Glyoxylate in Fatty-Acid Metabolism

Condensations of glyoxylate with fatty acids lead to alternate pathways of fatty-acid metabolism.

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One of the most perplexing problems of intermediary metabolism has been the nature of the mechanism by which some microbial cells derive all the energy they need, and synthesize the complex substances which they require, from compounds containing only two carbon atoms. One key to the solution of this problem was an understanding of how acetate was metabolized by such cells. For over 50 years, since Harden (*1*) suggested that two molecules of acetate condensed to form succinate, the enigma was never satisfactorily resolved. Harden's suggestion was later incorporated in a scheme, the Thunberg-Knoop dicarboxylic acid cycle (*2*), which seemed to account for a number of biochemical events related to the metabolism of acetate in bacteria. There was increasing evidence by 1955 that bacteria could also use the Krebs tricarboxylic acid cycle (*3*) for the same purpose. The net result of both mechanisms was complete oxidation of acetate to carbon dioxide and water. But the differences in the requirements of the two cycles to achieve the same result frustrated attempts to reconcile observations and theories into a unified concept acceptable to all workers. The pitfalls which impeded progress on this problem have been reviewed by Ajl (*4*), Elsden (*5*), and Kornberg (*6*).

The discoveries of isocitrate lyase by Olson (*7*) in 1954 and malate synthase by Wong and Ajl (*8*) in 1956 were crucial in the ultimate resolution of the problem. These two enzymes, acting in concert, form a cyclic mechanism, the glyoxylate bypass (*9*). The bypass provides an efficient means by which the cell can synthesize one mole of succinate from two moles of acetate, and thus serves to replenish the tricarboxylic acid cycle with four-carbon acids drained from the cycle during cellular biosynthesis. Reviews by Ajl (*4*), Krampitz (*10*), Krebs and Lowenstein (*11*), and Kornberg and Elsden (*12*) summarize this research and form a foundation for the work to be discussed here.

Several years ago a new and challenging aspect of intermediary metabolism became apparent when efforts in our laboratory were directed toward answering the question: Are there enzymes which catalyze the condensation of glyoxylate with short-chain fatty-acid esters of coenzyme-A (CoA) other than acetyl-CoA? If so, what products are formed, how are they metabolized, and what function do they have in bacterial physiology?

Condensation with Propionyl-CoA

It occurred to us that the biosynthesis of dicarboxylic acids of greater molecular weight than malic acid might be possible through reactions similar to that catalyzed by malate synthase, provided the cells were adapted to grow on compounds other than acetate. Thus, *Escherichia coli*, when grown aerobically in a mineral-salts medium containing propionate as the sole carbon source, contained an enzyme that catalyzed the formation of α-hydroxyglutaric acid from propionyl-CoA and glyoxylate (*13*). There was no disappearance of substrates or formation of condensation product in the presence of boiled enzyme. α-Hydroxyglutarate was produced only when both glyoxylate and propionyl-CoA were included in the reaction mixture. Analysis of incubation mixtures containing β-methylmalyl-CoA or β-methylmalate, adenosine triphosphate (ATP), and CoA in the presence of *E. coli* extracts failed to reveal α-hydroxyglutarate; only β-methylmalate could be recovered (*14*). This strongly suggested that α-hydroxyglutarate is produced by direct enzymatic condensation between glyoxylate and the β-carbon of propionyl-CoA, and that β-methylmalate is not an intermediate in this reaction.

α-Hydroxyglutarate synthase was found in high concentrations in cells grown on propionate, but not in those grown on acetate. It is specific for propionyl-CoA; in the presence of glyoxylate there is no reaction with the CoA esters of acetate, butyrate, or valerate (*13, 15*). The enzyme has been partially purified by fractionation with ammonium sulfate and subsequent chromatography on diethylaminoethyl cellulose. It requires Mg^{2+} for maximum activity and functions optimally at *p*H 8.5 (*15*).

Condensations with Butyryl- and Valeryl-Coenzyme A

A strain of *Pseudomonas aeruginosa*, orginally isolated from shake cultures in which butyrate was the sole source of carbon, contained an enzyme which catalyzed the condensation of glyoxylate with the α-carbon of butyryl-CoA to yield β-ethylmalic acid, but not α-hydroxyadipic acid or α-hydroxy-β-methylglutaric acid, the other theoretically possible condensation products (*16*). Highest specific activity was found in the fraction of cell-free extracts precipitated by 40-to-60-percent ammonium sulfate. Like α-hydroxyglutarate synthase, the enzyme requires Mg^{2+} and an alkaline *p*H (8.0 to 8.5) for maximum activity. β-Ethylmalic acid-C^{14} was demonstrable on chromatograms when either glyoxylate-1-C^{14} or (butyryl-3-C^{14})-CoA was included in complete reaction mixtures. If the enzyme or one of the substrates was omitted, β-ethylmalate was not detected. Final identification of the condensation product as β-ethylmalate

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was made by synthesizing the radioactive derivative 5-ethyluracil (*16*). Both α-hydroxyadipic and α-hydroxy-β-methylglutaric acids failed to react with urea to form a uracil derivative.

In analogy to the β-ethylmalate synthase reaction, crude extracts of valerate-grown *E. coli* catalyzed the disappearance of glyoxylate and valeryl-CoA, with the concomitant formation of β-*n*-propylmalate (*17*). If either substrate was omitted, or if boiled extract was used, the reaction did not occur. The enzyme was purified approximately 30-fold from these extracts by fractionation with ammonium sulfate and with acetone. β-*n*-Propylmalate synthase appears to be specific for valeryl-CoA since, in the presence of glyoxylate, no activity could be detected with the CoA esters of acetate, propionate, or butyrate. Likewise, compounds structurally related to glyoxylate (acetaldehyde, glycollate, glycoaldehyde, and glyoxal) could not replace glyoxylate in the presence of valeryl-CoA. β-*n*-Propylmalate synthase, like malate synthase, α-hydroxyglutarate synthase, and β-ethylmalate synthase, exhibits greatest activity at *p*H 8.5 in the presence of Mg^{2+}.

In experiments with 30-fold-purified enzyme preparations, β-*n*-propylmalate-C^{14} was formed when glyoxylate-1-C^{14} and unlabeled valeryl-CoA condensed, or when unlabeled glyoxylate and (valeryl-1-C^{14})-CoA were employed as substrates (*18*).

CoA esters of acetate, propionate, butyrate, and valerate are not the only compounds which condense enzymatically with glyoxylate. When radioactive glyoxylate was used in a very sensitive assay (described below), glyoxylate condensed with CoA esters of such diverse acids as isovaleric, caproic, lactic, crotonic, and β-hydroxybutyric. Enzymatic activity for all these condensations was present in extracts of valerate-grown *E. coli*, although the condensation with lactyl-CoA occurred more rapidly with extracts of propionate-grown *E. coli*. These reactions were not apparent when boiled extracts were used.

Condensation Products as CoA Esters

Throughout the early stages of this work we considered the malate synthase reaction as the model for the condensation of glyoxylate with CoA esters of propionate, butyrate, and valerate. Thus it was reasoned that these reactions could be assayed by the spectrophotometric method of Dixon and Kornberg (*19*). By this method the rate of condensation of acetyl-CoA and glyoxylate, accompanied by a cleavage of the thiol ester bond of acetyl-CoA and generation of reduced CoA, could be measured at 232 mμ.

The activity of the condensing enzymes was always considerably less than expected in view of the ease with which the free acid condensation products could be isolated. Results obtained with other assays which measured total release of sulfhydryl groups during the reactions were likewise disappointing.

We hypothesized that the condensation products were formed as CoA esters and that activity noted during assay could be largely due to enzymatic deacylation. The isolation and identification of the condensation products as the free acids could likewise have been the result of deacylation and, in addition, of hydrolytic effects on the thiol ester bonds during chemical work-up of the reaction mixtures. This view was substantiated by two further observations. First, the deproteinized reaction mixtures, after continuous extraction with ether, could be treated with alkali, again acidified, and again extracted with ether. The extracts were found to contain significant additional quantities of the condensation products as the free acids. (The strength of alkali used, length of contact with the ester, and temperature of reaction vary considerably, depending on the CoA ester to be hydrolyzed.) Second, the condensation products were poorly, or not at all, metabolized as the free acids by dialyzed extracts, whereas the CoA esters were readily metabolized.

Table 1. Influence of carbon source on occurrence of enzymes that catalyze the condensation of glyoxylate with coenzyme-A esters of fatty acids.

Inoculum	Carbon source	Specific activity*				Ratio of activities†
		Malate synthase	β-Ethylmalate synthase	α-Hydroxyglutarate synthase	β-*n*-Propylmalate synthase	
Acetate-adapted *E. coli*	Acetate	61.2	0.0	0.0	16.0	
Propionate-adapted *E. coli*	Propionate	59.0	4.8	10.5	15.7	1.0/2.2/3.3
Butyrate-adapted *P. aeruginosa*	Butyrate	56.8	11.8	9.3	15.8	1.0/0.8/1.3
Valerate-adapted *E. coli*	Valerate	44.6	38.1	40.1	38.4	1.0/1.1/1.0

* Disappearance of glyoxylate (mμmoles per 15 minutes per milligram of protein). † β-Ethylmalate synthase / α-hydroxyglutarate synthase / β-*n*-propylmalate synthase.

If the condensation products occurred as CoA esters, obviously the substrates and the products would both absorb at 232 mμ and the validity of the assay would be highly questionable. One way to quantitate these reactions is to measure the disappearance of radioactive glyoxylate as a function of the fatty-acid CoA ester; the residual radioactive glyoxylate could be isolated and counted as the *p*-nitrophenylhydrazone. An assay based on this concept (*20*) has proved eminently suitable for malate synthase and the other condensing enzymes. The method is specific and quantitative since the residual glyoxylic *p*-nitrophenylhydrazone is isolated chromatographically, and all the glyoxylate can be accounted for. The technique is sensitive to changes of less than 1 mμmole in glyoxylate concentrations; stoichiometry of the malate synthase reaction (disappearance of glyoxylate and formation of malate) was readily demonstrable.

With the new glyoxylate-C^{14} assay, crude extracts of *E. coli* grown separately on acetate, propionate, and valerate and of *P. aeruginosa* grown on butyrate were assayed for each of the glyoxylate-condensing enzymes. As shown in Table 1, malate synthase was present in high concentrations in cells grown on all fatty-acid carbon sources. That cells grown on acetate catalyzed only two of the four condensation reactions suggests that the condensations were catalyzed by separate enzymes rather than by a single enzyme having broad specificity. Further, a convincing argument for the heterogeneity of these enzymes was provided by the significant difference in the destruction by heat of their activities (*21*).

Formation and Metabolic Role of Glyoxylyl-CoA

The hypothesis that the condensation products in these reactions were formed as CoA esters, although useful for interpreting some of our observations, seemed deficient on thermodynamic grounds. Formation of CoA esters would appear more likely if both substrates in a given condensation reacted as the CoA esters. Glyoxylyl-CoA, however, was then an unknown compound, and we had no evidence to indicate that it participates in these reactions.

Fortuitously, the formation of a new radioactive metabolic intermediate formed from acetyl-CoA and C^{14}-labeled glyoxylate was detected chromatographically during development of the radioactive glyoxylate assay (*22*). After the eluted compound was treated with alkali, labeled glyoxylate was identified as the *p*-nitrophenylhydrazone derivative by co-chromatography with authentic glyoxylic *p*-nitrophenylhydrazone.

Coenzyme A was identified as the only other constituent by chromatography, from its ultraviolet absorption spectrum, and from its ability to replace authentic CoA in the phosphotransacetylase assay (*23*). The formation of the new metabolite, glyoxylyl-CoA, can be demonstrated in reaction mixtures, at *p*H 8.0 to 8.5, which contain glyoxylate, acetyl-CoA, Mg^{2+}, and extract of valerate-grown *E. coli*. It is undetectable in reactions in which acetyl-CoA is lacking or boiled extract is used. Either CoA and ATP or CoA esters of propionate, butyrate, and valerate are capable of substituting for acetyl-CoA.

The mechanism of glyoxylyl-CoA formation has not been elucidated, but it could involve a thiophorase transfer of CoA from the fatty-acid CoA ester to glyoxylate, in the following manner:

acetyl-CoA + glyoxylate ⟶ glyoxylyl-CoA + acetate

This proposal is experimentally verifiable with the use of labeled CoA. The enzymatic transfer of labeled CoA from acetyl-CoA-S^{35} to glyoxylate and the identification of glyoxylyl-CoA-S^{35} are currently being investigated in our laboratory.

If this basic reaction also occurs in the formation of condensation products resulting from interaction of glyoxylate with CoA esters of three-, four-, and five-carbon fatty acids, the mechanism using propionyl-CoA, for example, can be envisioned as in Fig. 1.

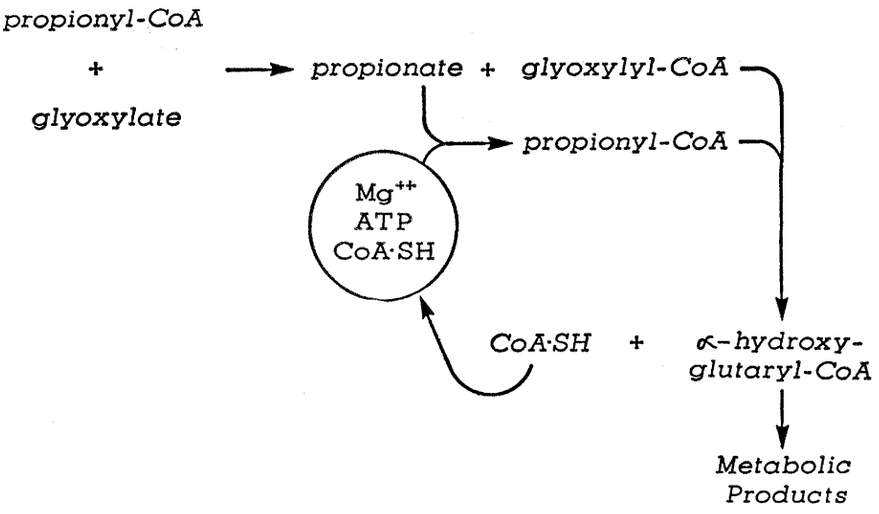

Fig. 1. Proposed mechanism for formation of condensation products as coenzyme-A esters.

Thus, formation of glyoxylyl-CoA and its ultimate condensation with acyl-CoA esters of fatty acids of larger molecular weight than acetate is thermodynamically acceptable, accounts for the metabolism of the condensation products as CoA esters, and requires no more CoA than does the condensation of acetyl-CoA with glyoxylate to form malate, since reduced CoA would be constantly regenerated in the process.

Metabolism of α-Hydroxyglutarate

Early attempts to determine the fate of α-hydroxyglutarate were concerned with its oxidation. The acid as the L-(+) isomer was found to reduce methylene blue in the presence of crude extracts obtained from propionate-grown *E. coli* supplemented with CoA, ATP, and Mg^{2+}. Virtually no dye reduction occurred in reactions lacking extract or the supplements, or when the D-(−) isomer was used as the substrate. The CoA ester of the L-(+) isomer was then prepared and, in its presence, the 60-to-75-percent ammonium sulfate fraction of the cell-free extract catalyzed the reduction of nicotinamide adenine dinucleotide (NAD); NAD phosphate (NADP) and ferricyanide were incapable of substituting for NAD. Repeated attempts to find the expected product of oxidation, α-ketoglutarate, were unsuccessful (*24*).

These data indicated that the L-(+)-α-hydroxyglutaryl-CoA was not oxidized directly, but only after it had been converted to another product in the metabolic pathway. When this possibility was explored, we found that disodium L-(+)-α-hydroxyglutarate was cleaved by undialyzed crude extracts of propionate-grown *E. coli*. The products were identified as lactate and acetate by paper and silicic acid chromatography (*25*). From 260 μmole of substrate, 119 μmole of acetate and 59 μmole of lactate were recovered. The lack of stoichiometry between the products suggested that lactate was further metabolized by the crude extracts and, indeed, later experiments showed that this was the case.

Since *E. coli* grown aerobically on propionate contains the tricarboxylic-acid cycle enzymes and isocitrate lyase, in addition to enzymes catalyzing both the synthesis and cleavage of α-hydroxyglutarate, a cyclic pathway of propionate metabolism was suggested (*25*) as shown in Fig. 2.

Efforts to investigate the further metabolism of lactate by *E. coli* seemed, on first inspection, to be of questionable value since the process has been so well studied. Unexpectedly, a dialyzed 65-to-75-percent ammonium sulfate fraction was found to catalyze the NAD-dependent oxidation of DL-lactyl-CoA (*26*). Lactate could not substitute for lactyl-CoA, and neither NADP nor ferricyanide could replace NAD as the hydrogen acceptor. A compound isolated from complete reaction mixtures, but not from controls, was distinguished from pyruvate by its chromatographic properties and the absorption spectrum of the 2,4-dinitro-

phenylhydrazone (DNP-hydrazone) derivative. The 65-to-75-percent ammonium sulfate fraction was devoid of lactate dehydrogenase activity, and lactyl-CoA did not serve as a substrate for crystalline lactate dehydrogenase. Hence the enzyme involved was not lactate dehydrogenase.

The unknown compound was identified as hydroxypyruvic aldehyde (*27*) by the following criteria: (i) It formed an osazone with 2,4-dinitrophenylhydrazine; (ii) it was degraded to glyoxylate and formaldehyde by periodic acid oxidation; and (iii) it formed a bisulfite addition product.

Hydroxypyruvic aldehyde was esterified by glutathione in the presence of a 40-to-50-percent ammonium sulfate fraction of *E. coli*, and by crystalline glyoxylase I (*28*); the ester was isolated and identified by its absorption spectrum. With the use of crude extracts of propionate-adapted *E. coli*, in which both glyoxylase I and glyoxylase II activity are present, we have shown the formation of glyceric acid from the isolated glutathione thiol ester of hydroxypyruvic aldehyde. Glyceric acid was identified by paper chromatography, and the identification was confirmed by treatment of eluates with 4,5-dihydroxy-2,7-naphthalene disulfonic acid. The absorption spectra of the derivative of the eluate and the derivative of authentic glyceric acid were identical.

Finally, crude extracts containing phosphoglycerate kinase and 3-phosphoglycerate kinase converted glycerate to 3-phosphoglycerate and subsequently to 1,3-diphosphoglycerate (*27*).

Prior to the initial report by Reeves *et al.* (*26*), hydroxypyruvic aldehyde had not been observed as a product of biological reactions, although Racker (*29*) had shown that the synthetic compound was a substrate for glyoxylase I. Thus, the suggestion that the function of the glyoxylase system is to detoxify methylglyoxal (*30*) is apparently not applicable in the case of hydroxypyruvic aldehyde. Since hydroxypyruvic aldehyde is produced enzymatically, the glyoxylase system may provide a mechanism for the entry of this compound either into the tricarboxylic acid cycle via pyruvate or into the glycolytic pathway.

The partial sequence of reactions involved in the metabolism of lactyl-CoA appeared to be as shown in Fig. 3. It is obvious that the formation of hydroxypyruvic aldehyde from lactyl-CoA is neither simple nor direct. To

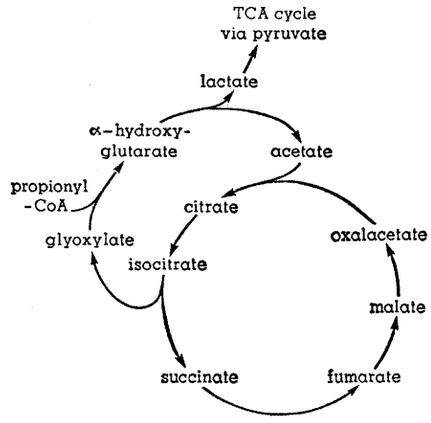

Fig. 2. Suggested pathway of propionate metabolism in *Escherichia coli*. [Redrawn from (*25*)]

elucidate this part of the pathway we began with the premise that lactyl-CoA was oxidized to pyruvyl-CoA (*31*). Indeed, extracts of propionate-grown *E. coli* did catalyze the formation of pyruvyl-CoA from C^{14}-labeled lactate in phosphate buffer supplemented with CoA, ATP, Mg^{2+}, and with reduced glutathione (*32*). Pyruvyl-CoA was isolated and identified as the radioactive hydroxamate by column and paper chromatography and was further characterized by formation and chromatography of the DNP-hydrazone. Under the same conditions lactyl-CoA was also isolated from unlabeled lactate; pyruvyl-CoA could not be detected unless the sensitivity of the procedure for identification was greatly increased by the use of radioactive lactate. It was thus apparent that *E. coli* extracts contained a lactyl-CoA synthetase and, in the absence of added NAD, had sufficient hydrogen acceptor to oxidize the lactyl-CoA to pyruvyl-CoA.

When either acetyl-CoA or propionyl-CoA was substituted for reduced CoA in the presence of lactate, lactyl-CoA formation could be demonstrated by isolation of the hydroxamic acid derivative. Hence lactyl-CoA may be synthesized by alternative means when provided with appropriate substrates.

Spectrophotometric Evidence

Direct spectrophotometric evidence was also obtained for the oxidation of lactyl-CoA by crystalline yeast alcohol dehydrogenase (*33*). In addition, spectrophotometric assay of lactyl-CoA oxidation, both by the crystalline yeast enzyme and by a 60-to-80-percent ammonium sulfate fraction of *E. coli* in the presence of NAD, established that there was no stereospecific requirement for either D-(−) or L-(+) isomers of lactyl-CoA; both isomers were approximately equally effective as substrates. The 60-to-80-percent ammonium sulfate fraction of *E. coli* contains a very active alcohol dehydrogenase but is devoid of lactate dehydrogenase activity. Furthermore, lactate dehydrogenase from *E. coli* has an extremely low affinity for the CoA ester of lactic acid under assay conditions optimum for this enzyme when lactic acid is used as substrate. That the formation of pyruvyl-CoA from lactyl-CoA is not catalyzed by lactate dehydrogenase was confirmed by the failure of lactyl-CoA to serve as a substrate for crystalline yeast lactate dehydrogenase. It is, therefore, highly im-

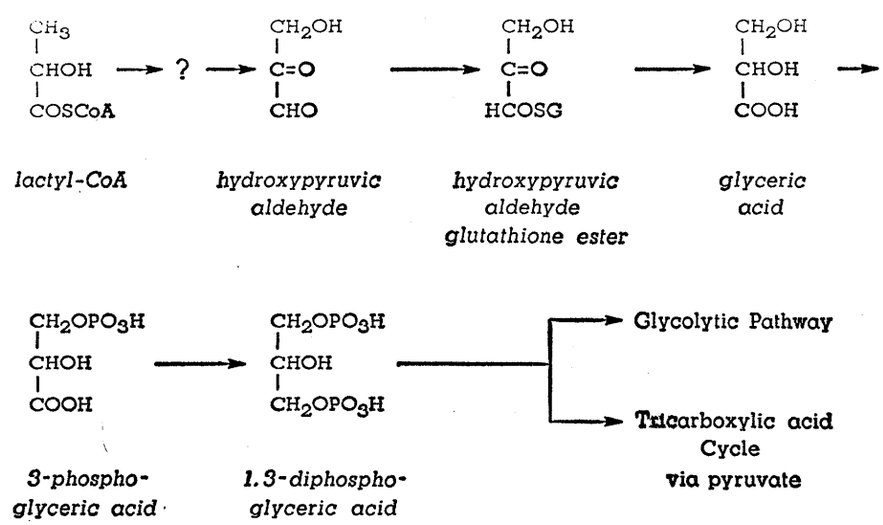

Fig. 3. Metabolism of lactyl-CoA in *Escherichia coli*.

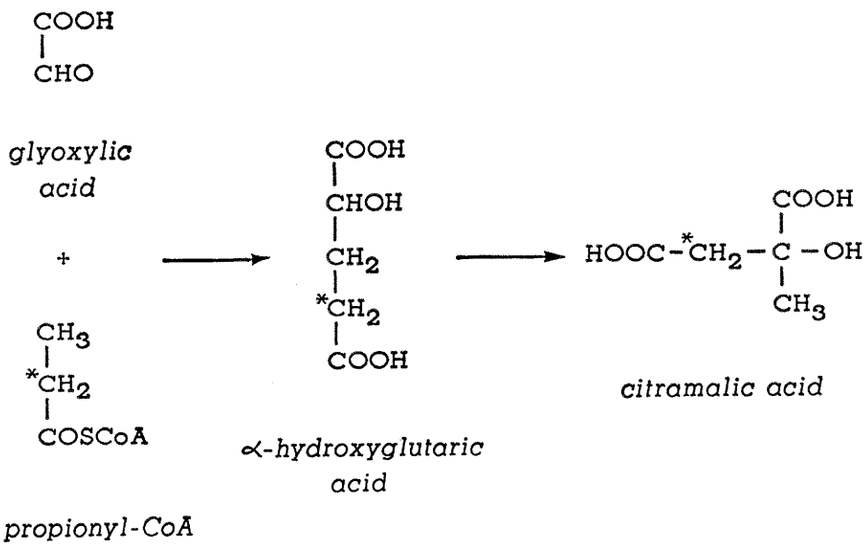

Fig. 4. Formation of citramalic acid by an isomerization mechanism.

probable that cellular oxidation of lactyl-CoA is catalyzed by lactate dehydrogenase.

These results recently became more easily understood, as will be discussed below, when it was found that cell-free extracts of propionate-grown *E. coli* contain enzymes which catalyze the propionyl-CoA-dependent formation of citramalate (α-methylmalate) from glyoxylate (*34*). Since the same extracts catalyze the biosynthesis of α-hydroxyglutarate from propionyl-CoA and glyoxylate, α-hydroxyglutarate may be enzymatically isomerized to citramalate.

Citramalate, found in complete reaction mixtures, but not in controls in which propionyl-CoA was lacking or in which boiled extract was used, was isolated by chromatography on Dowex-1-Cl^-. C^{14}-labeled citramalate formed from C^{14}-labeled glyoxylate was co-chromatographed with authentic citramalate; subsequent counting by scintillation spectrometry of segments cut sequentially along paper chromatograms showed that the radioactivity was fixed only in the citramalate position. Identification of citramalate was confirmed by degradation to acetone and chromatography of the DNP-hydrazone derivative.

From theoretical considerations, as well as previous reports by others (*35, 36*), the aldol cleavage of citramalate should yield pyruvate and acetate. We had already shown that α-hydroxyglutarate was cleaved by *E. coli* extracts to lactate and acetate, but had never been able to isolate pyruvate. We maintain that pyruvate is, in fact, one of the cleavage products but that it is rapidly converted to lactate by the very active lactate dehydrogenase present in crude extracts of *E. coli*. Data presented (*32*) indicate that a lactyl-CoA synthetase catalyzes the formation of lactyl-CoA and that this product is oxidized by alcohol dehydrogenase (or possibly by a specific lactyl-CoA dehydrogenase) to pyruvyl-CoA. The fact that the formation of pyruvyl-CoA can be demonstrated only with the use of labeled lactate indicates that this intermediate is actively metabolized and therefore cannot accumulate in appreciable amounts.

Experiments vital to the establishment of the isomerization of α-hydroxyglutarate to citramalate are currently being carried out. This type of isomerization would be completely analogous to the glutamate $\rightarrow$ β-methylaspartate (*37*) and methylmalonate $\rightarrow$ succinate (*38*) isomerizations. If this mechanism is applied to the formation of 1-methyl-1-hydroxysuccinic acid-2-C^{14} (citramalic acid) from glyoxylate plus (propionyl-2-C^{14})-CoA, the reaction sequence of Fig. 4 is evident.

By degradation of the C^{14}-labeled citramalate, the original position of the label in the citramalate could be determined and isomerization by means of a shift of two carbons confirmed. The labeled citramalate was degraded by the method illustrated in Fig. 5.

All the radioactivity was found in the CO_2 derived from acid treatment of $BaC^{14}O_3$; none was detected in acetone DNP-hydrazone. These qualitative observations indicate that the label in citramalate was derived from the carbon No. 2 of propionate. This is commensurate with an isomerization mechanism involving a two-carbon ($-CH_2COOH$) shift in α-hydroxyglutarate. Proof of this mechanism must await quantitative data from the use of (propionyl-2-C^{14})-CoA.

The proposal given in Fig. 6 for the biosynthesis of pyruvyl-CoA from hydroxyglutarate is consistent with the foregoing data. The conversion of pyruvyl-CoA to hydroxypyruvic aldehyde could be envisioned as in Fig. 7.

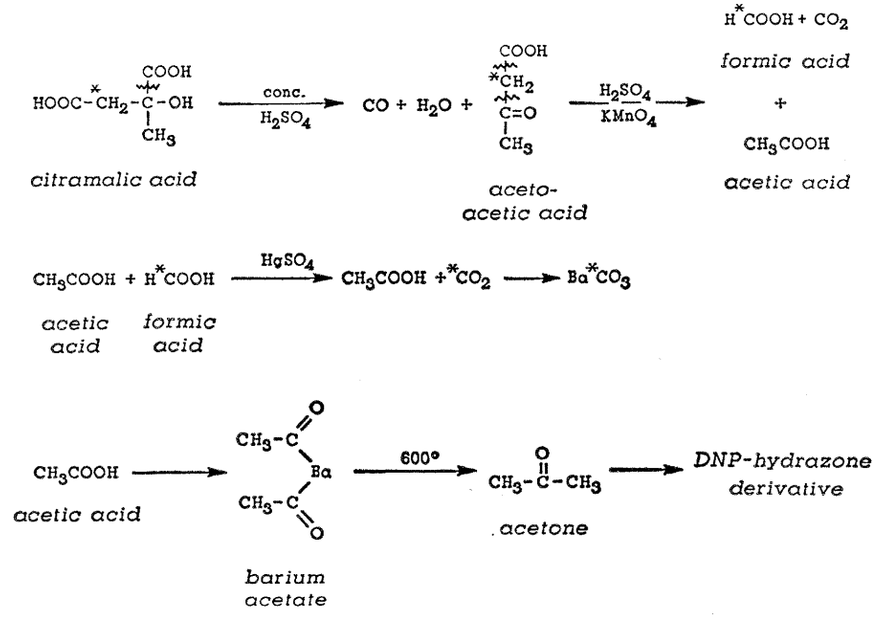

Fig. 5. Degradation of C^{14}-labeled citramalic acid.

There is a precedent for the reaction

$$R\text{–}\overset{\overset{O}{\|}}{C}\text{–}S\text{–}CoA \rightarrow R\text{–}CHO$$

Burton and Stadtman (*39*) reported an aldehyde dehydrogenase in *Clostridium kluyveri* which catalyzes the reversible NAD-dependent conversion of acetaldehyde to acetyl-CoA.

There is also evidence for the reaction

$$R\text{–}CH_3 \rightarrow R\text{–}CH_2OH$$

in the studies of Kusunose *et al.* (*40*), in which octanoate was oxidized to 8-hydroxyoctanoate in a psuedomonad.

When citramalate was formed as a consequence of the enzymatic condensation of propionyl-CoA and glyoxylate, a considerable quantity of succinate was produced. The origin of the succinate was investigated by incubating α-hydroxyglutarate-1-C^{14} with an extract of propionate-grown *E. coli*, CoA, ATP, Mg^{2+}, and NADP (*41*). The reaction was terminated by deproteinization with 50-percent sulfuric acid, and the reaction mixture was divided into two parts. To one part was added 2,4-dinitrophenylhydrazine; the resulting isolated DNP-hydrazone was chromatographically identical to succinic semialdehyde DNP-hydrazone. The other part was extracted with ether, and unlabeled succinic acid from the extract was readily identifiable chromatographically. Carbon dioxide formed during the enzymatic reaction was trapped as barium carbonate, which proved to be highly radioactive. Carbon dioxide produced from control reactions lacking CoA or ATP was only 25 percent of that formed in the complete system, an indication that the concentrations of endogenous CoA and ATP in the extract were low.

When the experiment was repeated with α-hydroxyglutarate-5-C^{14}, as expected, the CO_2 produced was unlabeled, whereas succinate and succinic semialdehyde DNP-hydrazone were highly radioactive. That succinic semialdehyde was a precursor of succinate was evident from the observation that *E. coli* extracts catalyzed a succinic semialdehyde–dependent reduction of NADP. This was measured spectrophotometrically and confirmed by direct chromatographic isolation of succinate from reactions in which succinic semialdehyde, *E. coli* extract, and NADP were present.

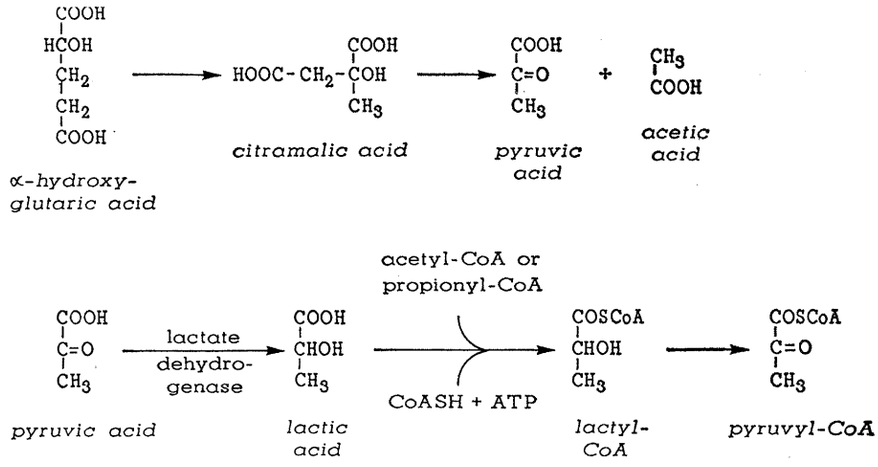

Fig. 6. Biosynthesis of pyruvyl-CoA from α-hydroxyglutaric acid.

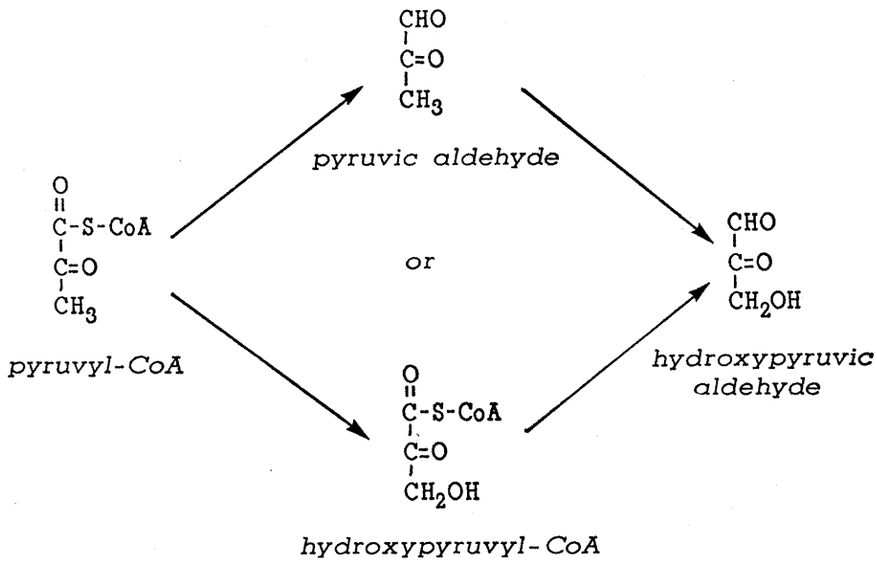

Fig. 7. Formation of hydroxypyruvic aldehyde from pyruvyl-CoA.

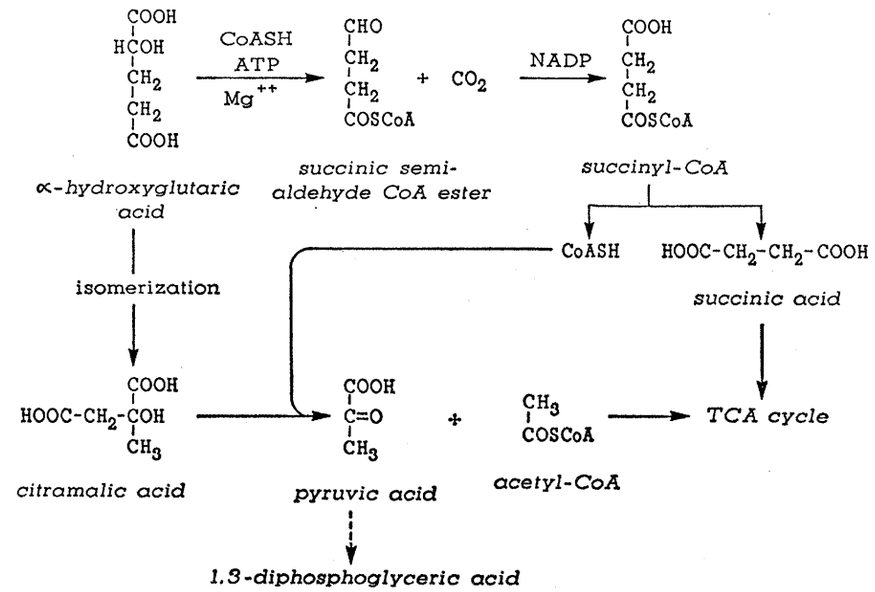

Fig. 8. Interrelation of succinyl-CoA and citramalic acid in the metabolism of α-hydroxyglutaric acid.

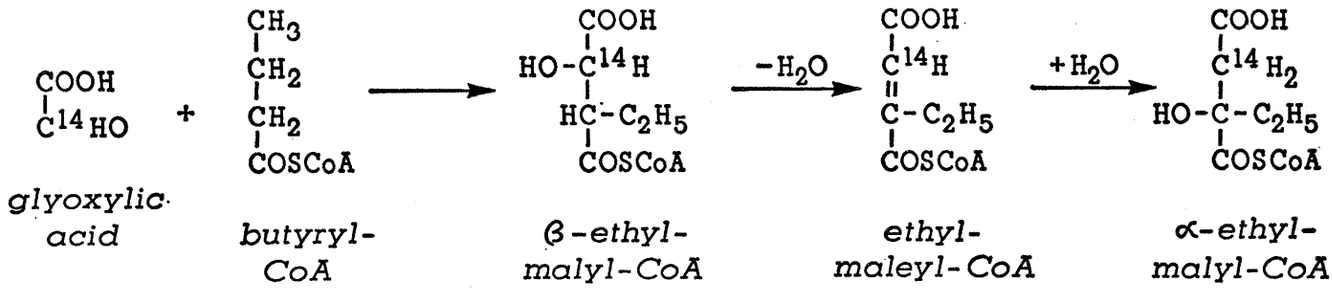

Fig. 9. Proposed pathway for the biosynthesis of α-ethylmalyl-CoA.

From recent work in Kornberg's laboratory (*36*) it appears that citramalate is cleaved to pyruvate and acetate only when the substrate exists as the CoA ester. High levels of succinate in *E. coli*, formed as a consequence of the condensation of glyoxylate and propionyl-CoA, may indicate that a significant proportion is present as succinyl-CoA. By a CoA transferase reaction succinyl-CoA would serve as a CoA donor to citramalate, as has already been shown by Cooper and Kornberg (*36*). The reaction sequence shown in Fig. 8 reflects these considerations and offers a basis for future experiments.

Metabolism of β-Ethylmalate

The structure of β-ethylmalate indicates at least two possibilities for its metabolism: dehydration and oxidation. To date our efforts have been mainly concerned with the former.

A parallel was recognized between the enzymatic hydration of fumarate to malate and the analogous, but previously unreported, hydration of ethylfumarate to β-ethylmalate. Ethylfumarate absorbs strongly and sharply in the ultraviolet region at 220 mμ. Fumarate has an absorption maximum at a wavelength of 208 mμ, and since its hydration can be assayed at a wavelength as high as 300 mμ (*42*) we also used the 300-mμ wavelength to observe a protein-dependent decrease in absorbance in the presence of ethylfumarate. The initial velocity was very low even at high concentrations of substrate. But initial velocity data showed that decrease in absorbance was at least 100-fold more rapid with ethylfumaryl-CoA as substrate than with the free acid (*43*).

The reaction was catalyzed by dialyzed crude extracts of butyrate-grown *P. aeruginosa* and the 20-to-40-percent ammonium sulfate fraction of the extract. This fraction contains no detectable fumarate hydratase activity. Among the metal ions tested, decrease in absorbance was stimulated only by Mn^{2+} and was most rapid at *p*H 9.0; above this *p*H, Mn^{2+} caused a nonenzymatic decrease in absorbance and was simultaneously oxidized. Optimum *p*H in the absence of Mn^{2+} was 9.5. The initial velocity of hydratase activity is a linear function of protein concentration, and the Michaelis constant, K_m, for ethylfumaryl-CoA is $5.3 \times 10^{-4} M$.

Rapid dehydration of both α- and β-ethylmalyl-CoA was also observed spectrophotometrically, but the free acids were poorly dehydrated even when very high substrate concentrations were used. In unexpected contrast to hydration of ethylfumaryl-CoA, dehydration of α- and β-ethylmalyl-CoA was most rapid at neutral *p*H, and Mn^{2+} was inhibitory. If ethylfumaryl-CoA was an intermediate in the conversion of β- to α-ethylmalyl-CoA and was in equilibrium with the hydration products, and one enzyme was responsible for the conversion, the optimum *p*H and Mn^{2+} stimulation should be the same for dehydration of both ethylmalyl-CoA esters and hydration of ethylfumaryl-CoA.

The apparent paradox was resolved when it was found that ethylmaleyl-CoA, but not the free acid, was readily hydrated to α-ethylmalate optimally at *p*H 7 and in the absence of Mn^{2+}, which was inhibitory. The hydration product of ethylfumaryl-CoA was β-ethylmalate, and not α-ethylmalate. These observations indicate that two different enzymes were involved, each having its own specificity for the *cis* and *trans* geometrical isomers. The hydration products, β- and α-ethylmalate, were isolated as the free acids, although the free acids are, in turn, dehydrated much more readily as CoA esters. It is probable that these compounds are metabolized and formed as CoA esters and that the ease with which they are isolated and identified as free acids is due to manipulative or enzymatic deacylation.

α-Ethylmalic acid was isolated and identified from reaction mixtures con-

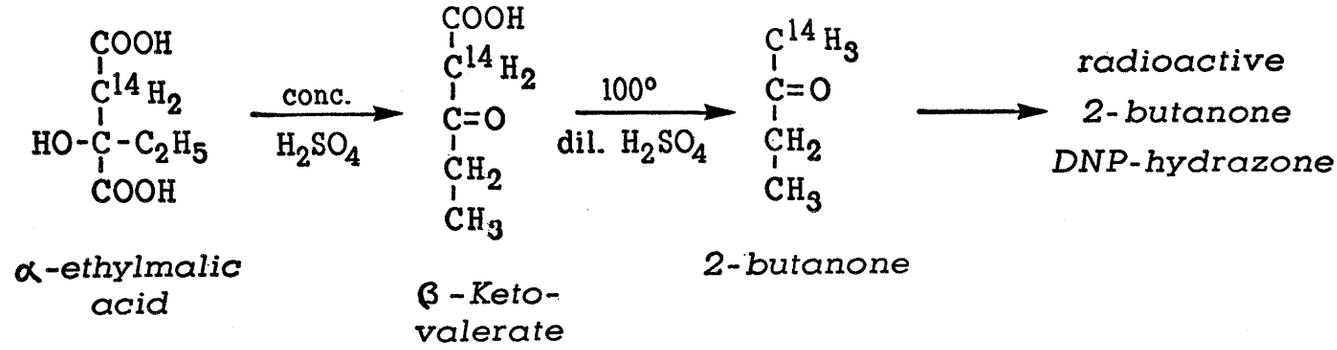

Fig. 10. Degradation of C^{14}-labeled α-ethylmalic acid.

taining β-ethylmalyl-CoA or ethylmaleyl-CoA as substrates, but not from those containing ethylfumaryl-CoA. The α-ethylmalate was degraded by concentrated sulfuric acid at room temperature and subsequent distillation at 100°C in the presence of dilute sulfuric acid. The distillate containing 2-butanone was collected in 2,4-dinitrophenylhydrazine. The resulting DNP-hydrazone chromatrographed identically with and had the same spectrum in ultraviolet light as authentic 2-butanone DNP-hydrazone. α-Ethylmalate-C^{14} was identified in the same manner from reaction mixtures containing both glyoxylate-2-C^{14} and butyryl-CoA as substrates in the presence of crude extracts of *P. aeruginosa*. The DNP-hydrazone derivative was radioactive as expected. Controls containing boiled extract failed to yield labeled α-ethylmalate.

These observations, coupled with the previous finding that β-ethylmalate is the product of the condensation of glyoxylate with butyryl-CoA, suggest the pathway shown in Fig. 9. The degradation of α-ethylmalate and formation of the hydrazone derivative are indicated in Fig. 10.

Upon the formation of an α-substituted malic acid, the pathways of the metabolism of α-hydroxyglutarate and β-ethylmalate seem to converge. It will be recalled that citramalate is produced after the condensation of propionyl-CoA and glyoxylate and probably arises from the isomerization of α-hydroxyglutarate. Since cleavage of citramalate to form acetate and pyruvate has been demonstrated (*35*, *36*), we were encouraged to expect a similar reaction with α-ethylmalate, leading to formation of α-ketobutyrate and acetate (*44*). An indication of the breakdown of α-ethylmalate was obtained when the CoA ester was incubated in the presence of Mg^{2+}, phenylhydrazine, and crude extract of *P. aeruginosa* at pH 7.0; a rapid increase in absorbance was recorded at 324 mμ, which is close to the absorption maximum for α-ketobutyrate phenylhydrazone. But when the free acid was substituted for the CoA ester, or when boiled extract was used, no increase in absorbance occurred. There was a similar lack of absorbance increase in the absence of phenylhydrazine. These experiments were repeated with β-ethylmalyl-CoA and free β-ethylmalic acid, and identical results were obtained. The reaction is based on the formation and trapping of α-ketobutyrate as the phenylhydrazone. This principle has been utilized effectively in assaying isocitrate lyase in which glyoxylate formed from isocitrate is trapped as the phenylhydrazone; the rate of increase of absorbance at 324 mμ is a measure of phenylhydrazone formation and thus of enzyme activity (*19*).

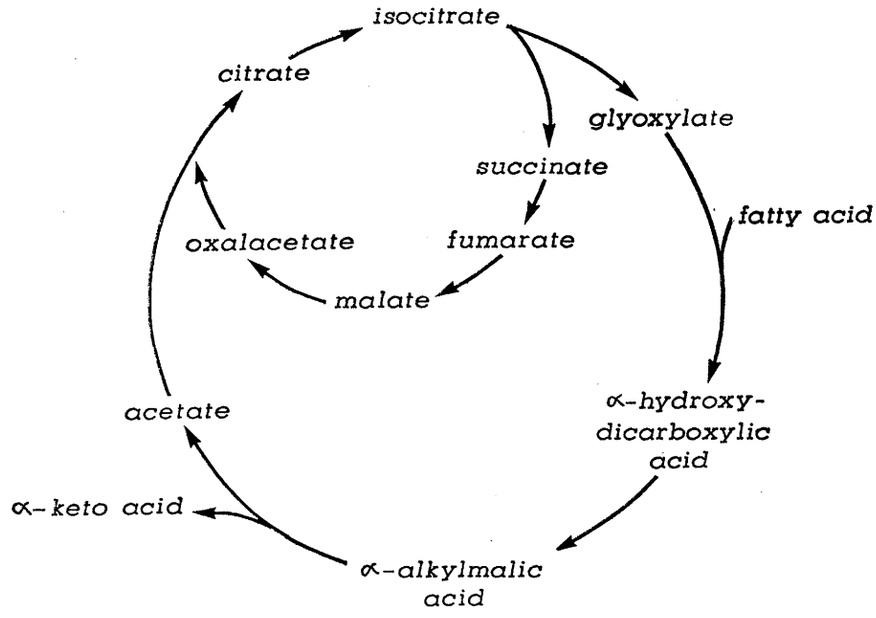

Fig. 11. "α-Alkylmalic acid cycle" in the bacterial metabolism of glyoxylate and fatty acids.

When the assay mixture employing β-ethylmalyl-CoA was further incubated and then deproteinized and treated with 2,4-DNP hydrazine, a DNP-hydrazone was formed. Purification of the hydrazone and analysis of it in the visible light region in alkaline solution provided a spectrum identical with that of authentic α-ketobutyric acid-2,4-DNP-hydrazone. The absorption maximum in both cases was 437 mμ. The identity of the hydrazones was confirmed by paper chromatography in several solvent systems.

The reverse reaction has been studied in greater detail. The 40-to-60-percent ammonium sulfate fraction of extracts of *P. aeruginosa* contains virtually all of the catalytic activity for synthesis of α-ethylmalate from α-ketobutyrate and acetyl-CoA (or of acetate in the presence of CoA, ATP, and Mg^{2+}). α-Ethylmalate is not detected in the absence of either of the substrates (or of CoA, or ATP) or enzyme. α-Ethylmalate is assayed fluorometrically, after treatment with sulfuric acid and reaction of the resulting keto acid with resorcinol, by the method of Strassman and Ceci (*45*). The K_m values for acetate and α-ketobutyrate are similar to those reported by Strassman and Ceci (*45*) for acetate and α-ketoisovalerate when bakers' yeast extract was used as the source of enzyme. Whereas Strassman and Ceci's system requires only catalytic quantities of CoA, the reaction catalyzed by *P. aeruginosa* needs substrate quantities for enzyme saturation. The K_m values for acetate and CoA in our system are $2.4 \times 10^{-4}M$ and $1.2 \times 10^{-4}M$, respectively. We consider this an indication that α-ethylmalate is produced as the CoA ester, although the compound is isolated from reaction mixtures almost entirely as the free acid. Here, as before, an enzymatic deacylation probably occurs, but as yet this has not been investigated in detail.

The formation of α-ethylmalate from α-ketobutyrate and acetate was also shown chromatographically. Areas of paper chromatograms in which the compound was located were removed and continuously extracted with ether. α-Ethylmalate, contained in the extract, was degraded by sulfuric acid and heat, and 2-butanone-2,4-DNP-hydrazone was synthesized. The R_F values of the derivative were indistinguishable from those of the DNP-hydrazone derivative of authentic 2-butanone in several solvent systems.

It was suggested earlier that β-ethylmalate might undergo oxidation at the hydroxyl group. We found that the 20-to-40-percent ammonium sulfate fraction of the crude extract of *P. aeruginosa* contains an enzyme which

oxidizes β-ethylmalyl-CoA in the presence of NAD (the free acid is oxidized at very low rates). When the substrate was prepared with the use of ethyl chloroformate in the mixed anhydride synthesis (*46*), rapid reduction of NAD was observed, and no cofactor was required or found stimulatory. But when the substrate was prepared with the use of methyl chloroformate, the initial rate of β-ethylmalyl-CoA oxidation was almost four times greater in the presence of Mg^{2+} than in its absence. A keto compound was readily isolated from reactions containing the substrate free of ethanol, that is, it was prepared with the use of methyl chloroformate, whereas this had not been possible when ethanol-contaminated substrate was used (*33*). The reason for this difference in the ability of the substrate to be oxidized became clear when we investigated further the synthesis of β-ethylmalyl-CoA and other CoA esters. Ethanol is formed as a byproduct of the synthesis of CoA esters by the mixed anhydride method in which ethyl chloroformate is used. Ethanol may interfere with the oxidation of CoA esters of acids containing hydroxyl groups by enzymes dependent on NAD. This interference is based on the ubiquity of alcohol dehydrogenase in cell-free extracts which, in the presence of ethanol and NAD, may mask the activity of CoA-ester dehydrogenases. The use of methyl chloroformate takes advantage of the facts that methanol (as a byproduct in the synthesis) is such a poor substrate for alcohol dehydrogenases from a number of sources and that its presence will not interfere with the assay of other dehydrogenases requiring NAD (*33*). The oxidation of β-ethylmalyl-CoA is being further studied in our laboratory.

The significance of the metabolism of β-ethylmalyl-CoA is speculative, since the pathway remains to be completely defined. However, there may be interrelations between this and other known metabolic reactions.

It appears that the formation of β-ethylmalate from glyoxylate and butyrate, with its further metabolism to acetate and α-ketobutyrate, is actually a mechanism for the reduction of glyoxylate and the oxidation of butyrate. This mechanism, based on consecutive dehydration of β-ethylmalate and rehydration of ethylmaleate to α-ethylmalate, is analogous to the interconversion of citrate and isocitrate by way of *cis*-aconitate. Although in propionate metabolism the mechanism is different, the result is similar: glyoxylate is reduced to acetate and propionate is oxidized to pyruvate. In both cases glyoxylate reduction can be considered a means for the conservation of acetate.

α-Ethylmalate is formed from β-ethylmalyl-CoA and ethylmaleyl-CoA, and also from the condensation of α-ketobutyrate with acetyl-CoA. The same reactions are utilized in the pathway by which β-isopropylmalic acid is synthesized from α-ketoisovaleric acid and acetyl-CoA by other microorganisms (*45, 47*). Except for the formation of isocitrate, the participation of glyoxylate in the formation of β-alkyl-substituted malic acids has not heretofore been investigated. Such acids serve as precursors of leucine, lysine, and glutamic acid according to the generalized mechanism proposed by Strassman and Ceci (*45*). Thus, condensations of appropriate CoA esters with glyoxylate might provide an alternative route to these β-substituted malic acids.

Metabolism of β-n-Propylmalate

In comparison with α-hydroxyglutarate and β-ethylmalate, little is known of the fate of β-*n*-propylmalate. But, since β-ethylmalate and β-*n*-propylmalate are homologs and are formed by the same condensation mechanism, their metabolism may be similar.

We do know that *cis*-enol oxalacetic acid can be isolated from prolonged incubations of reaction mixtures containing glyoxylate and *n*-valeryl-CoA in the presence of extracts of valerate-grown *E. coli* (*18*). The same compound is also isolated from incubations of *n*-propylmalate supplemented with CoA, ATP, and Mg^{2+}. Formation of *cis*-enol oxalacetate in the first system is dependent on the presence of active protein, glyoxylate, and *n*-valeryl-CoA. In the second system, active protein, CoA, and ATP are likewise essential.

When glyoxylate-1-C^{14} and unlabeled *n*-valeryl-CoA were used, the recovered oxalacetate was labeled. The reverse [with (*n*-valeryl-1-C^{14})-CoA], however, was not true. Clearly, the formation of oxalacetate from β-*n*-propylmalate does not occur by a simple splitting off of the propyl side chain. If this were the case, oxalacetate would be labeled when either of the substrates was labeled in the carbon No. 1 position.

β-*n*-Propylmalate might break down to acetate and α-ketovalerate in a way similar to that in which β-ethylmalate gives rise to acetate and α-ketobutyrate. One would then expect acetate to be labeled if glyoxylate-1-C^{14} and unlabeled valeryl-CoA were incubated together. The labeled acetate could then condense with glyoxylate to form malate which, in turn, could be oxidized to labeled oxalacetate. This could not occur if labeled valeryl-CoA was used, since the label would reside in α-ketovalerate but not in acetate. Admittedly, this scheme presupposes six sequential enzymatic reactions. But it should be noted that incubation periods ranged from 7 to 15 hours (incubations of 3 hours or less were insufficient to detect oxalacetate), supplemented crude extracts were used, and *p*H was neutral. Under these conditions such a scheme might be possible.

Enzyme Induction and Cellular Regulatory Mechanisms

Since the reactions catalyzed by α-hydroxyglutarate, β-ethylmalate, and β-*n*-propylmalate synthases closely resemble that catalyzed by malate synthase, the factors influencing their formation may be closely allied with those controlling the synthesis of malate synthase. Several mechanisms may operate to induce malate synthase. In *E. coli* grown on acetate, the enzyme appears to be induced by glyoxylate provided by the functioning of isocitrate lyase (*48*). In other organisms, malate synthase seems to be formed by a derepression mechanism, and in still others the enzyme is apparently constitutive (*12*). In a recent report (*49*) evidence is presented that two malate synthase isoenzymes are formed in an *E. coli* mutant grown on glycollate; one is considered as a derepressed enzyme, the second is thought to be induced by glycollate (*50*). In the following paragraphs we will consider the possibilities for induction of the other glyoxylate-condensing enzymes.

As has been discussed previously, these condensing enzymes are formed during growth on various short-chain fatty acids. If these enzymes are induced by glyoxylate, the metabolism of the fatty acids must proceed, at least in part, via a beta-oxidation mechanism to yield acetate. Acetate, then,

by inducing the formation of isocitrate lyase, presumably by lowering intracellular concentrations of phosphoenolpyruvate (*51*), could give rise to an intracellular pool of glyoxylate. This mode of induction is supported by the fact that in cells grown on glycollate the activity of these condensing enzymes is low. However, these enzymes are not detectable in cells grown on acetate (see Table 1). Since growth on acetate provides high concentrations of isocitrate lyase, the functioning of which would supply glyoxylate, these enzymes would be expected to be present in acetate-grown cells if glyoxylate were the inducer.

It is possible that each of the condensing enzymes is induced by the respective substrate fatty acid per se. However, this concept appears untenable in light of the fact that valerate-grown cells possess, in addition to the β-*n*-proplymalate synthase, β-ethylmalate and α-hydroxyglutarate synthases. Beta oxidation of valerate may give rise to propionate and acetate, but it is unlikely that under these conditions an appreciable amount of butyrate would accumulate. This conclusion is also supported by the fact that valerate-grown cells possess, in addition, enzymes which catalyze condensations of glyoxylate with β-hydroxybutyryl-CoA, crotonyl-CoA, isobutyryl-CoA, caproyl-CoA, and lactyl-CoA. The products of these condensation reactions have not as yet been identified.

Induction by Derepression

It is also possible to explain the formation of these condensing enzymes, under appropriate growth conditions, by a derepression mechanism. A greater degree of derepression of enzyme formation may result from growth on valerate than occurs during growth on acetate, propionate, or butyrate. This hypothesis might be investigated by determining the effect on the formation of these enzymes of various factors which influence growth on these substrates.

In most microorganisms studied, the glyoxylate-bypass enzymes are repressed when the products of their reactions, that is, succinate or malate, or substrates which can give rise to these products, that is, glucose, are available in the growth medium. It will be instructive to determine whether such growth conditions also repress the other glyoxylate-condensing enzymes, especially since the products of these glyoxylate-condensing reactions are biologically unique. The repression of malate synthase by succinate, for example, would not result in a cessation of malate formation, since malate may be formed from succinate via the tricarboxylic-acid cycle. In contrast, however, repression of the other glyoxylate-condensing enzymes by acids of the tricarboxylic-acid cycle would stop the formation of the products of the enzymes, since, it is generally thought, these products are not formed during the usual metabolic reactions involving succinate or other tricarboxylic acid intermediates. If the condensation products added to the growth media repressed their respective glyoxylate-condensing enzymes, for example, if α-hydroxyglutarate repressed formation of α-hydroxyglutarate synthase, but not of the butyrate or valerate-condensing enzymes, this would provide further evidence that the condensation reactions are catalyzed by separate enzymes.

Control of Enzyme Activities

In many biosynthetic pathways the operation of an enzyme is controlled by inhibition of enzyme activity as well as by repression of enzyme formation. Preliminary evidence indicates that β-ethylmalic acid, the product of the butyryl-CoA-glyoxylate condensation, inhibits malate synthase activity. If such feedback inhibition does exist amongst the various pathways generated by the condensation of glyoxylate with fatty acid CoA esters, it would constitute a significant control mechanism; the product of one enzymatic reaction might then inhibit the other glyoxylate-condensing reactions.

Kornberg (*52*) has shown that phosphoenolpyruvate is a potent inhibitor of isocitrate lyase from *E. coli* Bm. Succinate likewise inhibits this enzyme in *Micrococcus denitrificans* (*53*) and *Pseudomonas indigofera* (*54*). However, with isocitrate lyase of *E. coli* E 26, the strain used in our laboratories, neither phosphoenolpyruvate nor succinate at concentrations used by the workers cited above exhibited significant inhibition. Preliminary experiments indicate that α-hydroxyglutarate and β-ethylmalate, on the contrary, markedly inhibit isocitrate lyase activity. Thus there may be an efficient control mechanism whereby the accumulation of a product derived from glyoxylate may control the formation of that product by inhibiting a primary glyoxylate-yielding reaction. Such a regulatory system apparently does not exist in the isocitrate lyase-malate synthase system in *E. coli* since, as Umbarger (*55*) has shown, malate does not inhibit isocitrate lyase activity.

Conclusion

Numerous advances have been made and a new concept has evolved as a consequence of investigations on the role of glyoxylate in bacterial metabolism. The key reactions are those by which glyoxylate, probably as the coenzyme-A ester, condenses with CoA esters of fatty acids with higher molecular weights than acetate. That glyoxylate may play a highly important role in cellular physiology is further apparent from evidence that it also condenses enzymatically with CoA esters of isovalerate, caproate, lactate, crotonate, and β-hydroxybutyrate. The significance of these condensations (and the metabolism of the products) in the total economy of the bacterial cell is not yet clear. What is apparent, however, is that acyl-CoA synthetases for many organic acids exist in bacteria and that these activated compounds are probably more prevalent in intermediary metabolism than had been realized.

A most interesting observation is the conversion of glyoxylate to acetate via the glyoxylate-fatty acid condensation product. Thus yet another avenue is provided for the bacterial synthesis of acetate. This may be more clearly understood from the cycle of reactions shown in Fig. 11.

This "α-alkylmalic acid cycle" summarizes the essential biochemical features of the metabolism of glyoxylate as a function of both propionate and butyrate. The means differ by which α-hydroxyglutaric and β-ethylmalic acids are metabolized to citramalic and α-ethylmalic acids, respectively. But the fact that the latter are formed and undergo an aldol cleavage to yield acetate unifies these pathways and makes possible the generalized cyclic scheme. This proposed mechanism provides acetate and an α-keto acid by reduction of glyoxylate and oxidation of a fatty acid.

The inner cycle may be considered as a modified tricarboxylic-acid cycle,

the operation of which depends on isocitrate lyase to furnish glyoxylate necessary for the fatty-acid condensation reaction and also to provide succinate, the precursor of oxalacetate.

We have attempted to summarize data concerning a relatively unexplored area of intermediary metabolism. Specifically, these investigations relate to the central role of glyoxylate in the metabolism of fatty acids and other mono- and dicarboxylic acids in bacteria. Considerable progress has been made in elucidating the condensations of glyoxylate with various fatty-acid CoA esters and the metabolism of these condensation products. Future efforts must be directed towards evaluating the significance of these reactions in the growth and total economy of the bacterial cell.

References and Notes

1. A. Harden, *J. Chem. Soc.* **79**, 610 (1901).
2. T. Thunberg, *Skand. Arch. Physiol.* **40**, 1 (1920); F. Knoop, *Klin. Wochschr.* **2**, 60 (1923).
3. H. A. Krebs and W. A. Johnson, *Enzymologia* **4**, 148 (1937); H. A. Krebs, *Advan. Enzymol.* **3**, 191 (1943).
4. S. J. Ajl, *Bacteriol. Rev.* **15**, 211 (1951); *Physiol. Rev.* **38**, 196 (1958).
5. S. R. Elsden, in *Special Lectures in Biochemistry 1954–1955*, University College, London (Lewis, London, 1955), p. 17.
6. H. L. Kornberg, *Ann. Rev. Microbiol.* **13**, 49 (1959).
7. J. A. Olson, *Nature* **194**, 695 (1954).
8. D. T. O. Wong and S. J. Ajl, *J. Am. Chem. Soc.* **78**, 3230 (1956).
9. H. L. Kornberg and N. B. Madsen, *Biochim. Biophys. Acta* **24**, 651 (1957); H. L. Kornberg and H. A. Krebs, *Nature* **179**, 988 (1957).
10. L. O. Krampitz, in *The Bacteria*, I. C. Gunsalus and R. Y. Stanier, Eds. (Academic Press, New York. 1961), vol. 2, p. 209.
11. H. A. Krebs and J. M. Lowenstein, in *Metabolic Pathways*, D. M. Greenberg, Ed. (Academic Press, New York, 1960). vol. 1, p. 129.
12. H. L. Kornberg and S. R. Elsden, in *Advan. Enzymol.* **23**, 401 (1961).
13. H. C. Reeves and S. J. Ajl, *J. Bacteriol.* **84**, 186 (1962).
14. H. C. Reeves, W. J. Stahl, S. J. Ajl, *ibid.* **86**, 1352 (1963).
15. H. C. Reeves and S. J. Ajl, in Abstracts, Eighth International Congress for Microbiology, Montreal, Canada, 1962, p. 33.
16. R. Rabin, H. C. Reeves, S. J. Ajl, *J. Bacteriol.* **86**, 937 (1963).
17. K. Imai, H. C. Reeves, S. J. Ajl, *J. Biol. Chem.* **238**, 3193 (1963).
18. K. Imai, H. C. Reeves, R. Rabin, S. J. Ajl, *Biochim. Biophys. Acta* **81**, 367 (1964).
19. G. H. Dixon and H. L. Kornberg, *Biochem. J.* **72**, 3p (1959).
20. W. S. Wegener, H. C. Reeves, S. J. Ajl, *Anal. Biochem.* **11**, 111 (1965).
21. ———, *J. Bacteriol.* **90**, 594 (1965).
22. W. S. Wegener, H. C. Reeves, P. Furmanski, S. J. Ajl, *Bacteriol. Proc.* **1965**, P75 (1965).
23. G. D. Novelli, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York. 1957), vol. 3, p. 915.
24. H. C. Reeves and S. J. Ajl, unpublished observations.
25. ———, *Biochem. Biophys. Res. Commun.* **12**, 132 (1963).
26. H. C. Reeves, W. Stahl, C. Urbano, S. J. Ajl, *Bacteriol. Proc.* **1964**, P79 (1964).
27. H. C. Reeves and S. J. Ajl, *J. Biol. Chem.* **240**, 569 (1965).
28. E. Racker, *ibid.* **190**, 685 (1951).
29. ———, *Biochim. Biophys. Acta* **9**, 577 (1952).
30. ———, in *Glutathione, A Symposium*, S. P. Colowick, A. Lazarow, E. Racker, D. R. Schwarz, E. Stadtman, H. Waelsch, Eds. (Academic Press, New York, 1954), p. 171.
31. In attempts to isolate and identify NAD-dependent enzymatically formed pyruvate as the 2,4-dinitrophenylhydrazone derivative, it was noted that derivatives were formed which, when chromatographed on paper, were unlike known standards and gave blue colors when sprayed with alcoholic KOH. This phenomenon was first observed in a control mixture of tris-HCl, NAD, and crude cell-free extract. After addition of 2,4-dinitrophenylhydrazine and removal of protein, a flocculent precipitate was formed in the supernatant during overnight incubation at 37°C. Further work showed that the precipitate resulted from a nonenzymatic interaction of dinitrophenylhydrazine and NAD. Addition compounds of this carbonyl reagent with NADP, NADH, and NADPH were also isolated and their spectral properties described. See R. E. Megraw, H. C. Reeves, S. J. Ajl, *Nature* **205**, 502 (1965).
32. R. E. McGraw, H. C. Reeves, S. J. Ajl, *Bacteriol. Proc.* **1965**, P74 (1965); *J. Bacteriol.* **90**, 984 (1965).
33. R. Rabin, R. E. Megraw, H. C. Reeves, J. Carlin, S. J. Ajl, *Bacteriol. Proc.* **1965**, P72 (1965); R. Rabin, R. E. Megraw, H. C. Reeves, S. J. Ajl, *Life Sci.* **4**, 1233 (1965).
34. H. C. Reeves and S. J. Ajl, *Biochim. Biophys. Acta* **97**, 600 (1965).
35. J. Adler, S. F. Wang, H. A. Lardy, *J. Biol. Chem.* **229**, 865 (1957); S. F. Wang, J. Adler, H. A. Lardy, *ibid.* **236**, 26 (1961).
36. R. A. Cooper and H. L. Kornberg, *Biochem. J.* **91**, 82 (1964).
37. H. Eggerer, P. Overath, F. Lynen, E. R. Stadtman, *J. Am. Chem. Soc.* **82**, 2643 (1960).
38. H. Weissbach, J. Toohey, H. A. Barker, *Proc. Natl. Acad. Sci. U.S.* **45**, 521 (1959).
39. R. M. Burton and E. R. Stadtman, *J. Biol. Chem.* **202**, 873 (1953).
40. M. Kusunose, E. Kusunose, M. J. Coon, *ibid.* **239**, 1374 (1964).
41. H. C. Reeves, C. Urbano, S. J. Ajl, *Bacteriol. Proc.* **1965**, P73 (1965).
42. V. Massey, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1955), vol. 1 p. 730.
43. R. Rabin, J. Carlin, S. J. Ajl, *Bacteriol. Proc.* **1965**, P76 (1965); R. Rabin and S. J. Ajl, *Biochim. Biophys. Acta* **97**, 388 (1965).
44. M. Strassman, L. N. Ceci, B. E. Silverman, *Federation Proc.* **23**, 312 (1964).
45. M. Strassman and L. N. Ceci, *J. Biol. Chem.* **238**, 2445 (1963).
46. M. Flavin, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press. New York, 1963), vol. 6, p. 538.
47. C. Jungwirth, S. R. Gross, P. Margolin, H. E. Umbarger, *Biochemistry* **2**, 1 (1963); S. R. Gross, R. O. Burns, H. E. Umbarger, *ibid.* p. 1046; J. M. Calvo, M. G. Kalyanpur, C. M. Stevens, *ibid.* **1**, 1157 (1962).
48. H. C. Reeves and S. J. Ajl, *J. Bacteriol.* **83**, 597 (1962).
49. P. Falmagne, E. Vanderwinkel, J. M. Wiame, *Arch. Intern. Physiol. Biochim.* **71**, 813 (1963).
50. E. Vanderwinkel, P. Liard, F. Ramos, J. M. Wiame, *Biochem. Biophys. Res. Commun.* **12**, 157 (1963).
51. H. L. Kornberg, in Abstracts, Sixth International Congress of Biochemistry, New York, 1964, vol. 9, p. 678.
52. J. M. Ashworth and H. L. Kornberg, *Biochim. Biophys. Acta* **73**, 519 (1963); H. L. Kornberg, *Angew. Chem. Inter. Ed. Eng.* **4**, 558, (1965).
53. H. L. Kornberg, J. F. Collins, D. Bigley, *Biochim. Biophys. Acta* **39**, 9 (1960).
54. W. V. Howes and B. A. McFadden, *J. Bacteriol.* **84**, 1216 (1962).
55. H. E. Umbarger, *Federation Proc.* **19**, 52 (1960).

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