruvate synthase and is constitutive for isocitrate lyase) on acetate (wild type, □; IL^c–1, ○) or pyruvate (wild type, △; IL^c–1, ●) at 30°.

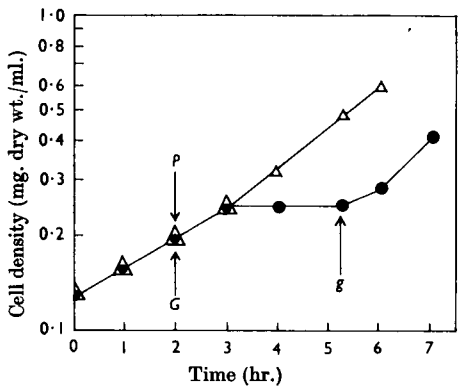


Fig. 14. Effect of 10 mM-pyruvate (△), or of 10 mM-glucose followed by 5 mM-glyoxylate (●), on the growth on acetate of *E. coli* PN–1/1 R. See Fig. 9 for details.

also added. Hence it is apparent that, in these organisms, isocitrate lyase can be inhibited by glucose or a catabolic product of glucose, but not by pyruvate. These observations are thus consistent with the view that phosphoenolpyruvate exerts *in vivo* the 'fine' control over the glyoxylate cycle which can be readily demonstrated with experiments *in vitro*.

Concluding remarks

It has been my aim, in this lecture, to survey the evidence so far available for the view that the operation of the glyoxylate cycle is both necessary and sufficient to account for the necessary replenishment of the tricarboxylic acid cycle during the growth of *E. coli* on acetate, and that the glyoxylate cycle is subject to control exerted over the synthesis and the activity of isocitrate lyase. Although much remains to be learned about these regulatory mechanisms, the available data are consistent with the hypothesis that both types of control are mediated through the intracellular concentrations of the same compound, phosphoenolpyruvate. Phosphoenolpyruvate is the starting material for gluconeogenesis, for the formation of cell-wall components and for the synthesis of aromatic amino acids, of serine, of glycine, of histidine, of the pentose moieties of nucleic acids (and doubtless of other materials); phosphoenolpyruvate is also the major C_3 compound produced from the glyoxylate and tricarboxylic acid cycles (for reviews see Krebs & Kornberg, 1957; Krebs, 1964). It is thus not unduly fanciful to regard phosphoenolpyruvate as an (if not the) end product of the glyoxylate cycle, and hence to view the control of that cycle as a further example of the negative-feedback systems so frequently encountered in living matter.

I would like to end on a personal note. You have done me great honour in naming me as the first holder of the Colworth Medal, and I am deeply grateful. In accepting this award, I wish also and gladly to acknowledge the debt that I owe to the many people who have helped me in this work. In particular, I wish to express my thanks to Dr John Ashworth, Dr Ronald Cooper and Mrs Janet Smith, with whom I have been privileged to work over the past 4 years. And I wish also to record the debt that I owe to Professor Sir Hans Krebs. It is obvious that the glyoxylate cycle would not have formed the subject of this lecture if he had not done the real pioneer work, on the tricarboxylic acid cycle, beforehand. It is also obvious to me that I would not be standing before you tonight if I had not had the benefit of Sir Hans's guidance and encouragement over the past 20 years. To adapt a remark of Isaac Newton's, I am ever aware that, if I have been so fortunate as to catch at least a glimpse of new territory, it is because I have stood on the shoulders of this giant.

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