β-ETHYLMALATE SYNTHETASE

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ABSTRACT

RABIN, ROBERT (Albert Einstein Medical Center, Philadelphia, Pa.), HENRY C. REEVES, AND SAMUEL J. AJL. β-Ethylmalate synthetase. J. Bacteriol. **86**:937–944. 1963.—*Pseudomonas aeruginosa*, when grown in an inorganic salts medium with butyrate as the sole carbon source, produced an enzyme which catalyzed the condensation of butyryl coenzyme A (CoA) and glyoxylate to form β-ethylmalic acid. Maximal activity was found in the 40 to 60% $(NH_4)_2SO_4$ fraction of cell-free extracts. Mg^{++} was stimulatory, and the optimal pH was 8.0 to 8.5. β-Ethylmalic acid-C^{14} was demonstrable in paper chromatograms when either glyoxylate-*1*-C^{14} or butyryl CoA-*3*-C^{14} was included in complete systems incubated anaerobically. When either glyoxylate, butyryl CoA, or enzyme was omitted, β-ethylmalate was not detected. Further evidence that β-ethylmalic acid-C^{14} was the product of the condensation of butyryl CoA-*3*-C^{14} and nonlabeled glyoxylate was provided by synthesis of the derivative 5-ethyluracil-C^{14}. The derivative was recrystallized to constant specific activity and identified by its melting point and spectral properties.

The discovery of malate synthetase (EC4.1.3.2.) in *Escherichia coli* by Wong and Ajl (1956) and its significance in acetate metabolism have been extensively reviewed (Wong and Ajl, 1957; Kornberg, 1959; Krebs and Lowenstein, 1960; Kornberg and Elsden, 1961). Because the enzymatic condensation of glyoxylate with acetate may be the prototype of a more widely occurring reaction than is now known, our laboratory has been investigating the enzymatic condensations of glyoxylate with other monocarboxylic acids. As a result, Reeves and Ajl (1962) have recently reported that α-hydroxyglutaric acid is formed by the condensation of glyoxylate with propionate. This reaction is catalyzed by α-hydroxyglutarate synthetase obtained from aerobically grown, propionate-adapted *E. coli*. The subsequent finding (Reeves et al., 1963; Reeves and Ajl, 1963) that acetate and lactate are enzymatically formed from the condensation product indicates a possible new metabolic pathway in propionate-grown cells.

Recent work by Imai, Reeves, and Ajl (1963) has demonstrated that glyoxylate and valerate enzymatically condense in the presence of extract from valerate-grown *E. coli* to form β-*n*-propylmalic acid.

The present study, preliminary results of which have been presented (Rabin, Reeves, and Ajl, 1963), was undertaken to extend the earlier observations concerning the role of glyoxylate in acetate- and propionate-adapted *E. coli* to butyrate-adapted *Pseudomonas aeruginosa*. The experimental approach, as presented in this report, is based on the incubation of C^{14}-labeled glyoxylate or butyryl coenzyme A (CoA) in the presence (or absence) of cell-free extracts derived from butyrate-grown bacteria. Paper chromatography of the deproteinized, ether-extracted incubation mixtures shows the presence of β-ethylmalic acid-C^{14} (which is not formed in appropriate controls).

MATERIALS AND METHODS

Organism. The bacterium used was isolated in our laboratories in pure culture. It was a small, motile, gram-negative rod which produced a soluble blue-green pigment in common liquid or solid culture media. The pigment was extractable with chloroform and changed to an insoluble red pigment when the chloroform extract was acidified. The bacterium grew well at room temperature, at 37 C, and at 42 C on ordinary media, and rapidly liquefied gelatin. Reaction in litmus milk

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was alkaline. Lactose was not fermented and H_2S was not produced in Friewer Shaughnessy Medium (Difco). These are typical characteristics of *P. aeruginosa*, and the organism was provisionally identified as such.

Growth and handling of cells. Stock cultures were stored at 4 C on agar slants of a mineral salts medium (Reeves and Ajl, 1960) containing 0.2% sodium butyrate as the sole carbon source. Growth on slants was washed off into flasks of the identical medium without agar, and the cultures were incubated at 37 C on a rotary shaker for 24 to 36 hr. These were transferred to 8 liters of the same medium and incubated with forced aeration for 48 to 72 hr. After being harvested, cells were kept at −20 C until extracts were prepared. From a 10% [5 g (wet weight)/50 ml] suspension of cells in a solution of 0.001 M $MgCl_2$ and 0.005 M tris(hydroxymethyl)aminomethane (Mg-tris buffer) at pH 8.0, cell-free extract was made by sonic treatment in a Raytheon sonic oscillator (10 kc, 250 w) at maximal output (cup at 3 C) for 6 min followed by centrifugation at 14,500 × *g* for 30 min at 2 C. The supernatant fluid was fractionated by addition of solid $(NH_4)_2SO_4$, and the protein in each fraction was dissolved in a small volume of Mg-tris buffer.

Assays. β-Ethylmalate synthetase was assayed in a Cary model 14 recording spectrophotometer by the method of Dixon and Kornberg (1959) for malate synthetase. Butyryl CoA was determined by the method of Lipmann and Tuttle (1945). Protein was determined spectrophotometrically by use of the formula of Warburg and Christian as modified by Layne (1957).

Chromatography. Chromatography was done by the ascending method at 24 C with strips (1.5 in. wide) of Whatman no. 1 filter or chromatography paper. Distribution of C^{14} in air-dried chromatograms was determined by cutting 0.5-in. segments in succession from spotting line to solvent front. Each segment was counted in a liquid scintillation spectrometer (Packard Tri-Carb model 314 EX). The scintillation fluid contained: 2,5-diphenyloxazole, 1.5 g; 1,4-bis-2-(5-phenyloxazoleyl)-benzene, 75 mg; toluene, 150 ml; and absolute ethanol, 75 ml. A 10-ml amount of scintillation fluid was used in each counting vial. After the segments were counted, the chromatograms were redrawn to scale, and percentage distribution of isotope was calculated at various R_F values. Measurements of C^{14} distribution were also made with a Scanogram II chromatogram scanner (Atomic Accessories, Inc., Valley Stream, N.Y.)

Chemicals. α-Hydroxyadipic acid was obtained from Siegfried Ltd., Zofingen, Switzerland. α-Hydroxy-β-methylglutaric acid, disodium salt, was obtained from Cyclo Chemical Corp., Los Angeles, Calif. β-Ethylmalic acid was synthesized by the hydrolysis of diethyl-β-ethylmalate, which was in turn prepared by the reduction of ethyl ethoxalylbutyrate by catalytic hydrogenation (Scherp, 1946). The ethyl ethoxalylbutyrate was synthesized by the Claisen condensation of diethyl oxalate and ethyl butyrate (Cox and McElvain, 1943). Powdered sodium used in the condensation was prepared by the method of Vogel (1948). Butyryl CoA was synthesized by the method of Simon and Shemin (1953); when the reaction was completed, excess butyric anhydride was removed by several ether extractions. Sodium glyoxylate-*1*-C^{14} monohydrate was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Butyric anhydride-*3*-C^{14} used in the synthesis of butyryl CoA-*3*-C^{14} was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Evidence for enzymatic condensation of butyryl CoA and glyoxylate. Dixon and Kornberg (1959) showed that 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. Since the thiol ester bond in acyl CoA derivatives of monocarboxylic acids absorbs strongly at 232 mμ, the hydrolysis of this bond can be followed by measuring the decrease in optical density at this wavelength. This method was found suitable for the present work. Typical results of assays of β-ethylmalate synthetase in various $(NH_4)_2SO_4$ fractions (dialyzed 12 hr against Mg-tris buffer at pH 8.0) are shown in Table 1. Each reaction was followed for 6 min, and the data are based on the differences observed between complete systems and control without enzyme. The 40 to 60% fraction consistently showed the greatest specific activity. However, the activity was subsequently found to be 50% higher in nondialyzed fractions. The nondialyzed 40 to 60% fraction, like the dialyzed one, could not be stored longer than 24 hr at 2 C without being inactivated; storage at −20 C for 24 hr resulted in considerable loss of activity. Because of their lability, cell-free extracts were prepared, fractionated, and

used (undialyzed) on the same day in all experiments. A strong butyryl CoA deacylase, present in the crude cell-free extract, was found only in the 20 to 40% $(NH_4)_2SO_4$ fraction. In Table 1, the specific activity of the 20 to 40% fraction is corrected for deacylase activity. When $MgCl_2$ was omitted, the specific activity was reduced by 50%. Specific activity was maximal at pH 8.0 and 8.5; at pH 7.0, activity was completely lost.

Condensation products. Three possible products may be formed by the condensation of butyryl CoA and glyoxylic acid (Fig. 1). If the alpha carbon of butyrate and the carbonyl group of glyoxylate condense, the expected product is β-ethylmalic acid. Beta- and gamma-carbon condensations would be expected to yield, respectively, α-hydroxy-β-methylglutaric acid and α-hydroxyadipic acid.

TABLE 1. *Assay of β-ethylmalate synthetase in cell-free extract of Pseudomonas aeruginosa**

Ammonium sulfate fraction	Butyryl CoA cleaved per mg of protein per 6 min
%	μmoles
0–20	0
20–40	0.009
40–60	0.052
60–100	0

* Cuvette contents: 2.25 ml of 0.1 M tris buffer (pH 8.0), 8.4 μmoles of $MgCl_2$, 0.2 μmoles of butyryl CoA, 0.02 ml of $(NH_4)_2SO_4$ fraction, and 2.5 μmoles of sodium glyoxylate monohydrate. Total volume: 2.48 ml. The assay procedure was described by Dixon and Kornberg (1959).

Formation of β-ethylmalic acid from labeled glyoxylate and nonlabeled butyryl CoA. A number of experiments were done, with incubation times varying from 30 min to 6 hr, with mixtures of the complete system which contained $MgCl_2$, butyryl CoA, glyoxylate, and 40 to 60% $(NH_4)_2SO_4$ protein fraction at pH 8.0; controls minus butyryl CoA, glyoxylate, and protein fraction were incubated simultaneously. None of the deproteinized ether extracts of these mixtures revealed any of the possible condensation products when paper chromatograms were sprayed with alcoholic bromocresol green solution. To obtain a more sensitive method for detecting minute quantities of condensation product, C^{14}-labeled glyoxylate and unlabeled butyryl CoA were paired in certain incubations, and unlabeled glyoxylate and C^{14}-labeled butyryl CoA were paired in other incubations. Incubations were carried out at 37 C in Thunberg tubes which had been alternately evacuated and flushed with N_2 several times. After incubation, protein was precipitated with tungstic acid and removed by centrifugation. The clear supernatant fluids were adjusted to pH 2 to 3 with 10 N NaOH and continuously ether-extracted in a Kutcher-Steudel type extractor (Bellco Glass, Inc., Vineland, N.J.) for 36 hr. After the ether was evaporated, the residues in each flask were dissolved in 1.0 ml of water, and paper chromatography was carried out as described. Labeled α-hydroxy-β-alkyldicarboxylic acid accumulated from labeled glyoxylate with increased incubation time, and no accumulation occurred in the controls (Fig. 2). The solvent

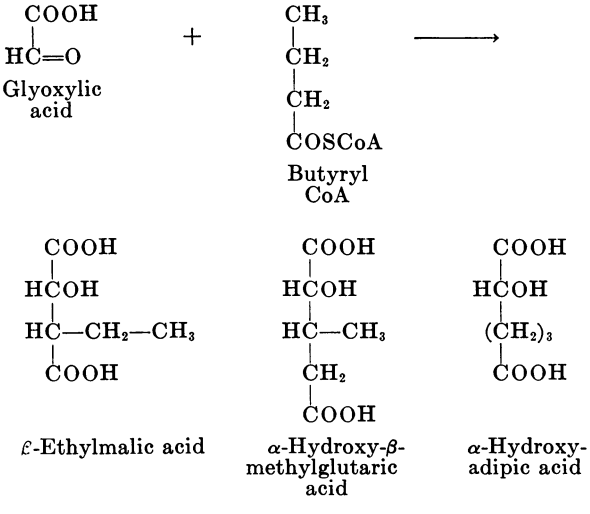

FIG. 1. *Possible products resulting from the enzymatic condensation of glyoxylic acid and butyryl CoA.*

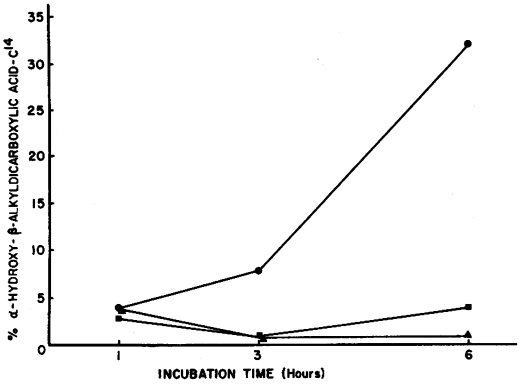

FIG. 2. *Formation of α-hydroxy-β-alkyldicarboxylic acid-C^{14} during incubation of systems containing Na glyoxylate-1-C^{14}. The complete test system contained 1.0 ml of tris buffer (0.5 M, pH 8.0), 40 μmoles of $MgCl_2$, 1.25 ml of 40 to 60% $(NH_4)_2SO_4$ fraction of Pseudomonas aeruginosa cell-free extract, 5.4 μmoles of butyryl CoA, 0.25 μmoles of Na glyoxylate monohydrate-1-C^{14}. Total volume: 5.65 ml. After incubation at 37 C, the system was deproteinized with tungstic acid and continuously ether-extracted for 36 hr. Samples of the aqueous solutions of the ether-soluble residues were chromatographed on Whatman no. 1 chromatography paper by the ascending method at 24 C with ethyl acetate-acetic acid-water (4:1:5), upper phase. Equal-sized segments cut from spotting line to solvent front were counted in a liquid scintillation spectrometer. Figure shows percentage of C^{14} found at R_F of β-ethylmalic and α-hydroxy-β-methylglutaric acids from complete system (●), control minus butyryl CoA (■), and control minus 40 to 60% $(NH_4)_2SO_4$ fraction (▲). Data for each time period were obtained from separate incubations with freshly made enzyme preparations; their specific activities for the 1-, 3-, and 6-hr incubations were 0.08, 0.08, and 0.09 μmole of butyryl CoA cleaved per mg of protein per 6 min, respectively.*

system used differentiated α-hydroxyadipic acid from β-ethylmalic and α-hydroxy-β-methylglutaric acids, but the β-alkyl acids showed the same R_F. This was typical of the results obtained with a large number of solvent systems. Increased radioactivity was never detected at the R_F of α-hydroxyadipic acid.

In Fig. 3 are shown reconstructed chromatograms from a 3-hr incubation period corresponding to the data for the same time shown in Fig. 2. With the described isotope-counting technique, it was possible to account for 80 to 85% of the total radioactivity in each chromatogram in well-defined areas. It is evident that α-hydroxy-β-alkyldicarboxylic acid accumulated in the complete system E, but not in controls C_2 (minus butyryl CoA) or C_3 (minus enzyme).

Identification of labeled β-ethylmalic acid from incubations with labeled glyoxylate. Good separation of the two α-hydroxy-β-alkyl acids was possible only in the solvent system ether-benzene-formic acid-water (21:9:7:2), a modification (by K. Imai in our laboratory) of one described by Adelberg (1953). Figure 4 shows a chromatogram, reconstructed to scale, developed in this solvent system. Peak radioactivity occurred in the area of β-ethylmalic acid. The activity adjacent to the α-hydroxy-β-methylglutaric acid spot is apparently due to slight trailing of β-ethylmalic acid.

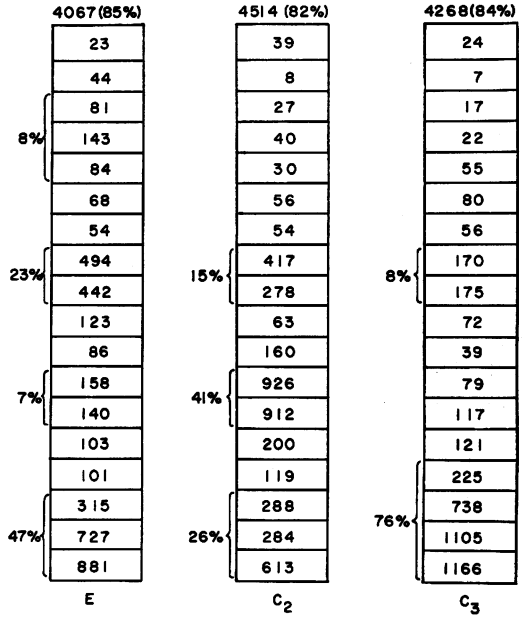

FIG. 3. *Reconstructed (to scale) radiochromatograms from 3-hr incubation period (see Fig. 2 for details). Counts per minute minus background are shown in each segment. Brackets indicate well-defined areas of radioactivity; accompanying percentages were calculated from the total counts per minute shown at the top of each chromatogram. The sums of the individual percentages are shown in parentheses. Reading from bottom to top, the lowest bracketed area corresponds to authentic glyoxylate, the next area in E and C_2 corresponds to authentic malate, the next area is unknown, and the topmost area in E only is identical with β-ethylmalic and α-hydroxy-β-methylglutaric acids.*

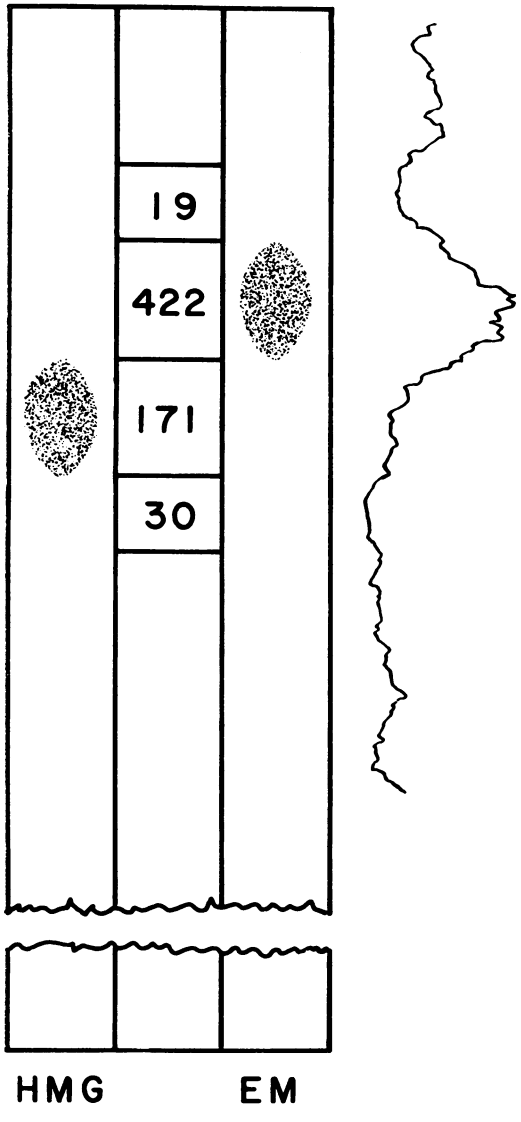

FIG. 4. *Location of authentic and biosynthetic radioactive β-ethylmalic acids and their separation from α-hydroxy-β-methylglutaric acid by paper chromatography. The Whatman no. 1 filter paper was divided lengthwise in three sections; the left section was spotted with authentic α-hydroxy-β-methylglutaric acid (HMG), and the right section with authentic β-ethylmalic acid (EM). The middle section was spotted with a sample of an aqueous solution of the ethereal extract of the deproteinized complete system after 6 hr of incubation (see Fig. 2 for contents of complete system). After ascending chromatography at 24 C in ether-benzene-formic acid-water (21:9:7:2), the side sections were removed, autoclaved for 10 min, and sprayed with bromocresol green to locate the acids. The middle section was scanned with an automatic radiochromatogram scanner. The record of the scanned section is shown with the right edge of the chromatogram as the abscissa of the recording paper. Segments of the middle strip corresponding to the authentic acid spots, and the segments adjacent to these, were cut out and counted in the scintillation spectrometer. Counts per minute minus background are shown in the segments.*

Formation of labeled β-ethylmalic acid from incubations with labeled butyryl CoA. After a 6-hr incubation of the complete system with labeled butyryl CoA and unlabeled glyoxylate, β-ethylmalic acid accounted for 20% of the total radioactivity on chromatograms developed in the modified Adelberg solvent. On chromatograms prepared from simultaneously incubated controls minus glyoxylate and minus enzyme, the figures were 3% and 5%, respectively.

If β-ethylmalic acid could be easily crystallized, it would be possible to mix the biosynthesized labeled compound with carrier acid and recrystallize it to constant specific radioactivity. But the acid occurs normally as a syrup and solidifies incompletely in an amorphous form only when kept in the cold in vacuo over a strong desiccant for at least a week. However, a derivative, 5-ethyluracil, crystallizes easily, has a definite melting point, and has characteristic absorption properties in the ultraviolet region. To obtain β-ethylmalic acid-C^{14} for the synthesis of the derivative, a radiochromatogram was prepared from the ethereal extract of a complete system, containing labeled butyryl CoA, after 6 hr of incubation. Authentic β-ethylmalic acid was spotted on Whatman no. 1 filter paper alongside the radioactive sample, and was developed in the modified Adelberg system. The area of the radiochromatogram adjacent to the spot of β-ethylmalic acid was cut out and continuously ether-extracted for 72 hr. The ether was evaporated, and 350 mg of unlabeled β-ethylmalic acid were added to the labeled acid residue. The mixture was reacted with 300 mg of urea (recrystallized from hot 95% ethanol) dissolved in 1.5 ml of fuming H_2SO_4, according to the method of Scherp (1946). The reaction involved is shown below; the probable positions of C^{14} in the compounds originating from butyryl CoA are indicated by the asterisk.

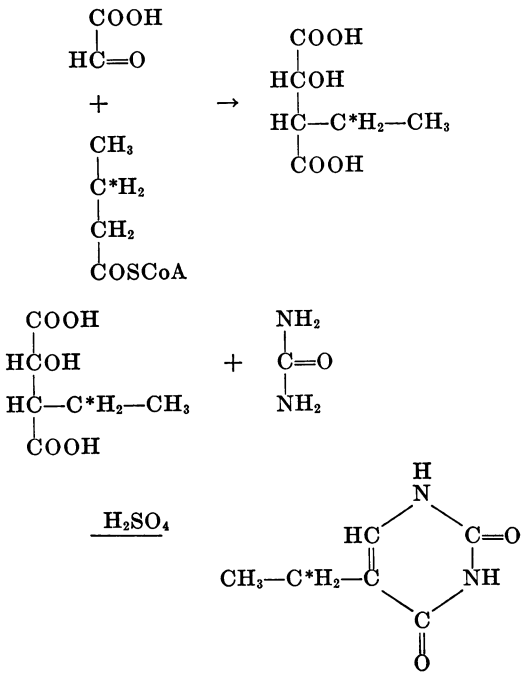

Table 2 summarizes the purification of 5-ethyluracil by recrystallizations from hot water. The melting point of the twice-recrystallized product agrees closely with those reported: 301 to 303 C with decomposition by Ulbricht (1959); 300 C with sublimation by Guyot and Mentzer (1958); 300 to 303 C by Burckhalter and Scarborough (1955); 300 C with decomposition by Johnson and Menge (1906); darkens at 300 C and melts at 303 C by Tafel and Thompson (1907). That radioactivity did not decrease during purification indicates that it was derived from β-ethylmalic acid-C^{14} synthesized during the incubation. Further evidence that radioactivity in the derivative came from β-ethylmalic acid was provided by the observation that we were unable to form a uracil-type compound from α-hydroxyadipic or α-hydroxy-β-methylglutaric acids and urea.

Absorption spectra of the twice-recrystallized 5-ethyluracil were determined at pH 2 and 12 in a Cary spectrophotometer. Spectral properties of the compound, compared with those of uracil and 5-methyluracil (thymine), and the melting points of the compounds are shown in Table 3. The similarity of the spectral data and distinctive melting point of each compound provide additional evidence that 5-ethyluracil was the derivative synthesized.

DISCUSSION

The data presented herein clearly indicate that glyoxylate condenses with the alpha carbon of butyrate to form β-ethylmalate, and that this reaction is catalyzed by an enzyme present in cell-free extracts of *P. aeruginosa* which was adapted to grow on butyrate. To our knowledge, this reaction and its product, β-ethylmalate, have not been previously reported.

TABLE 2. *Purification of 5-ethyluracil-C^{14}*

Compound	Color	Melting point (unc)	Counts per min per mmole
		C	
Initial product	Light tan	302–305*	519
First recrystallization	Very light tan	301–304*	545
Second recrystallization	White	301–303*	580

* With decomposition.

TABLE 3. *Comparative spectral properties and melting points of 5-ethyluracil, 5-methyluracil, and uracil*

Compound	Melting point (unc)	Absorption spectrum				
		pH	Maximum	Minimum	250/260	280/260
	C		*mμ*	*mμ*		
5-Ethyluracil	301–303*	2	264	234	0.70	0.54
		12	290	245	0.67	1.35
5-Methyluracil	318–321*	2	264	233	0.67	0.53
		12	291	244	0.65	1.31
Uracil	335*	2	259	227	0.84	0.17
		12	284	241	0.71	1.40

* With decomposition.

The data are analogous to those of Imai et al. (1963) who, with *E. coli* extracts, found that the alpha carbon of valerate is involved in the condensation with glyoxylate to form the higher homologue, β-*n*-propylmalate. As Reeves and Ajl (1962) have shown, *E. coli* extracts also possess an enzyme which condenses propionate and glyoxylate, but the apparent point of condensation is the beta carbon of propionate, resulting in α-hydroxyglutarate.

Although it is apparent that glyoxylate may have hitherto unknown metabolic roles, the significance of condensations involving butyrate and valerate must await the results of work in progress. The significance of the acetate and glyoxylate condensation is well known and, indeed, clarifies our understanding of growth of *E. coli* on acetate. Similarly, data recently presented by Reeves et al. (1963) and Reeves and Ajl (1963) showing that lactate and acetate arise from α-hydroxyglutarate have led them to postulate that the acetate is available for utilization in the Krebs cycle and pyruvate arises by oxidation of lactate by well-known reactions.

The extension of the work with *E. coli* to *P. aeruginosa* indicates that the condensation of glyoxylate and short-chain fatty acids may occur widely in microorganisms able to grow aerobically and adaptively on these acids.

Acknowledgments

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Addendum in Proof

Since this manuscript was submitted, the organism provisionally identified as *Pseudomonas aeruginosa* was confirmed by Rudolph Hugh of the George Washington University School of Medicine, Washington, D. C.

Literature Cited

Adelberg, E. A. 1953. Quantitative determination of 1,2-glycols in mixtures. Anal. Chem. **25**: 1553.

Burckhalter, J. H., and H. C. Scarborough. 1955. The synthesis of uracils as anticonvulsants. J. Am. Pharm. Assoc. **44**:545–550.

Cox, R. F. B., and S. M. McElvain. 1943. Ethyl ethoxalylpropionate. Org. Syn. Coll. Vol. **2**: 272–273.

Dixon, G. H., and H. L. Kornberg. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**:3p.

Guyot, A., and C. Mentzer. 1958. New synthesis of thymine. Compt. Rend. **246**:436–438.

Imai, K., H. C. Reeves, and S. J. Ajl. 1963. Enzymatic condensation of valeryl-CoA and glyoxylate. Bacteriol. Proc., p. 103.

Johnson, T. B., and G. A. Menge. 1906. Researches on pyrimidins: 5-ethylcytosin. J. Biol. Chem. **2**:105–115.

Kornberg, H. L. 1959. Aspects of terminal respiration in microorganisms. Ann. Rev. Microbiol. **13**:49–78.

Kornberg, H. L., and S. R. Elsden. 1961. The metabolism of 2-carbon compounds by microorganisms. Advan. Enzymol. **23**:401–470.

Krebs, H. A., and J. M. Lowenstein. 1960. The tricarboxylic acid cycle. Metab. Pathways **1**:129–203.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 447–454. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.

Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. **159**:21–28.

Rabin, R., H. C. Reeves, and S. J. Ajl. 1963. Utilization of glyoxylate by butyrate-grown bacteria. Bacteriol. Proc., p. 104.

Reeves, H. C., and S. Ajl. 1960. Occurrence and function of isocitritase and malate synthetase in bacteria. J. Bacteriol. **79**:341–345.

Reeves, H. C., and S. J. Ajl. 1962. Alpha-hydroxyglutaric acid synthetase. J. Bacteriol. **84**:186–187.

Reeves, H. C., and S. J. Ajl. 1963. Enzymatic formation of lactate and acetate from α-hydroxyglutarate. Biochem. Biophys. Res. Commun. **12**:132–136.

Reeves, H. C., W. J. Stahl, C. Urbano, and S. J. Ajl. 1963. Enzymatic cleavage of *alpha*-hydroxyglutarate. Bacteriol. Proc., p. 103.

Scherp, H. W. 1946. Convenient syntheses of thymine and 5-methylisocytosine. J. Am. Chem. Soc. **68**:912–913.

Simon, E. J., and D. Shemin. 1953. The preparation of S-succinyl coenzyme A. J. Am. Chem. Soc. **75**:2520.

Tafel, J., and H. B. Thompson. 1907. Electrolytische Reduktion der Äthyl-barbitursäuren. Chem. Ber. **40**:4489–4497.

VOGEL, A. I. 1948. A textbook of practical organic chemistry. Longmans, Green & Co., Inc., New York.

ULBRICHT, T. L. V. 1959. Syntheses with pyrimidine-lithium compounds. Tetrahedron **6**:225–231.

WONG, D. T. O., AND S. J. AJL. 1956. Conversion of acetate and glyoxylate to malate. J. Am. Chem. Soc. **78**:3220.

WONG, D. T. O., AND S. J. AJL. 1957. Significance of the malate synthetase reaction in bacteria. Science **126**:1013–1014.