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Alternate Pathways of Metabolism of Short-Chain Fatty Acids

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INTRODUCTION

When microorganisms are grown on various media, certain metabolites act as key intermediates in catabolic and anabolic processes. Glyoxylic acid appears to occupy such a position during the growth of *Escherichia coli* on short-chain fatty acids. Numerous investigations, many of them in our laboratories, have been carried out to determine the relationship between glyoxylate and fatty acid metabolism.

These studies were initiated by the discovery of isocitrate lyase by Smith and Gunsalus (179) and malate synthase by Wong and Ajl (221). The simultaneous action of these two enzymes produces a cyclic mechanism, the glyoxylate bypass (97); this mechanism serves to replenish C_4 acids drained from the tricarboxylic acid cycle for cellular biosynthesis when microorganisms are grown on acetate.

The central role of glyoxylate during the growth of microorganisms on acetate is now well documented. For the past several years, we have attempted to assess the importance of this metabolite during growth of *E. coli* on other short-chain fatty acids. These studies disclosed a series of new reactions involving condensations of glyoxylate with various short-chain fatty acid acyl-coenzyme A (CoA) esters. This review summarizes the metabolic pathways initiated by these condensation reactions, the regulation of condensing-enzyme formation, and the proposed significance of these pathways. It is not the primary aim of this presentation to review all of the literature concerning glyoxylate-fatty acid interactions; instead, it is our intention to emphasize and to bring up to date the findings concerning the relationship between glyoxylate and fatty acid metabolism. For this reason, the reader will find more than the usual amount of experimental detail included in this paper.

ROLE OF GLYOXYLATE DURING GROWTH ON ACETATE

Physiological Significance and Regulation of the Glyoxylate Bypass

For more than 50 years, the mechanism by which microorganisms utilize acetate as the sole carbon source during growth remained unex-

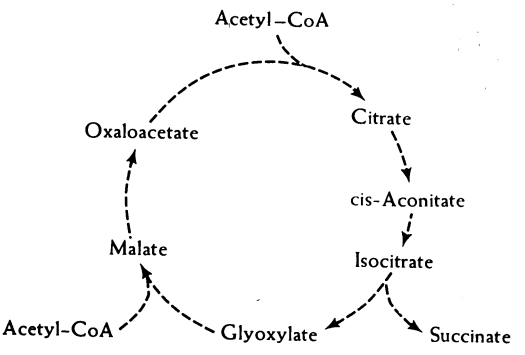


FIG. 1. *Glyoxylate cycle.*

plained. Growth cannot occur on acetate if the C_4 acids drained from the tricarboxylic acid cycle for biosynthetic reactions are not replenished. When anaerobic microorganisms grow on acetate, C_4 acids may be formed via successive carboxylation of C_2 compounds (4, 8, 134, 192–194, 219, 223); however, in aerobic microorganisms, such reactions cannot satisfy the C_4 requirement, and the intermediates are synthesized by the operation of the glyoxylate cycle (97).

The glyoxylate bypass is a shunt of the tricarboxylic acid cycle and consists of two enzymes, isocitrate lyase and malate synthase. Isocitrate lyase (20, 138, 173, 179) catalyzes the reversible aldol cleavage of $D_s(+)$ isocitrate to succinate and glyoxylate. Malate synthase (221) catalyzes the condensation of glyoxylate with acetyl-CoA to form malate. The net effect of the glyoxylate cycle (Fig. 1) is the formation of 1 mole of C_4 acid from 2 moles of acetate. The investigations which led to the formulation of the concept of the glyoxylate bypass have been summarized previously (3, 85, 94, 108, 109).

The glyoxylate bypass has been demonstrated in a variety of microorganisms (94). This mechanism has also been described in more recent studies of bacteria (32, 53, 93, 126, 128, 160, 196, 220), fungi (40, 80, 161, 197, 217), algae (27, 55, 60, 62, 63, 72, 133, 151, 190), protozoa (65, 117, 152), nematodes (163–165), and plant tissues (11, 16, 17, 23, 64, 73, 75, 124, 136, 175, 176). The operation of the glyoxylate cycle has not been demonstrated in animal tissues, although one enzyme of the bypass, malate synthase, was reported in the rat liver (47).

In the last few years, we have begun to understand the factors regulating the glyoxylate bypass (86, 88–92, 147). The operation of the glyoxylate cycle is controlled mainly by the regulation of isocitrate lyase, the first enzyme of the bypass.

As noted previously, the isocitrate lyase reaction is reversible, but the equilibrium favors isocitrate cleavage rather than isocitrate synthesis. The physiological significance of the reversible nature of the reaction has not been demonstrated, but it is conceivable that, under certain conditions (i.e., when the cell has a readily available source of glyoxylate), the enzyme may act in the direction of isocitrate synthesis. In the following discussion, the regulation and significance of the cleavage reaction are considered.

Isocitrate lyase is regulated in a crude manner by control of enzyme synthesis or more finely by control of enzyme activity. Initially, acetate was thought to directly induce isocitrate lyase formation, since high enzyme activity was apparent only during growth on acetate or acetate precursors. Kornberg (87), who studied enzyme induction in a citrate synthase-less mutant of *E. coli*, indicated that this is not the case. When this *E. coli* mutant was grown in a proline or glutamate medium, the addition of acetate did not stimulate isocitrate lyase formation. Therefore, Kornberg proposed that acetate does not directly induce isocitrate lyase but rather acts to derepress enzyme synthesis by removal of a repressor close to oxalacetate. Two isocitrate lyase enzymes are formed by *Neurospora crassa* (177); one enzyme is derepressed by acetate, whereas the other enzyme is constitutive.

By use of a variety of metabolic mutants, Kornberg showed that, in *E. coli*, phosphoenolpyruvate (PEP) is a repressor of isocitrate lyase (88, 91, 92); however, it is likely that enzyme synthesis is also controlled by metabolites other than PEP. In *Mima polymorpha* (12), acetate induced isocitrate lyase in succinate growth media; furthermore, succinate itself appeared to weakly induce enzyme formation. Similar findings have been reported for *Achromobacter* (162) and *Pseudomonas indigofera* (127). In *Tetrahymena pyriformis* (117), the control of isocitrate lyase was very sensitive to changes in the culture. When this organism was grown aerobically in peptone media, the level of isocitrate lyase was low; enzyme activity was only slightly increased by subsequent aerobic incubation in acetate media. In contrast, incubation under static conditions resulted in high enzyme activity, and, in the latter cultures, the addition of acetate did not further promote enzyme formation. Thus, it appears that, in *Tetrahymena*, the levels of intracellular metabolite repressors of isocitrate lyase are controlled more by changes in the conditions of the cultures, particularly the oxygen content, than by the presence of acetate.

One must exercise caution, therefore, in attempting to generalize about the regulation of the glyoxylate bypass. It is likely that multiple regulatory controls exist for both isocitrate lyase and for malate synthase and that different organisms will be shown to exhibit different regulatory behaviors.

Phosphoenolpyruvate is not only thought to be a repressor of isocitrate lyase formation but it also has been reported to inhibit the activity of this enzyme noncompetitively when assayed in cell-free extracts of acetate-grown *E. coli* (6). Data obtained by use of mutants lacking PEP carboxylase or PEP synthase suggest that PEP also inhibits enzyme activity in vivo (91). When the mutant which is incapable of carboxylating PEP to oxalacetate was grown on acetate, the addition of pyruvate to the medium resulted in cessation of growth. Under these conditions, PEP accumulation was considered to inhibit isocitrate lyase activity and thus to arrest the utilization of acetate for growth. In contrast, cultures of PEP synthase-less mutants, which lacked the ability to form PEP from pyruvate, continued to grow on acetate after the addition of pyruvate.

The in vitro inhibition of isocitrate lyase by PEP appears to be influenced by the nature of the buffer employed for enzyme assay. Preliminary experiments (R. Rabin, H. C. Reeves, and S. J. Ajl, *unpublished data*) suggest that, although PEP is a powerful inhibitor of isocitrate lyase purified from *P. aeruginosa* assayed in imidazole buffer, PEP only weakly inhibits enzyme activity in phosphate or tris(hydroxymethyl)aminomethane (Tris)-chloride buffer. Rao and McFadden (145) reported that, in addition to PEP, several other phosphorylated compounds also inhibit isocitrate lyase. When the purified enzyme obtained from *P. indigofera* was assayed in Tris-chloride buffer at an inhibitor concentration of 1 mM, PEP, fructose-1,6-diphosphate, and 3-phosphoglyceric acid inhibited isocitrate lyase at rates of 20, 19, and 25%, respectively. In addition, a number of dicarboxylic acids, including succinate, maleate, itaconate, glycolate, and oxalate, also inhibited enzyme activity (145). Further studies by Reeves and Ajl (*unpublished data*) suggest that phosphocreatine, uridine diphosphoglucose, and guanosine monophosphate also inhibit isocitrate lyase. A number of other phosphorylated compounds were tested, but they proved to be noninhibitory.

Less is known about the regulation of malate synthase. In *E. coli* and *Pseudomonas* sp. malate synthase is repressed by products of glucose catabolism and by growth on tricarboxylic acid intermediates, but the degree of repression is less severe than for isocitrate lyase (86, 94). The factors which regulate enzyme activity are unknown. As in the case of isocitrate lyase, however, it is difficult to generalize about the regulation of malate synthase, since the degree of fluctuation in the activity of this enzyme varies considerably in different organisms.

The mechanism of the malate synthase reaction was studied recently by Eggerer and Klette (36). Essentially, the enzyme catalyzed the enolization of acetyl-CoA and the hydrolysis of malyl-CoA. The enolization of acetyl-CoA was dependent on Mg^{++} or other divalent ions and was greatly stimulated by glyoxylate or other α-ketoacids. In the natural system, the acetyl-CoA carbanion immediately reacted with the carbonyl group of the glyoxylate-Mg^{++}-enzyme complex to form enzyme-bound malyl-CoA, which was then hydrolyzed to the free acid. No acetyl-enzyme was formed between acetyl-CoA and malate synthase.

Two malate synthase enzymes have been demonstrated in *E. coli* (38, 39, 203–205); one enzyme is formed during growth on acetate, and the other enzyme is formed during growth on glycolate. This is of interest since malate synthase has an anaplerotic function in the glyoxylate bypass but a respiratory function in the dicarboxylic acid cycle during growth on glycolate (86).

The malate synthase formed during growth on acetate appeared to be controlled by a derepression mechanism, whereas the enzyme formed during growth on glycolate may be induced by glyoxylate (203). An interesting observation relevant to this concept was made in *Rhizopus nigricans* (218). When this fungus was grown in a glucose medium and then transferred to either an acetate or a glycolate medium, malate synthase was induced. The addition of Zn^{++} to the acetate medium markedly facilitated enzyme induction but did not stimulate enzyme formation in the glycolate medium. This metal may stimulate growth of *Rhizopus* via a primary effect on RNA synthesis (216). In addition Zn^{++} was shown to relieve glucose repression and to stimulate acetate derepression of isocitrate lyase formation (217). It is proposed that (i) Zn^{++} stimulates acetate derepression of malate synthase formation by facilitating the utilization of repressor metabolites, and (ii) the fact that Zn^{++} does not stimulate enzyme formation in glycolate media suggests that glyoxylate induction is not accomplished by a similar mechanism. In *Rhizopus* (218), as in *E. coli* (39), the malate synthase enzymes formed during growth on glycolate and acetate, respectively, can be differentiated by the rate of thermal inactivation.

CONDENSATIONS OF GLYOXYLATE WITH OTHER SHORT-CHAIN FATTY ACIDS

Elucidation of the Condensation Reactions

Attempts have been made to determine whether dicarboxylic acids having higher molecular weights than malic acid are formed by reactions analogous to that catalyzed by malate synthase. The condensations studied are shown in Fig. 2.

The first reaction, that of glyoxylate with acetyl-

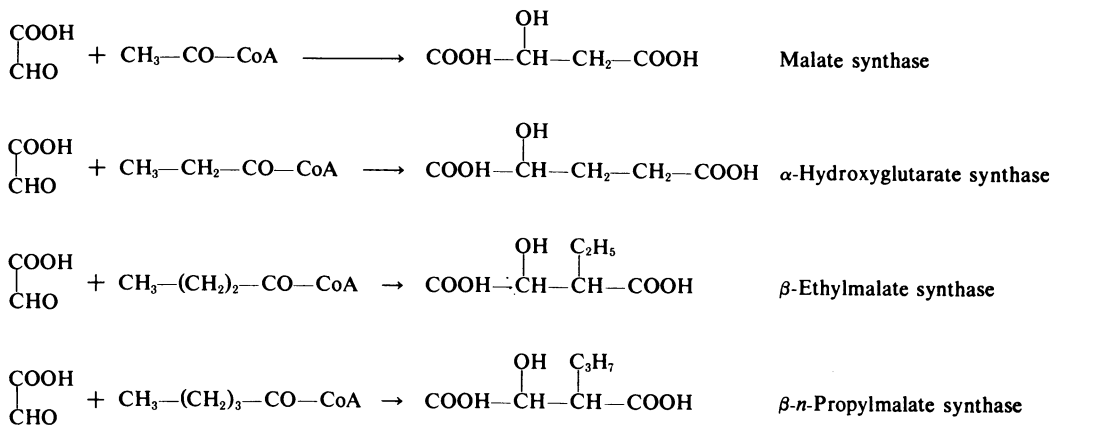


FIG. 2. *Condensation of glyoxylate with a series of short-chain fatty acid acyl-CoA esters.*

CoA to form malate, was discussed. The condensation of glyoxylate with propionyl-CoA to form α-hydroxyglutarate was first demonstrated in extracts of propionate-grown *E. coli* (148). This product was identified as the free acid by paper chromatography (154) and as the lactone derivative by thin-layer chromatography (14). An interesting aspect of this condensation concerns the reaction mechanism. If α-hydroxyglutarate is the initial product, then glyoxylate must condense with the β-carbon of propionyl-CoA. However, a β-carbon condensation is less likely chemically than is an α-carbon condensation. Condensation of glyoxylate with the α-carbon of propionyl-CoA would yield β-methylmalate. Under the conditions employed, however, this product was not detected; furthermore, neither β-methylmalate, β-methylmalate plus CoA.SH and ATP, nor β-methylmalyl-CoA was metabolized to α-hydroxyglutarate (154). These findings were confirmed recently by Trop and Pinsky (195).

It is possible, however, that the condensation of propionyl-CoA with glyoxylate may proceed in a manner analogous to that of fatty acid biosynthesis (153). For example, propionyl-CoA may condense with glyoxylate by an α-carbon condensation mechanism to yield β-methylmalate as a protein-bound intermediate. This protein-bound β-methylmalate may isomerize to form protein-bound α-hydroxyglutarate, and this substance may then be released as the free acid.

The next condensation reaction shown, that of glyoxylate with butyryl-CoA to form β-ethylmalate, was first demonstrated in extracts of *P. aeruginosa* grown in butyrate-mineral salts media (142). Subsequently, it was shown that *E. coli* also catalyzes this reaction. β-Ethylmalate is the product of an α-carbon condensation and has been identified by paper chromatography as both the free acid and the 5-ethyluracil derivative.

The last reaction shown is the condensation of glyoxylate with *n*-valeryl-CoA to form β-*n*-propylmalate (66). This product was demonstrated by use of extracts of valerate-grown *E. coli* and was identified by paper chromatography as the free acid; it is also the product of an α-carbon condensation.

The condensations of glyoxylate with propionyl-, butyryl-, and valeryl-CoA are analogous to that catalyzed by malate synthase, but several important differences should be noted. First, the activities of α-hydroxyglutarate and β-ethylmalate synthase formed during growth on propionate and butyrate, respectively, are considerably lower than the activity of malate synthase found in acetate-grown cells. Moreover, growth on propionate and butyrate also results in high activity of malate synthase, suggesting that these substrates may be metabolized principally via acetate (209). The formation of these synthase enzymes during growth on valerate will be considered later. There is indirect evidence (143) that these condensation products are formed as acyl-CoA or protein-bound esters which yield free acids by hydrolysis rather than as free acids themselves.

The technique of differential heat inactivation was used to determine whether these condensation reactions are catalyzed by a single enzyme possessing broad fatty acid acyl-CoA specificity. The reactions were assayed by measuring the respective fatty acid acyl-CoA-dependent utilization of ^{14}C-glyoxylate (210). The respective activities exhibited markedly different rates of thermal inactivation (211) when crude enzyme extracts of valerate-grown *E. coli* were used. In addition, malate and β-*n*-propylmalate synthases were formed under growth conditions in which α-hydroxyglutarate and β-ethylmalate synthase activity can not be detected (211). These data suggest that the condensations are not catalyzed by a single enzyme possessing a single

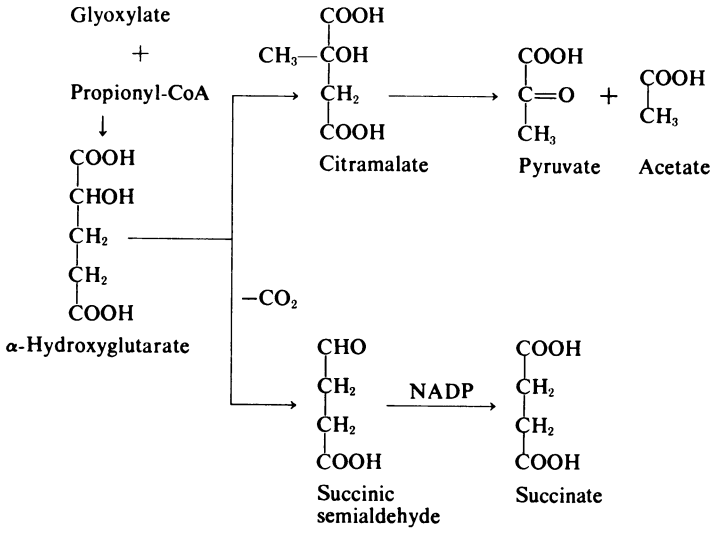


FIG. 3. *Further metabolism of α-hydroxyglutarate.*

catalytic site; however, the data do not preclude the possibility of two or more enzymes possessing multiple catalytic sites.

FURTHER METABOLISM OF THE CONDENSATION PRODUCTS

α-Hydroxyglutarate

α-Hydroxyglutarate may be metabolized by at least two alternate pathways (Fig. 3). The first pathway involves a decarboxylation of α-hydroxyglutarate to form succinic semialdehyde (155), which is further oxidized to succinate. Succinate may also be formed from α-hydroxyglutarate via an NAD-dependent oxidation of α-hydroxyglutarate to α-ketoglutarate. Attempts to demonstrate the formation of α-ketoglutarate from α-hydroxyglutarate in *E. coli* have been unsuccessful, but this reaction was demonstrated in *Rhodopseudomonas spheroides* (137).

A second pathway for the metabolism of α-hydroxyglutarate involves an isomerization followed by aldol cleavage. Early experiments showed that incubation of α-hydroxyglutarate, CoA.SH, ATP, and extracts of propionate-grown *E. coli* resulted in the formation of acetate plus lactate (149). This result was clarified by the finding that the above extracts catalyzed the formation of citramalic acid (α-methylmalate) from glyoxylate and propionyl-CoA (143). Citramalyl-CoA is known to be metabolized to acetate plus pyruvate by aldol cleavage (28). It was proposed that α-hydroxyglutarate is isomerized to citramalate and then cleaved to pyruvate and acetate. In the crude extracts employed, pyruvate probably was reduced to lactate. It is likely that this enzymatic sequence involved acyl CoA-ester intermediates rather than free acids. In these reactions, the L (+) isomer, but not the D (−) isomer, of α-hydroxyglutarate was biologically active.

An interesting aspect of the latter pathway is the further metabolism of lactate itself. Extracts of propionate-grown *E. coli* catalyzed the conversion of lactate, as the acyl-CoA ester, to hydroxypyruvic aldehyde (150). Pyruvyl-CoA (129) appeared to be an intermediate in this sequence. Subsequently, hydroxypyruvic aldehyde was metabolized to 1,3-diphosphoglyceric acid (150). The latter reaction was accomplished by glyoxylase I and II, phosphoglycerokinase, and 3-phosphoglycerokinase. Although the significance of these reactions has not been assessed, such a pathway may be important for cellular biosynthesis during growth on substrates metabolized via lactate.

β-Ethylmalate

β-Ethylmalic acid is the product of the condensation of glyoxylate with the α-carbon of butyryl-CoA (142). The further metabolism of this substance is diagrammed in Fig. 4. α-Ethylmalic acid is also formed when glyoxylate and butyryl-CoA are incubated with extracts of *P. aeruginosa* and when β-ethylmalic or ethylmaleic acids are used as substrates (141, 144). The *trans* isomer, ethylfumaric acid, is not enzymatically hydrated. Two pairs of racemates of β-ethylmalic acid, *threo* and *erythro*, are formed during chemical synthesis (144). Both pairs have been separated, crystallized, identified by NMR analysis, and tested as substrates for enzymatic isom-

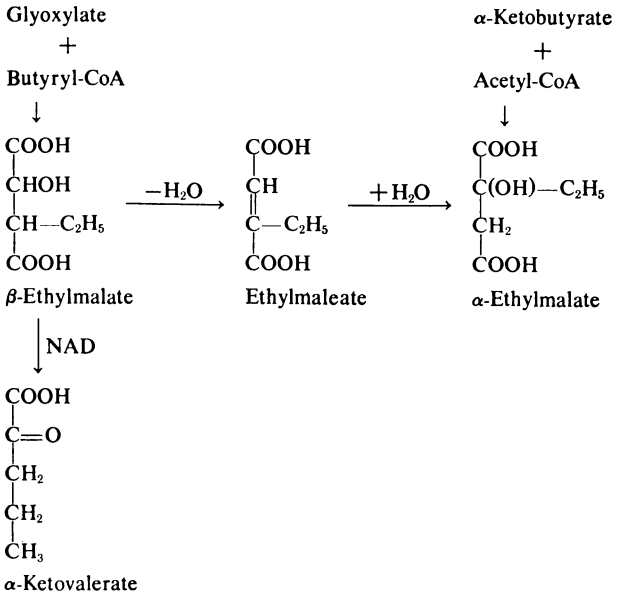


FIG. 4. *Further metabolism of β-ethylmalate.*

erization and oxidation. Only one racemate, the DL-erythro-β-ethylmalic acid, is biologically active (R. Rabin, *unpublished data*).

It was originally thought that β- and α-ethylmalic and ethylmaleic acids were metabolized as CoA esters (143). However, the ease with which ethylmaleic acid is hydrated, the inability of the CoA thiol ester of β-ethylmalic acid to be oxidized (whereas the free acid is readily oxidized), and the formation of free α-ethylmalic acid from acetyl-CoA and α-ketobutyrate argue against involvement of CoA esters of these compounds as substrates. β-Ethylmalic acid is oxidized to α-ketovalerate by an NAD-linked dehydrogenase which requires Mg^{++} or Mn^{++} and K^{+} and is optimally active at *p*H 9.0 (144).

These reactions are not unique for α- or β-ethylmalate but are similar to the reactions which occur during leucine biosynthesis (18, 56, 74, 187, 207). Experiments on cofactor requirements, heat inactivation, and gel electrophoresis indicate that the oxidation of β-ethylmalate to α-ketovalerate is probably catalyzed by β-isopropylmalate dehydrogenase. Similarly, the formation of α-ethylmalate by the condensation of acetyl-CoA with α-ketobutyrate appears to be catalyzed by α-isopropylmalate synthase. This conclusion is based on the observed strong inhibition of the condensations by L-leucine, failure to demonstrate reversibility with free α-ethylmalic acid, and incomplete specificity of the reaction with respect of α-keto acid substrates. Although the conversion of β- to α-ethylmalate has not been examined in detail, this reaction also appears to be similar to the isomerization of β- to α-isopropylmalate. In both instances, only the *cis* isomer of the unsaturated acid is hydrated to the α-substituted malic acid, and the apparent equilibrium favors the formation of the α-substituted compound rather than the β-substituted compound. These findings (144) suggest that ethyl-substituted malic acids are metabolized by enzymes which are very similar, if not identical, to those of the leucine pathway.

It is also of interest that β-*n*-propylmalate is formed from the enzymatic condensation of glyoxylate with valeryl-CoA (66), and α-*n*-propylmalate results from the condensation of acetyl-CoA with α-ketovalerate (188). These findings indicate that a metabolic relationship may exist between the α- and β-*n*-propylmalic acids which is similar to that found between the α- and β-ethylmalic acids. β-*n*-Propylmalate may be metabolized to *cis-enol* oxalactate, but the intermediates in this conversion are unknown (67).

Although these observations do not define the role of the ethylmalic acids in bacterial metabolism, Ingraham et al. (69) suggested that the following reactions form part of the pathway of *n*-butyl alcohol formation by glucose-fermenting yeast: acetyl-CoA + α-ketobutyrate → α-ethylmalate → β-ethylmalate → α-ketovalerate. α-Ketovalerate then is decarboxylated and reduced to form *n*-butyl alcohol by well-known reactions. By a similar process, higher alcohols, such as *n*-amyl and isoamyl, are also formed (25, 59, 68,

TABLE 1. *Formation of condensing enzymes during growth on short-chain fatty acids*[a]

Carbon source	Specific activity[b]			
	Malate synthase	α-Hydroxyglutarate synthase	β-Ethylmalate synthase	β-*n*-Propylmalate synthase
Acetate.....	63.2	—[c]	—[c]	12.9
Propionate...	59.6	5.9	4.2	13.1
Butyrate.....	61.2	5.6	6.2	15.2

[a] *E. coli* E-26 was cultured as indicated and was assayed for the various glyoxylate-condensing enzymes. From Wegener et al. (209).

[b] Measured as mμmoles of glyoxylate-*1*-^{14}C utilized per 15 min per mg of protein.

[c] No activity detectable.

206). In the intact rat and in rat-liver homogenates, α-ketovalerate is metabolized to butyrate and then to acetate (78). Significant formation of *n*-butyl alcohol and the higher alcohols would not be expected in *P. aeruginosa*. Thus, the metabolism of β-ethylmalate and perhaps of β-*n*-propylmalate may merely reflect a broad specificity of the enzymes of the leucine pathway, a characteristic that may be of relatively minor consequence in nonfermentative metabolism. On the other hand, it is also possible that β-ethylmalate is a precursor of the amino acid norvaline. Norvaline is catabolized by both yeast (206) and the rat (78), and, under certain conditions, it may serve as a substrate for endogenous metabolism in bacteria (33, 130).

FACTORS REGULATING FORMATION OF THE GLYOXYLATE-CONDENSING ENZYMES

Effect of Carbon Source on Formation of the Condensing Enzymes

Unlike malate synthase, which is induced by growth on acetate, the other condensing enzymes are not induced by their respective fatty acid substrates (209). Growth of *E. coli* on propionate or butyrate results in the formation of all of the described enzymes (Table 1). Although such conditions effect high activity of malate synthase, only low activities of α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases are formed. If these enzymes were induced by their respective fatty acid substrates, one would expect that, during growth on propionate or butyrate, malate synthase would be induced by acetate, since propionate and butyrate probably are metabolized via this substrate. However, one would expect α-hydroxyglutarate synthase, not β-ethylmalate or β-*n*-propylmalate synthase, to be formed during growth on propionate, since it is unlikely that growth on this substrate results in appreciable accumulations of butyric or valeric acids. Similarly, one would expect β-ethylmalate synthase, not α-hydroxyglutarate or β-*n*-propylmalate synthase, to be formed during growth on butyrate.

When cells are grown on acetate, α-hydroxyglutarate and β-ethylmalate synthase activity cannot be detected, but such conditions do result in malate and β-*n*-propylmalate synthase activity. It is not clear why growth on propionate or butyrate, but not on acetate, results in the formation of low activities of α-hydroxyglutarate and β-ethylmalate synthases. It is possible that growth on the former substrates gives rise to a small percentage of mutant cells which possess high activities of these enzymes. The low activity of β-*n*-propylmalate synthase formed under all of these conditions may represent a constitutive level of enzyme formation.

In contrast to propionate and butyrate, growth of *E. coli* on valerate results in high activity of α-hydroxyglutarate synthase (Table 2). Growth on caproate, which would be expected to be metabolized by β-oxidation to acetate plus butyrate, resulted in enzyme activity comparable to that found on butyrate, whereas growth on heptanoate, which would be expected to be metabolized by β-oxidation to acetate plus valerate, was similar to growth on valerate (209).

Selection of Constitutive Mutants During Growth on Valerate

Since growth on valerate results in high activity of the glyoxylate-condensing enzymes, it was of

TABLE 2. *Effect of carbon source on formation of α-hydroxyglutarate synthase*[a]

Carbon soure	Specific activity of α-hydroxyglutarate synthase[b]
Glucose.....	—[c]
Succinate.....	—[c]
Lactate.....	—[c]
Acetate.....	—[c]
Propionate.....	4.7
Butyrate.....	4.3
Valerate.....	48.1
Caproate.....	6.1
Heptanoate.....	42.6

[a] *E. coli* E-26 was cultured as indicated and assayed for α-hydroxyglutarate synthase. From Wegener et al. (209).

[b] Measured as mμmoles of glyoxylate-*1*-^{14}C utilized per 15 min per mg of protein.

[c] No activity detectable.

TABLE 3. *Effect of cell type on formation of α-hydroxyglutarate synthase*[a]

Carbon source	Cell type	Specific activity[b]			
		Malate synthase	α-Hydroxyglutarate synthase	β-Ethylmalate synthase	β-n-Propylmalate synthase
Acetate	E-26	63.9	—[c]	—[c]	12.9
	E-26V	64.5	39.1	41.6	58.7
Propionate	E-26	60.7	4.1	3.0	10.7
	E-26V	61.1	45.6	39.6	56.6
Glucose	E-26	48.7	—[c]	—[c]	3.2
	E-26V	50.1	29.6	22.3	45.1

[a] *E. coli* E-26 and E-26V were cultured as indicated and were assayed for the various glyoxylate-condensing enzymes. From Wegener et al. (209).

[b] Measured as mμmoles of glyoxylate-*1*-^{14}C utilized per 15 min per mg of protein.

[c] No activity detectable.

interest to determine whether this fatty acid induced enzyme formation or whether such growth conditions resulted in the production of a mutant population. The data shown in Table 3 indicate that the latter possibility is true. Wild-type *E. coli* E-26 was employed in these studies; E-26V was derived from E-26 by culture in a valerate medium. In contrast to E-26, E-26V showed high activities of α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases when cultured in acetate, propionate, or glucose media. Extensive subculture of E-26V in complex media did not result in a loss of the capacity to form these enzymes. It is proposed that growth of *E. coli* on valerate results in the formation of a mutant strain (E-26V) which is permanently derepressed with respect to formation of α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases.

E-26 and E-26V form equal activities of malate synthase; earlier studies (146) indicated that this enzyme is constitutive in *E. coli* E-26. These studies also suggested that there may be two β-*n*-propylmalate synthases; one may be associated with a constitutive malate synthase, whereas the other may be coordinantly regulated with α-hydroxyglutarate and β-ethylmalate synthase. A genetic analysis of the relationship between these enzymes is essential for a more complete interpretation of these data.

An interesting aspect of growth on valerate is that the cell population formed is markedly influenced by inoculum size (Fig. 5). Cells cultured in valerate media from a high inoculum form principally small colonies when plated on valerate-agar, whereas cells cultured from a low inoculum primarily give rise to large colonies on valerate-agar (209). The respective colony types differ in the ability to form the condensing enzymes (Table 4). The large-colony type is considered mutant and is designed at E-26V-M; the small-

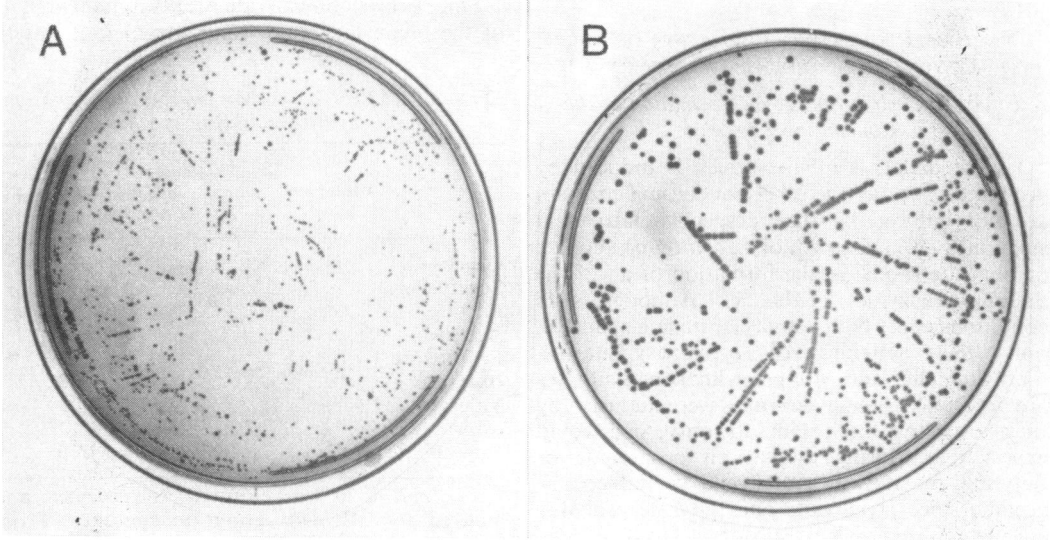


FIG. 5. *Differentiation of "adapted" and mutant cells on valerate-agar. (A) Plated from a valerate culture grown from a small inoculum of Escherichia coli E-26 (initial turbidity 0 to 1 Klett units). (B) Plated from a similar culture grown from a large inoculum (initial turbidity 20 Klett units). From Wegener et al. (209).*

TABLE 4. *Comparison of properties of "adapted" and mutant cells*[a]

Cell type	Colony size on valerate-agar	Specific activity of α-hydroxy-glutarate synthase[b]	Time required for growth in valerate media
E-26	—[c]	—[c]	180–190
E-26V-M	Large	28–31	24–30
E-26V-A	Small	3–5	90–100
E-26V-A*[d]	—[c]	—[c]	180–190

[a] *E. coli* E-26 was cultured in valerate medium and then was plated on valerate-agar. Appropriate colony types were selected, cultured in glucose-mineral salts media, and assayed for α-hydroxy-glutarate synthase. The ability of these colony types to initiate growth when transferred from Trypticase Soy Broth to a valerate-mineral salts medium was also determined. From Wegener et al. (209).

[b] Measured as mμmoles glyoxylate-*1*-^{14}C utilized per 15 min per mg of protein.

[c] No activity detectable; no growth detectable.

[d] E-26V-A* was derived by subculture of E-26V-A on Trypticase Soy Broth followed by reisolation on Trypticase Soy Agar.

colony type will be referred to as an adapted population and is designated E-26V-A. Although mutant colonies formed high activity of α-hydroxyglutarate synthase when cultured in glucose media, the adapted-cell type formed only low enzyme activity under these conditions.

The E-26V-A isolates, when subcultured in complex media and reisolated (designated E-26V-A*), showed no detectable α-hydroxy-glutarate synthase activity. As discussed later, valerate-adapted cultures give rise to the mutant cell type at a high frequency. The low activity of α-hydroxyglutarate synthase in glucose cultures of the E-26V-A cell type probably reflects the formation of a low percentage of mutants from the adapted cells, and the adapted cells are probably indistinguishable from E-26 with respect to α-hydroxyglutarate synthase activity. The E-26V-M, E-26V-A, and E-26V-A* isolates also exhibited differential growth rates in valerate-mineral salts media. E-26V-A grew quickly on valerate medium; however, it grew less quickly than did E-26V-M but had a shorter lag than did E-26; E-26V-A* grew at the same rate as did E-26.

In summary, growth on valerate may give rise to at least two different cell populations. Valerate mutants form large colonies on valerate-agar, possess a short lag on valerate-mineral salts medium, and are constitutive for the formation of α-hydroxyglutarate synthase. Such cells can be subcultured extensively in complex medium without the loss of these characteristics. Valerate-adapted cells form low α-hydroxyglutarate synthase activity in glucose media and, when subcultured in complex medium and reisolated, lose both the capacity to grow with a short lag on valerate medium and the capacity to form α-hydroxyglutarate synthase (209).

Studies on the Mechanism of Mutagenesis During Growth on Valerate

The mechanism by which growth on valerate results in the formation of a mutant population is not clearly understood. Mutagenesis on valerate does not occur simply by selection of a spontaneously occurring mutant; instead, it appears to involve the intermediate formation of a valerate-"adapted" population which exhibits a high frequency of conversion to the mutant cell type (46, 209).

By use of a Luria-Delbrück fluctuation analysis (120), the valerate-independent rate of mutation of E-26 to the E-26V cell type was calculated to be about 5×10^{-10} mutations per cell per generation (46). It is unlikely, therefore, that the selection of a spontaneously occurring mutant by valerate can account for the high rate of mutagenesis observed during growth in valerate medium. Under the same conditions, valerate-adapted cells were converted to the E-26V mutant cell type at a very high frequency. These results suggest that cells which are "adapted" to growth on valerate possess a genotypically altered capacity to undergo conversion to the mutant cell type.

The E-26V mutant grows with only a short lag when cultured on various fatty acids, whereas E-26 exhibits an extremely long lag when cultured on these substrates. This difference was employed to study the time course of mutant formation during growth on valerate (Fig. 6). In this experiment, E-26 was cultured in valerate medium from a low inoculum, and, at various intervals during growth, samples were removed, transferred to Trypticase Soy Broth, and then cultured in tubes of valerate and butyrate media. Cells removed from the initial valerate culture during the exponential phase of growth grew quickly when subsequently transferred to either valerate or butyrate media, whereas samples removed after up to 50 hr of incubation were similar to the parent E-26 strain. In contrast, samples removed after 50 to 180 hr of incubation showed a progressively increased capacity to grow on valerate but not on butyrate.

These data suggest that cumulative exposure of E-26 to valerate causes a genotypic change which results in a high frequency of conversion of cells to the mutant cell type. This alteration is

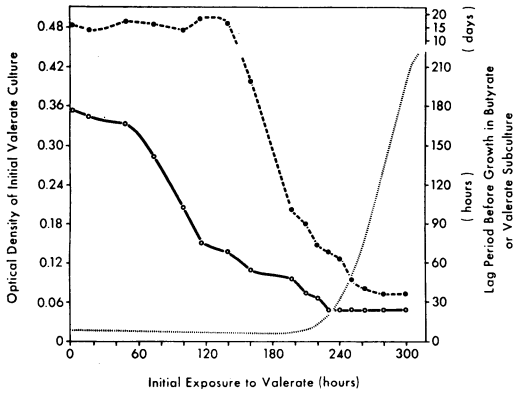


FIG. 6. *Cumulative effect of valerate on the formation of the E-26V mutant. The growth curve of E. coli E-26 in valerate media is shown by the dotted line. At various intervals, samples of the culture were removed, subcultured on Trypticase Soy Broth, and then cultured in valerate and butyrate media. Secondary growth time (right ordinate) expresses the time required for the respective samples to initiate growth in valerate* (○) *and butyrate* (●) *media. From Furmanski et al. (46).*

a function of valeric acid specifically, and, after incubation in valeric acid, the change is maintained as a stable characteristic, even when cells are subsequently subcultured in complex media. It is suggested (46) that, initially, the specific effect of valerate is to produce an "adapted cell" population; this population then undergoes a change, independently of valerate, to produce a mutant cell. Since it is difficult to conceive of valerate as a specific mutagen active on cells in an additive manner, this property must be characteristic of adapted cells per se. The precise nature of the "adapted" cell population is obscure.

Earlier, the assumption was made that the properties characterizing the valerate-grown population resulted from a genotypic alteration. However, it must be pointed out that there is no proof that a genetic change has taken place. This fact can only be established by genetic recombination experiments. Therefore, it is not possible to disregard the alternative possibility that the properties of valerate-grown cells are the consequence of a valerate-dependent phenotypic alteration which is subsequently maintained in the absence of this fatty acid. This type of "irreversible induction" phenomenon may explain the high rate of reversion of glycine-dependent *E. coli* mutants to glycine independence in cells cultured in media containing certain α-hydroxy acids (224). It is not our intent to endorse this explanation for our results, although we have not ruled it out as a possibility.

Further Properties of Valerate Mutants

E-26 and E-26V were compared with respect to the activities of a number of other enzymes, but no significant differences were found (209). Likewise, E-26 and E-26V exhibited similar growth rates in acetate, glycolate, lactate, glucose, and succinate media. However, as noted previously, these strains showed marked differences when cultured in mineral salts media containing various short-chain fatty acids (Table 5). It is of interest that the "valerate-adapted" population grew quickly on valerate, but did not possess a selective advantage on other short-chain fatty acids (209).

Because of the low activities of α-hydroxyglutarate and β-ethylmalate synthases formed during growth of the parent E-26 strain on propionate and butyrate, the metabolic significance of these enzymes in the parent strain is questionable. However, the activities of these enzymes were significantly higher when the mutant E-26V strain was grown on these substrates, and, in addition, this mutant displayed a much shorter lag on propionate, butyrate, valerate, caproate, and heptanoate than did the parent strain. Although it is doubtful that these enzymes are essential to the growth of E-26V on short-chain fatty acids, it is possible that they function in initiation of growth by providing alternate pathways of fatty-acid metabolism. The role of α-hydroxyglutarate synthase during growth on propionate is discussed in the next section. It is also possible, however, that the E-26V mutant possesses genotypically modified physiological properties which are unrelated to glyoxylate metabolism. This may apply particularly to the selective

TABLE 5. *Comparison of E-26 and E-26V in regard to growth on short-chain fatty acids*[a]

Growth substrate	Duration of lag	
	E-26	E-26V
	days	*hr*
Propionate	3–4	36
Butyrate	5–15[b]	24
Valerate	5–6	24
Caproate	5–15[b]	24
Heptanoate	5–6	24

[a] Suspensions of *E. coli* E-26 and E-26V which were grown in Trypticase Soy Broth were cultured in mineral salts medium containing the fatty acid (0.20%) indicated. Duration of lag is the time required to effect an increase in turbidity from 0 to 1 to 10 Klett units.

[b] Varies considerably.

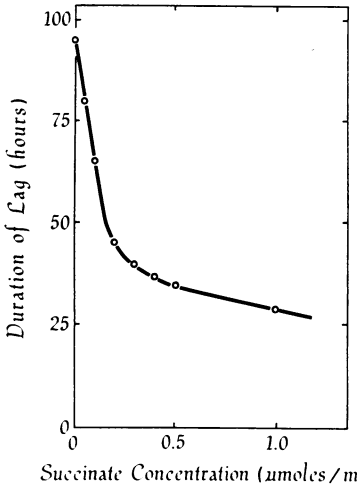


FIG. 7. *Effect of succinate addition on adaptation to propionate. Washed suspensions of Escherichia coli E-26 grown on Trypticase Soy Broth were inoculated into 0.20% propionate media containing various concentrations of succinate. The duration of lag expresses the time required to effect an increase in turbidity from 0 to 1 to 10 Klett units. From Wegener et al. (213).*

growth advantage of E-26V on butyrate and valerate, since preliminary observations suggest that this mutant possesses an increased capacity to oxidize these substrates (Wegener and Ajl, *unpublished data*).

FACTORS REGULATING GROWTH ON PROPIONATE

To study the function of these condensing enzymes, we undertook a study of the factors influencing the growth of *E. coli* on short-chain fatty acids. Although the metabolism of propionic acid has been extensively studied in a variety of systems (22, 52, 70, 118, 159, 182, 183, 189, 199, 226), little is known of the mechanisms by which aerobic organisms, such as *E. coli*, grow when this substrate is supplied as the sole carbon source.

C_4 Acid Requirement for Growth Initiation During Adaptation to Propionate

First, we considered why there is such a long lag before *E. coli* grows in propionate-mineral salts media. This extended lag is apparent only during adaptation to propionate; when cultures are grown on this substrate and then are washed and inoculated into a fresh propionate medium, growth is initiated quickly. Growth on propionate is an adaptive rather than a mutational process, since such cultures lose the capacity to initiate rapid growth on this substrate after subculture in complex media (213).

To determine whether growth initiation is limited by the capacity of cells to form C_4-acid intermediates from propionate, the experiment shown in Fig. 7 was performed (213). It is evident that the lag preceding growth can be considerably reduced by adding limiting concentrations of succinate to propionate media. Although these concentrations of succinate are by themselves sufficient to support only marginal growth, such cultures grew luxuriously and, moreover, exhibited a monophasic pattern of growth. The ability to shorten the lag during adaptation to propionate is not specific for succinate but is met by a variety of substrates including glucose and lactose. These sugars often cause catabolite repression of enzyme formation, but, in this system, they appear to provide a pool of intermediary metabolites necessary for enzyme induction. The addition of α-hydroxyglutarate to propionate media also stimulated growth (213).

The following observations appear to be related: (i) E-26V mutants form high activity of α-hydroxyglutarate synthase and exhibit a shortened lag during growth on propionate; (ii) α-hydroxyglutarate is metabolized to succinate; (iii) both

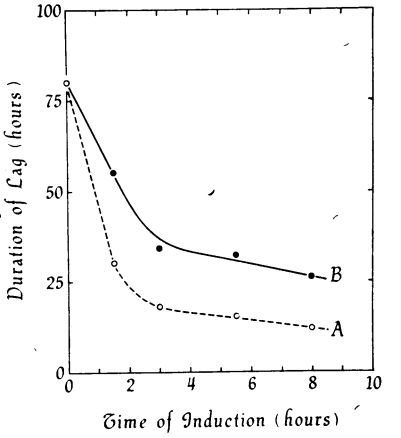


FIG. 8. *Adaptation to growth on propionate. In system A, Escherichia coli E-26 was grown on succinate and then inoculated into a medium containing 0.20% propionate and 0.01% succinate. At the times indicated, samples of the culture were withdrawn, washed, and inoculated into unsupplemented propionate media. System B was identical, except that the inducing medium contained 0.20% succinate and 0.01% propionate. The duration of lag expresses the time required in unsupplemented propionate media to effect an increase in turbidity from 0 to 1 to 10 Klett units. From Wegener et al. (213).*

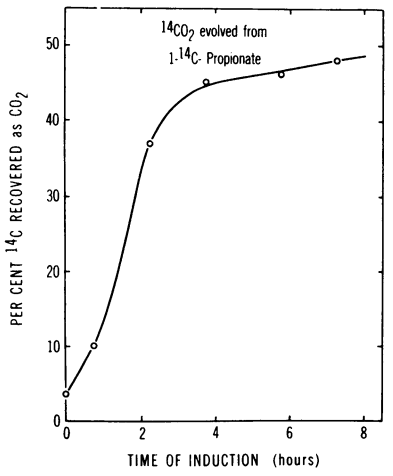


FIG. 9. *Adaptation to oxidize propionate. A succinate-grown suspension of Escherichia coli E-26 was inoculated at high initial turbidity into a medium containing 0.20% propionate and 0.01% succinate. At the times indicated, samples of the culture were withdrawn, and the cells were harvested, washed, and incubated in mineral salts buffer containing propionate-1-^{14}C. The incubation mixture was aerated at 37 C, and respired $^{14}CO_2$ was trapped by bubbling effluent air into tubes containing hyamine hydroxide. To quantitate the $^{14}CO_2$ which evolved, portions of the trapping solution were analyzed (212) by use of a liquid scintillation spectrometer. The time of incubation in the inducing medium is plotted versus the percentage of the initial propionate-^{14}C activity recovered after a 20-min incubation period. From Wegener et al. (213).*

α-hydroxyglutarate and succinate, when added to propionate media, considerably reduce the lag period of wild-type E-26 cultures. These findings support the hypothesis that adaptation to propionate in *E. coli* E-26 is limited by the capacity of cells to form C_4 acids directly from propionate.

It is postulated that the addition of C_4 acids to propionate media permits the induction of enzymes required for growth on propionate. This hypothesis is supported by the data shown in Fig. 8. It is likely that the experimental conditions facilitated adaptation to propionate by inducing enzymes involved in propionate oxidation. If this is the case, one would expect cells to exhibit an increased capacity to oxidize propionate after incubation in the inducing medium. Such a relationship was observed in the experiment shown in Fig. 9. A relatively short period of incubation in media containing propionate plus succinate was sufficient to induce enzymes catalyzing the oxidation of propionate to CO_2 (213).

Propionate Oxidation

Propionate may be oxidized to acetate by several different pathways: oxidation via malonic semialdehyde (35, 50, 51, 156, 225), malonic semialdehyde-CoA (198, 201), or lactate (9, 10, 24, 113, 114, 116, 121, 180, 200). Propionate may also be metabolized to succinate by several pathways.

In the malonic semialdehyde pathway, propionate is first activated to propionyl-CoA, then dehydrogenated to acrylyl-CoA, and hydrated to β-hydroxypropionyl-CoA. β-Hydroxypropionyl-CoA is deacylated to the free acid and oxidized to acetyl-CoA plus CO_2 via malonic semialdehyde. These reactions are important in plant tissues (50, 51) and constitute the principal pathway of propionate metabolism in *Prototheca zopfii* (19, 119).

In *Clostridium kluyveri* (198, 201), propionate is metabolized by the malonic semialdehyde-CoA pathway. In this pathway, propionyl-CoA again is dehydrogenated to acrylyl-CoA and hydrated to β-hydroxypropionyl-CoA. In contrast to the former pathway, however, β-hydroxypropionyl-CoA is oxidized directly to malonic semialdehyde-CoA. The latter is oxidized to malonyl-CoA and hence to acetyl-CoA plus CO_2.

In *C. propionicum* (24, 116) and in *Peptostreptococcus* (9, 10, 113, 114), the synthesis of propionate proceeds by a mechanism in which acrylate and lactate or their acyl-CoA esters are intermediates. The lactate pathway of propionate oxidation also operates in species of *Pseudomonas* (180, 200) and in animal tissues (121, 200).

Propionate may also be metabolized by conversion to succinate. It is well known that propionyl-CoA is carboxylated to methylmalonyl-CoA (42, 43, 44, 61, 77, 186, 191) and then isomerized to succinyl-CoA (21, 41, 77, 125, 157, 158, 181). This pathway is significant in animal tissues and in a number of microorganisms, including *Rhodospirillum* (103), *Propionibacterium* (184, 222), *Ochromonas* (5), *Micrococcus* (178), and *Rhizobium* (34). Propionate may also be metabolized to succinate via the α-hydroxyglutarate pathway (143). These pathways for the metabolism of propionate are diagrammed in Fig. 10.

The pattern of $^{14}CO_2$ evolution from specifically labeled carbon atoms of propionate can be used to differentiate between these pathways (212). The results of a radiorespirometric experiment designed to determine the major pathway of propionate oxidation in *E. coli* E-26 are shown in Fig. 11. Both the rate and the final cumulative percentage of ^{14}C activity recovered as $^{14}CO_2$ were greatest for propionate-*1*-^{14}*C*, intermediate for propionate-*2*-^{14}*C*, and lowest for propionate-*3*-^{14}*C* (212). The radiorespirometric pattern which would be expected if propionate-*1*-^{14}*C*, -*2*-^{14}*C*, or -*3*-^{14}*C* was metabolized via the described pathways is summarized in Table 6. Of the pathways

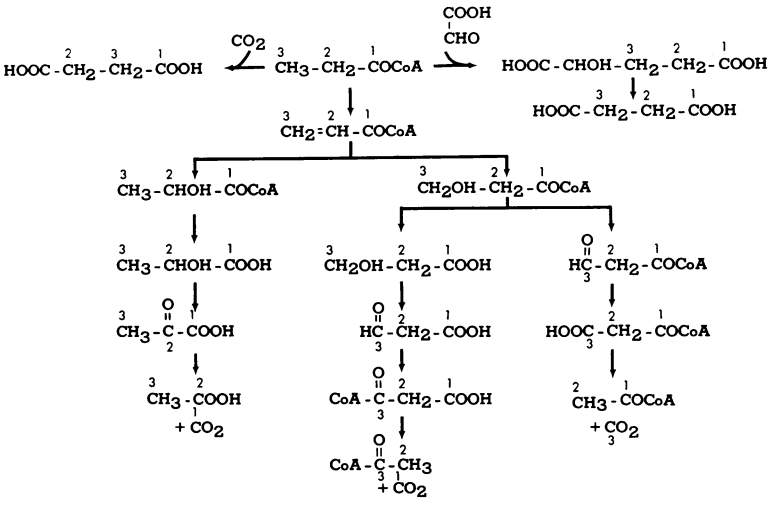


FIG. 10. *Pathways of propionate oxidation.*

listed, only the first can account for a greater recovery of ^{14}C activity as $^{14}CO_2$ from propionate-2-^{14}C than from propionate-3-^{14}C. The demonstration of propionyl-CoA synthetase, propionyl-CoA dehydrogenase, acrylyl-CoA hydratase, and both lactyl-CoA and lactate dehydrogenase activities in extracts of propionate-grown E-26 is in accord with the operation of the lactate pathway. The intermediates in this reaction sequence, propionyl-CoA, acrylyl-CoA, and lactyl-CoA, were identified as hydroxamate derivatives by paper chromatography. Furthermore, the accumulation of pyruvate was demonstrated when extracts were incubated with propionyl-CoA plus an appropriate electron acceptor (215).

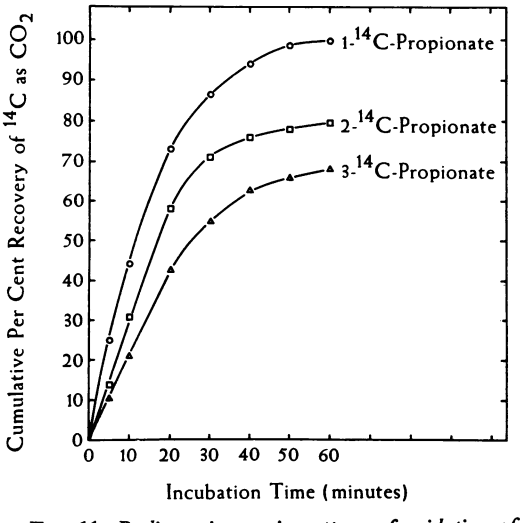


FIG. 11. *Radiorespirometric pattern of oxidation of specifically labeled propionate. A washed suspension of propionate-grown Escherichia coli E-26 was incubated in a mineral salts buffer containing propionate-1-^{14}C, -2-^{14}C, or -3-^{14}C. Respired $^{14}CO_2$ was trapped and quantitated by the method described in Fig. 9. From Wegener et al. (212).*

Significance of Isocitrate Lyase During Growth on Propionate

The results presented above indicate that adaptation of *E. coli* to growth on propionate involves the inductive formation of enzymes catalyzing the oxidation of propionate to CO_2. In addition, it has been shown that a major pathway of propionate metabolism in adapted cells is oxidation to acetate via lactate. Theoretically, a significant difference in the metabolism of cells cultured on propionate, as opposed to acetate, is the fact that C_4 acids may be formed by carboxylation reactions during aerobic growth on the former substrate but not on the latter substrate.

Since our studies strongly suggest that *E. coli*

TABLE 6. *Expected radiorespirometric pattern of $^{14}CO_2$ evolution during the oxidation of specifically labeled propionate*[a]

No.	Pathway	Expected $^{14}CO_2$ evolution pattern
1	Lactate	1 > 2 > 3
2	Malonic semialdehyde	1 > 3 > 2
3	Malonic semialdehyde-CoA	3 > 1 > 2
4	Propionyl-CoA carboxylase	1 > 3 = 2
5	α-Hydroxyglutarate	1 > 3 = 2

[a] From Wegener et al. (212).

E-26 metabolizes propionate primarily by oxidation to acetate rather than by conversion to C_4 acids, the glyoxylate bypass, which is essential to growth of *E. coli* on acetate, would be predicted to be significant during growth on propionate. This prediction is supported by the fact that, in *E. coli* E-26, the formation of isocitrate lyase immediately precedes growth on propionate (214). The hypothesis is contested, however, by Kornberg's experiments (7, 102) with isocitrate lyase-less mutants derived from *E. coli* W. Such mutants were unable to grow on acetate but grew on propionate at rates similar to the growth rates of the parent organism. Therefore, we decided to reevaluate these experiments, using the cultural parameters described in the preceding pages. The isocitrate lyase-less mutant employed (*E. coli* M-18) was derived from *E. coli* W and was obtained from H. L. Kornberg, (Dept. of Biochemistry, Univ. of Leicester, England).

The results obtained from our investigation of Kornberg's studies emphasized the fact that it is essential to specifically define the cultural parameters when attempting to assess the physiological significance of isocitrate lyase during growth on propionate (208). Kornberg's studies were performed using mineral salts media containing relatively low concentrations (5 mM) of propionate (H. L. Kornberg, *personal communication*). In contrast, we routinely employed mineral salts media containing significantly higher (21 mM) concentrations of propionate. The growth characteristics of the isocitrate lyase-less mutant, *E. coli* M-18, were quite different when cultured in media containing different concentrations of propionate. At the lower substrate concentration, both the mutant and parent strains grew at similar rates (as previously reported). However, under our cultural conditions, *E. coli* M-18 exhibited a marked disadvantage on propionate. During adaptation to this substrate, the lag of M-18 was 5 to 6 times longer than that of *E. coli* W; furthermore, even after adaptation to propionate, M-18 grew at a rate which was only 20 to 30% of the rate observed with the parent strain. No deficiency in M-18 was apparent when cells were cultured on glucose, succinate, or lactate, and, in addition, acetate revertants of M-18 grew in media containing 21 mM propionate at rates equal to those of *E. coli* W.

These results indicate that the substrate concentration at which *E. coli* is cultured markedly influences the pathway by which propionate is metabolized. At a higher substrate concentration, propionate is metabolized principally by oxidation to acetate; under these conditions isocitrate lyase is physiologically significant to cellular growth. At a lower substrate concentration, isocitrate lyase is not physiologically significant to growth. The observations made with *E. coli* M-18 are supported by the finding that *E. coli* W exhibits a 10- to 15-fold lower activity of isocitrate lyase when grown on 5 mM propionate than when cultured in media containing 21 mM propionate. It is probable that, at the lower substrate concentration, *E. coli* W possesses an increased capacity to metabolize propionate to C_4 acids via carboxylation reactions. The importance of carboxylation reactions during adaptation to higher concentrations of propionate is discussed in the next section.

Significance of CO_2 Fixation to Growth on Propionate

Perhaps the most important difference between the growth of *E. coli* on propionate and the growth of *E. coli* on acetate is the time required to initiate growth during adaptation to these substrates. Adaptation to propionate appears to be regulated by the rate of formation of C_4 acids from propionate. There are two possible ways that succinate may be formed directly from this fatty acid. One method involves the α-hydroxyglutarate pathway; the second method involves carboxylation reactions. By the latter means, succinate may arise either by carboxylation of propionyl-CoA or by carboxylation of a metabolite derived from propionate, e.g., pyruvate or phosphoenolpyruvate.

As noted previously, the propionyl-CoA carboxylase pathway is a major route of propionate metabolism in several microorganisms. The carboxylation of propionyl-CoA is biotin-dependent, and the subsequent isomerization of methylmalonyl-CoA to succinyl CoA requires B_{12}-coenzyme. These aspects have been reviewed (77).

TABLE 7. *Effect of HCO_3^- and vitamin B_{12} on adaptation of Escherichia coli to propionate*[a]

Additions to medium	Duration of lag (hr)
None	90
HCO_3^-	65
B_{12}	70
Succinate	65
B_{12} + HCO_3^-	55
B_{12} + HCO_3^- + succinate	30

[a] *E. coli* E-26 was cultured from Trypticase Soy Broth to propionate (0.20%)-mineral salts media supplemented with vitamin B_{12} (crystaline cyanocobalamine), 0.5 μg/ml; $KHCO_3$, 5 μmoles/ml; or succinate, 0.1 μmole/ml. Duration of lag is the time required to effect an increase in turbidity from 0 to 1 to 10 Klett units. From Wegener et al. (213).

TABLE 8. *Effect of succinate and HCO_3^- on the ability of Escherichia coli E-26VP$^-$ to initiate growth on propionate*[a]

Primary growth conditions	Secondary growth conditions	Lag (hr)
TSB[b]	Propionate	—[c]
TSB	Propionate + succinate	65
Propionate + succinate	Propionate	35
Propionate + succinate then subcultured in TSB	Propionate	—[c]
TSB	Propionate + HCO_3^-	115
Propionate + HCO_3^-	Propionate	30
Propionate + HCO_3^- then subcultured in TSB	Propionate	86

[a] *E. coli* E-26VP$^-$ was cultured as described. The mineral salts medium contained 0.20% propionate ±0.02% succinate or ±1.2 $(10)^{-3}$ M $KHCO_3$. Lag is the time required in the secondary growth medium to effect an increase in turbidity from 0 to 1 to 10 Klett units. From Kolodziej et al. (84).

[b] TSB = Trypticase Soy Broth.

[c] No growth detectable after 10 days.

When E-26 is grown on substrates such as acetate, lactate, succinate, and glycolate, a requirement for exogenous cofactors is not apparent. Nevertheless, it is possible that adaptation to propionate may be limited by a deficiency in the synthesis of one or more essential cofactors. Of those tested, only vitamin B_{12} reduced the lag preceding adaptation to growth on propionate (Table 7). It is noteworthy that the addition of HCO_3^- to the medium also stimulated growth and that B_{12}, HCO_3^-, and succinate were additive in producing this effect (213).

It is possible then that the limited capacity of E-26 to form C_4 acids necessary for adaptation to propionate may result from low activity of the propionyl-CoA carboxylase pathway. The finding that the in vitro specific activity of propionyl-CoA carboxylase in extracts of propionate-grown E-26 is considerably less than the activity reported in extracts of animal tissues agrees with this suggestion (W. S. Wegener and S. J. Ajl, *unpublished data*). Alternatively, the operation of the propionyl-CoA carboxylase pathway may be limited by a deficiency in the synthesis of vitamin B_{12} or the B_{12}-coenzyme.

The importance of carboxylation reactions to adaptation to propionate is emphasized in a mutant derived from *E. coli* E-26V. This mutant (E-26VP$^-$) grew at the same rate as the parent strain on acetate, glycolate, succinate, lactate, glucose, butyrate, and valerate medium, but it did not grow on propionate (84). The possibility that the mutant was unable to oxidize propionate to acetate was ruled out, since valerate-grown cultures of E-26VP$^-$ oxidized propionate at a rate equal to that of the parent strain. The mutant did not possess a lesion in the enzymes of the tricarboxylic acid cycle or glyoxylate bypass, since such defects would not permit growth on acetate. The mutant and parent strains also possessed equal activities of the enzymes of the α-hydroxyglutarate pathway.

To test the possibility that E-26VP$^-$ possessed a deficiency solely in initiating growth on propionate, the mutant was cultured in a propionate medium supplemented with a limiting concentration of succinate (Table 8). The addition of succinate restored the ability of E-26VP$^-$ to grow on propionate; moreover, such cells now grew when subsequently transferred to unsupplemented propionate media. These conditions resulted in the adaptation to propionate rather than the selection of a revertant population, since the ability to grow in unsupplemented propionate media was lost when the cells were first subcultured in complex media.

The addition of HCO_3^- to the medium also stimulated the adaptation of E-26VP$^-$ to propionate (Table 8), but vitamin B_{12} did not show a similar effect. As was the case in the propionate plus succinate system, propionate plus HCO_3^--grown cultures initiated growth rapidly when subsequently transferred to an unsupplemented propionate medium. However, in contrast to the former system, the latter conditions selected a revertant population, since such cultures were not repressed by subculture in complex media. By serially transferring the mutant from Trypticase

TABLE 9. *Incorporation of $^{14}CO_2$ by Escherichia coli E-26V and E-26VP*[a]

Growth phase (Klett units)	^{14}C incorporation	
	E-26V	E-26VP$^-$
5	2,391	498
50	689	394
100	238	249

[a] *E. coli* E-26V and E-26VP$^-$ were cultured from Trypticase Soy Broth to a 0.20% propionate plus 0.01% succinate medium. At the turbidities indicated, samples were withdrawn, harvested, and incubated for 5 min in a propionate medium containing $NaH^{14}CO_3$ (1 μc; 0.04 μmole). ^{14}C incorporation into cellular material was determined by filtering the cell suspension on a Millipore filter, washing with HCl, and assaying the residue for radioactivity. From Kolodziej et al. (84).

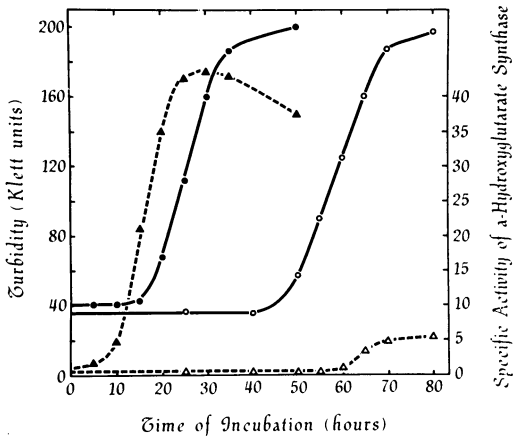


FIG. 12. *Comparison of E-26 and E-26V with respect to formation of α-hydroxyglutarate synthase during growth on propionate. Escherichia coli E-26 and E-26V were cultured under restricted oxygen in glucose-citrate media, aseptically harvested and washed, and then inoculated at an initial turbidity of 40 Klett units into carboys containing 0.20% propionate-mineral salts medium. At the times indicated, samples were withdrawn, and enzyme extracts were prepared and assayed for α-hydroxyglutarate synthase. Symbols: ○, growth of E-26; ●, growth of E-26V; △, formation of α-hydroxyglutarate synthase in E-26; ▲, formation of α-hydroxyglutarate synthase in E-26V. From Wegener et al. (214).*

Soy Broth to propionate plus HCO_3^- and then back again to Trypticase Soy Broth, cultures which grew in unsupplemented propionate media within 24 hr were obtained. These results suggested that E-26VP$^-$ possesses a deficiency in carboxylation reactions necessary for growth on propionate but not on acetate.

Evidence for this hypothesis was provided by the experiment shown in Table 9. When grown on propionate plus limiting succinate, the parent culture incorporated considerably more $^{14}CO_2$ than did the mutant culture, and, in both strains, the amount of $^{14}CO_2$ incorporated into cell material was markedly influenced by the growth phase. The data indicated that carboxylation reactions are most active during growth initiation and decline as growth proceeds. Although such in vivo experiments cannot define the specific lesion characterizing the E-26VP$^-$ mutant, they strongly imply that adaptation of *E. coli* to propionate requires CO_2-fixation reactions (84).

Significance of the α-Hydroxyglutarate Pathway During Growth on Propionate

It will be recalled that the growth of *E. coli* E-26 on valerate resulted in the selection of a mutant population, E-26V, which was constitutive for the formation of glyoxylate-condensing enzymes. In addition, this mutant possessed a growth advantage on a series of short-chain fatty acids. One possible approach to assessing the significance of these enzymes is to determine whether the ability of this mutant to grow quickly on short-chain fatty acids is correlated with the formation of these enzymes (214).

In Fig. 12, *E. coli* E-26 and E-26V are compared with respect to growth and α-hydroxyglutarate synthase formation in propionate-mineral salts media. E-26V initiated growth on propionate considerably more quickly than did E-26; in addition, the strains differed both in the level of activity and in the pattern of α-hydroxyglutarate synthase formation. E-26V possessed higher enzyme-specific activity and, more importantly, enzyme activity increased sharply immediately before growth. The activity of α-hydroxyglutarate synthase was considerably lower in E-26 and was not apparent until the mid to late phase of growth.

The activities and patterns of formation of isocitrate lyase and malate synthase were similar in E-26 and E-26V (Fig. 13 and 14). To facilitate comparison of the relative rates of formation of these enzymes, the activities of isocitrate lyase and malate synthase at various phases of growth are expressed as a percentage of the respective maximal specific activities obtained. In both

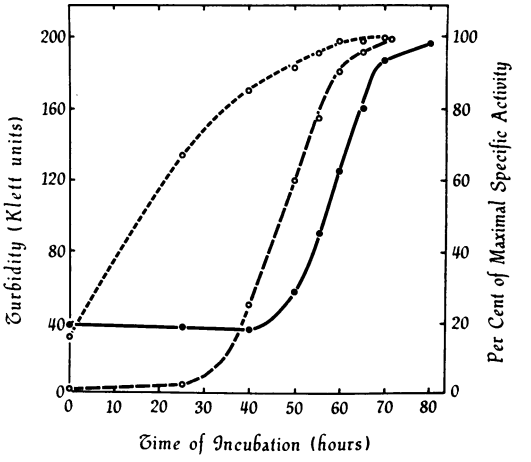


FIG. 13. *Comparison of isocitrate lyase and malate synthase formation in E-26. The procedure was described in Fig. 12. Enzyme activities at various times during growth are expressed as a percentage of the respective maximal specific activities obtained. Symbols: solid line, growth of E-26; long-dashed line, isocitrate lyase; short-dashed line, malate synthase. From Wegener et al. (214).*

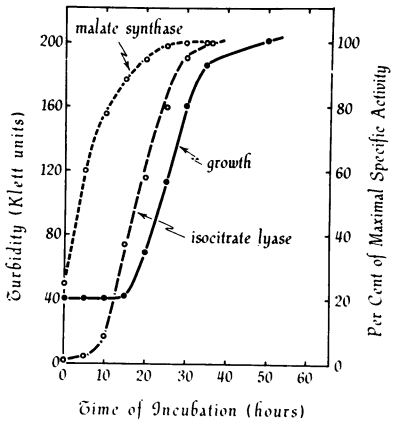


FIG. 14. *Comparison of isocitrate lyase and malate synthase formation in E-26V. The procedure was described in Fig. 12. Enzyme activities at various times during growth are expressed as a percentage of the respective maximal specific activities obtained. From Wegener et al. (214).*

strains, isocitrate lyase was formed immediately before growth. This is in accord with the concept that isocitrate lyase is physiologically significant during the growth of wild-type *E. coli* on propionate. Growth in glucose-citrate media resulted in repression of malate synthase, but enzyme activity increased during incubation in propionate-mineral salts media. This increase in enzyme activity occurred before the initiation of exponential growth and probably represents enzyme derepression due to protein turnover.

These data agree with the concept that E-26V possesses a selective advantage during adaptation to propionate owing to its capacity to form a higher activity of α-hydroxyglutarate synthase. It is proposed that the α-hydroxyglutarate pathway is physiologically significant in *E. coli* E-26V as a mechanism for the formation of C_4 acids directly from propionate. The increased ability of the mutant to form such C_4 intermediates confers an advantage in the synthesis of the adaptive enzymes involved in the oxidation of propionate to acetate. In the parent E-26 strain, the physiological significance of this pathway as a mechanism for the formation of C_4 acid intermediates is questionable, since only a low activity of α-hydroxyglutarate synthase was formed.

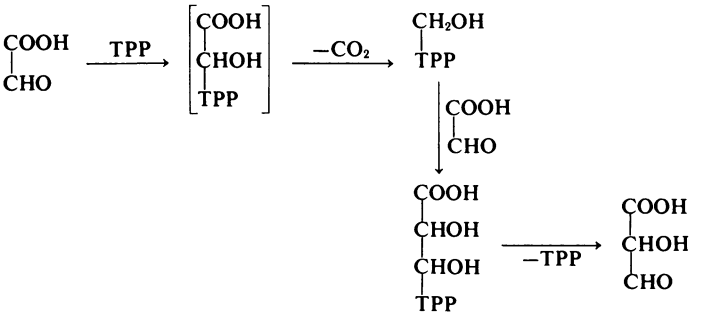
OTHER GLYOXYLATE CONDENSATION REACTIONS

Glyoxylic acid, which is an extremely reactive compound, participates in a variety of biochemical reactions. The previous discussion dealt with condensations of glyoxylate with fatty acid acyl-CoA esters. This section will consider other biological glyoxylate condensation reactions; however, reactions such as amination, transamination, oxidation, reduction, or interaction with thiols will not be included here. The biological significance, rather than chemical aspects, of these condensations will be emphasized. Figure 15 summarizes the reactions discussed.

Condensation with Glyoxylate

The thiamine pyrophosphate (TPP)-dependent condensation of two molecules of glyoxylate to form tartronic semialdehyde plus CO_2 is catalyzed by glyoxylic carboligase (26, 31, 95, 104–106, 202). Tartronic semialdehyde is then reduced by tartronic semialdehyde reductase to glyceric acid (96, 107). Both enzymes are induced in bacteria grown on glycolate, and their combined operation, referred to as the glycerate pathway, is essential for synthesis of cell constituents during growth on this substrate (86, 94).

The proposed mechanism (71, 83) of the glyoxylate carboligase reaction is diagrammed below. It is suggested that glyoxylate and TPP interact to form hydroxymethyl-TPP, which then reacts with a second molecule of glyoxylate to form tartronic semialdehyde. TPP-activated glyoxylate is a probable intermediate in the formation of hydroxymethyl-TPP.



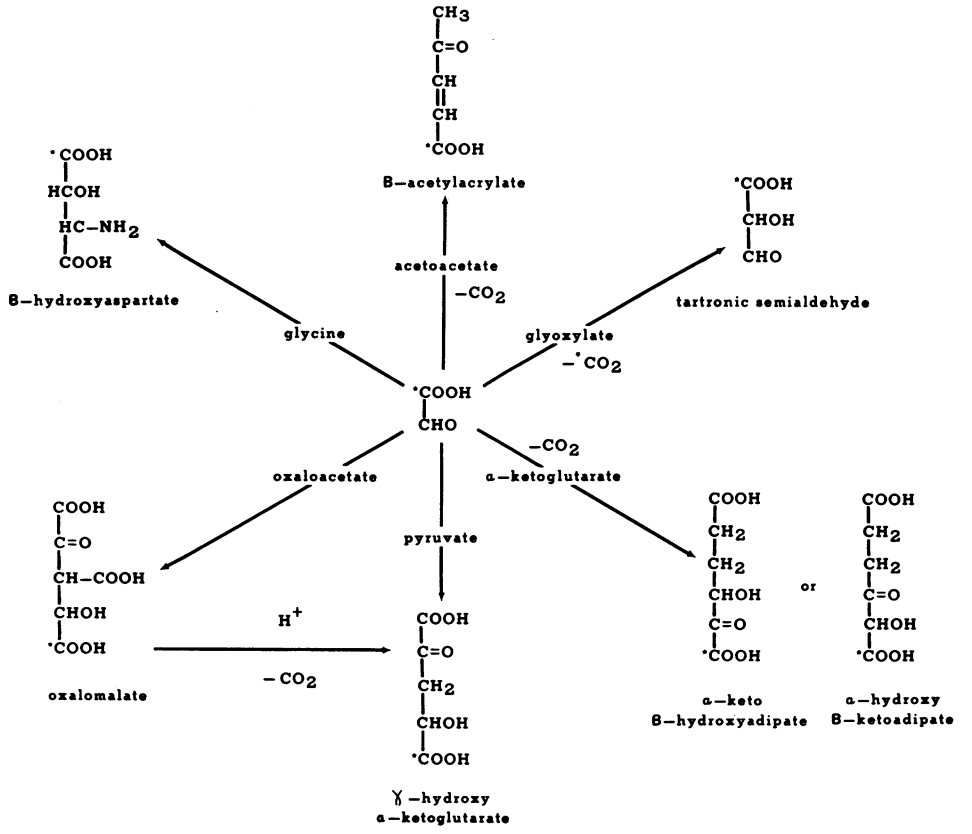


FIG. 15. *Various glyoxylate condensation reactions.*

It has been shown that flavine adenine dinucleotide (FAD) is a component of purified glyoxylate carboligase (57, 58). This prosthetic group is dissociated from the apoenzyme by acid ammonium sulfate fractionation, and the inactive apoenzyme is reactivated by FAD. Although the reaction mechanism would not appear to involve flavine as an oxidation-reduction mediator, it has been inferred that this prosthetic group may provide a mechanism for regulating enzyme activity (58). Glyoxylic carboligase is inactivated by dithionite, and the inactivation is relieved by O_2 or FAD. Enzyme activity may then be controlled in vivo by factors influencing the oxidation-reduction state of the flavine prosthetic group.

Condensation with Glycine

An alternate mechanism for cellular biosynthesis during growth on glycolate has been demonstrated in *Micrococcus denitrificans*. This organism does not possess the enzymes of the glycerate pathway and forms its C_4 acids from glyoxylate via the β-hydroxyaspartate pathway (48, 49, 98–101). In this pathway, glycine (formed by direct amination or transamination of glyoxylate) is condensed with glyoxylate to form erythro β-hydroxyaspartate (β-hydroxyaspartate aldolase), and the latter substance is converted to oxalacetate (β-hydroxyaspartate dehydratase). The nonenzymatic formation of β-hydroxyaspartate from glyoxylate plus glycine also has been reported (131).

Condensation with Pyruvate

In animal tissues, L-hydroxyproline is catabolized to γ-hydroxy-L-glutamic acid (1, 2, 13). The further metabolism of this compound has been studied in several laboratories (15, 54, 110–112, 122, 123) and has been shown to proceed by transamination to D_s-γ-hydroxy-α-ketoglutarate (HKG), followed by cleavage to glyoxylate plus pyruvate. Both the transaminase and aldolase reactions are reversible. Rat liver aldolase is specific for glyoxylate and pyruvate and relatively specific for HKG; both optical antipodes of HKG are formed as well as cleaved by the enzyme. Maitra and Dekker (123) reported that the equilibrium of the aldolase reaction favors cleavage; they suggested that the principal function of this enzyme may be in the catabolism of hydroxyproline.

Kuratomi and Fukunaga (111) purified a rat

liver enzyme which catalyzes the reversible cleavage and formation of HGK; however, the equilibrium of this reaction favors the synthesis of HKG. In addition, glyoxylate and pyruvate condense nonenzymatically to form HKG (172); this reaction occurs at 40 C (*p*H 7.4) and is stimulated by Mg^{++}. The enzymatic condensation is not activated by Mg^{++}.

In plant tissues, HKG is enzymatically decarboxylated to malate (140); this reaction is catalyzed by a peroxidase and by an α-keto acid dehydrogenase. These reactions may be physiologically significant as a pyruvate-catalyzed mechanism for the oxidation of glyoxylate.

Condensation with Oxalacetate

As in the case of pyruvate, glyoxylate may condense either enzymatically or nonenzymatically with oxalacetate to form oxalomalate. Oxalomalate is unstable at an acid *p*H and is spontaneously decarboxylated to HKG.

The enzymatic reaction has been studied in *Acetobacter suboxydans* and may be involved in glutamate biosynthesis (174). The enzymatic reaction may be distinguished from the nonenzymatic reaction; the former occurs at *p*H 6.0 in the absence of Mg^{++}, whereas the nonenzymatic condensation has an optimal *p*H of 7.5 to 8.5 and requires Mg^{++} (174).

The nonenzymatic condensation of glyoxylate with oxalacetate may be physiologically significant as a mechanism for regulating the activity of the tricarboxylic acid cycle (166–169, 172). Kleinzeller (79) first reported that glyoxylate inhibited respiration in animal tissues. Subsequently, Ruffo et al. (30, 167, 170) demonstrated that the inhibition of respiration by glyoxylate was enhanced by oxalacetate and was accompanied by the accumulation of citrate. This observation is in accord with the finding that oxalomalate competitively inhibits aconitase (171) and also inhibits the oxidation of citrate and pyruvate by rat liver mitochondria (167). This inhibition does not appear to result from an inhibition of oxidative phosphorylation, since the P:O ratio was unaffected (167). Payes and Laties (115, 139) reported that HKG also inhibited aconitase from plant tissues and, in addition, was an inhibitor of both isocitric and succinic dehydrogenase. Subsequent studies by Ruffo et al. (172) suggested that oxalomalate is 10-fold more effective as an inhibitor of aconitase and isocitrate dehydrogenase than is HKG.

Condensation with Acetoacetate

The nonenzymatic condensation of glyoxylate with acetoacetate to form β-acetylacrylate, as described by Ellington et al. (37), proceeds at a neutral *p*H and is accelerated by Mg^{++}. Although the physiological significance of this reaction is doubtful, it may account for the observed antiketogenic properties of glyoxylate in rat liver slices.

Condensation with α-Ketoglutarate

A glutamate-dependent decarboxylation of glyoxylate to form *N*-formylglutamate was reported by Nakada and Sund in rat liver (135). Subsequently, the synergistic decarboxylation of glyoxylate and α-ketoglutarate has been described (29, 185).

The condensation of glyoxylate with α-ketoglutarate to form α-keto β-hydroxyadipate was characterized in *Aspergillus niger* by Franke et al. (45) and in *R. spheroides* by Okuyama et al. (137). In the latter organism, α-keto β-hydroxyadipate was decarboxylated to α-hydroxyglutarate and then oxidized to regenerate α-ketoglutarate. Similar reactions occur in rat liver mitochondria (76).

A different glyoxylate-α-ketoglutarate condensation to form α-hydroxy β-ketoadipate was reported in rat liver mitochondria by Koch et al. (81). This condensation may be assayed by measuring the decarboxylation of α-hydroxy β-ketoadipate (in the presence of H^+ or 4 amino-antipyrine) to δ-hydroxylaevulinic acid. The mechanism of this condensation appears to involve decarboxylation of α-ketoglutarate to active succinic semialdehyde followed by condensation with glyoxylate. The reaction was studied in tissues of patients with primary hyperoxaluria (82), a genetic disorder of glyoxylate metabolism characterized by increased urinary excretion of oxalate, glycolate, and glyoxylate. Cytoplasmic extracts of liver, kidney, and spleen tissues from such patients showed a significantly lower activity of this enzyme than did control tissues, suggesting that the condensation may be of physiological significance in the metabolism of glyoxylate by animal tissues.

A similar condensation was described in *Mycobacterium takeo* (132). Isocitrate lyase, but not malate synthase, was detected in this strain, and it has been suggested that the glyoxylate-α-ketoglutarate condensation may serve a biosynthetic function.

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