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Formation of Lactyl-Coenzyme A and Pyruvyl-Coenzyme A from Lactic Acid by *Escherichia coli*

ROBERT E. MEGRAW, HENRY C. REEVES, AND SAMUEL J. AJL
Department of Biochemistry, Albert Einstein Medical Center, Philadelphia, Pennsylvania

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ABSTRACT

MEGRAW, ROBERT E. (Albert Einstein Medical Center, Philadelphia, Pa.), HENRY C. REEVES, AND SAMUEL J. AJL. Formation of lactyl-coenzyme A and pyruvyl-coenzyme A from lactic acid by *Escherichia coli*. J. Bacteriol. **90**:984-988. 1965.—Cell extracts of propionate-adapted *Escherichia coli* were found to contain a lactyl-coenzyme A (CoA) synthetase which catalyzes the formation of the CoA thiolester from lactate, CoA, and cofactors. The extracts also catalyzed the nicotinamide adenine dinucleotide-dependent oxidation of lactyl-CoA. The product of this oxidation was identified chromatographically as pyruvyl-CoA.

Reeves and Ajl (1962) reported that cell extracts of propionate-grown *Escherichia coli*, strain E-26 contain an enzyme which catalyzes a β-condensation of propionyl-coenzyme A (CoA) and glyoxylate to yield α-hydroxyglutarate. It was subsequently noted that these extracts contained an enzyme which would cleave this α-hydroxy acid to yield lactate and acetate (Reeves and Ajl, 1963). Stoichiometric quantities of acetate were recovered, but considerably less than theoretical amounts of lactate were recovered, suggesting that lactate was being metabolized further. Further investigations revealed that lactate, as the CoA thiolester, was being converted to hydroxypyruvic aldehyde (Reeves and Ajl, 1965*a*). We began the present study on the premise that the metabolism of lactyl-CoA to pyruvyl-CoA was the first reaction to take place in this metabolic series. In this communication, we report that this reaction does indeed occur, and that these same extracts contain an enzyme that catalyzes the synthesis of lactyl-CoA from lactate and CoA. We have named this enzyme lactyl-CoA synthetase (Commission on Enzymes, 1961).

MATERIALS AND METHODS

Cultures and cultural methods. E. coli E-26 was used as a source of enzyme throughout this study. Stock cultures were maintained on slants of Trypticase Soy Agar (BBL). To obtain the cells, 8 liters of a mineral-salts medium (Reeves and Ajl, 1960) containing 0.5% propionate as sole carbon source were inoculated with three 100-ml starter flasks of cultures growing logarithmically in the same medium. After incubation at 37 C with vigorous aeration for 32 hr, the cells were harvested, and cell-free extracts were prepared as previously described (Reeves and Ajl, 1962).

Chemicals. CoA and nicotinamide adenine dinucleotide (NAD) were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Sodium lactate-*1-C*^{14} was a product of Nuclear Research Chemicals, Inc., Orlando, Fla., and D- and L-lactic acids, Ca^{++} salts, and lactic dehydrogenase were purchased from Calbiochem. The calcium salts of lactic acid were converted to their free acids by treatment with Dowex-50 (H^+). The lactic acids were ether-extracted and dried over $CaCl_2$ in vacuo prior to use in preparation of lactyl-CoA. Dowex-1 (Cl^-) and Dowex-50 (H^+) were products of the J. T. Baker Chemical Co., Phillipsburg, N.J., and *p*-tolylsulfonylmethylnitrosamide was purchased from K & K Laboratories, Inc., Jamaica, N.Y. All other organic and inorganic compounds used were of the best grade available.

Chromatographic methods. Strips of Whatman no. 1 paper (38 by 40 mm) were used for paper chromatography. Chromatograms were developed by use of the ascending technique in hydrometer cylinders (63 by 425 mm) containing 35 to 40 ml of solvent at 24 C. After locating the hydroxamate by spraying with 5% ferric chloride in 0.1 N methanolic HCl, C^{14} incorporation was determined by cutting out the spots and placing them in 10 ml of toluene-ethyl alcohol scintillation fluid [6 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis-2 (5-phenyl-oxayloyl) benzene in 600 ml of toluene and 300 ml of absolute ethyl alcohol] and counting in a Packard Tri-Carb liquid scintillation spectrometer.

Column chromatography was performed on a

column (2.5 by 27 cm) of Dowex-1 (Cl^-). Samples were run into the column, and the resin bed was overlaid with 10 ml of water. A reservoir containing water was connected, and 4-ml fractions were collected at the rate of 20 to 24 ml/hr.

Preparation of CoA esters and authentic hydroxamates. Lactyl-CoA was prepared by the mixed anhydride method described by Flavin (1957) as modified by Rabin et al. (1965). Propionyl-CoA and acetyl-CoA were prepared from their respective anhydrides according to the method of Simon and Shemin (1953). Pyruvate hydroxamate was obtained from its mixed anhydride, prepared with freshly neutralized hydroxylamine, or from pyruvic methyl ester, prepared with diazomethane by the *p*-tolylsulfonylmethylnitrosamide method described by Fieser (1957).

Assay methods. CoA esters and mixed anhydrides were assayed quantitatively by the method of Lipmann and Tuttle (1945). Protein was determined spectrophotometrically by the method of Warburg and Christian (1941) as modified by Layne (1957).

RESULTS

Enzymatic synthesis of lactyl-CoA from lactic acid. To demonstrate the formation of lactyl-CoA from lactic acid, the following reaction mixture was used: phosphate buffer (*p*H 6.0), 50 μmoles; DL-lactic acid, 50 μmoles; adenosine triphosphate (ATP), 5 μmoles; CoA, 2 μmoles; reduced glutathione, 8 μmoles; $MgCl_2$, 5 μmoles; and cell extract, 14 mg of protein in a total volume of 1.11 ml. Four control reaction mixtures were also used: (i) complete but with boiled extract, (ii) complete minus ATP, (iii) complete minus CoA, and (iv) complete minus substrate. All five tubes were then treated in an identical manner. The reaction mixture was incubated for 1 hr at 37 C, and 200 μmoles of freshly neutralized hydroxylamine were added and allowed to react for 15 min. After this time, 20 N H_2SO_4 was added to reduce the tube contents to *p*H 2.5 to 3.0. The mixture was then transferred to a Kutsher-Steudel apparatus and continuously ether-extracted for 18 hr. The ether residue was dissolved in 0.1 ml of water and samples were spotted on chromatograms. Under these experimental conditions, a spot corresponding to lactate hydroxamate was readily demonstrable in the complete system (Table 1). A very faint spot observed in samples spotted from control systems was presumed to be endogenous lactyl-CoA present in the undialyzed crude extract.

Using pyruvate as substrate in the above system, however, we failed to detect any pyruvate hydroxamate spot, indicating that crude cell extracts do not possess the necessary enzymes to synthesize pyruvyl-CoA from the free acid, CoA, and the other cofactors described.

TABLE 1. *Chromatographic identification of lactyl-CoA**

Solvent system	R_F	
	Experimental hydroxamate	Authentic lactic acid hydroxamate
n-Amyl alcohol–acetic acid–water (4:1:5)	0.33	0.33
Octanol–formic acid–water (3:1:3)	0.13	0.13
Water-saturated *n*-butanol	0.43	0.43

* As hydroxamate derivative. Experimental details given in text.

In further experiments of this type, all components of the reaction mixture were doubled and, after incubating for 1 hr, divided into two equal portions. One portion was treated with 200 μmoles of hydroxylamine immediately. The other portion was incubated for an additional 15 min after the addition of 10 μmoles of NAD. After the additional incubation period, it was likewise treated with hydroxylamine. The hydroxamate of lactyl-CoA was present in the tube lacking NAD. It did not appear on chromatograms spotted with samples of the reaction mixture containing NAD, and, furthermore, no spot for the hydroxamate of pyruvyl-CoA was demonstrable. This suggested that pyruvyl-CoA was, for the most part, further metabolized.

Oxidation of lactyl-CoA. Having shown the enzymatic synthesis of lactyl-CoA by demonstrating its hydroxamic acid derivative, we then looked for the oxidation of chemically synthesized lactyl-CoA. Figure 1 shows the results of a spectrophotometric assay after NAD reduction at 340 mμ.

Both D and L isomers of lactyl-CoA were oxidized, whereas the oxidation of free lactic acid was negligible. The reduction of NAD was also followed by use of methanol as substrate. Methanol was included because it is a by-product of methyl chloroformate used in the chemical synthesis of lactyl-CoA (Rabin et al., 1965). Small amounts of methanol and lactic acid contamination persisted even though the CoA ester preparation was ether-extracted four to six times.

The use of D and L isomers of lactic acid in the preparation of lactyl-CoA was to determine whether the enzyme in *E. coli* extracts displays absolute stereospecificity for this CoA ester. The data indicate such a specificity does not exist for *E. coli*, although a slightly greater affinity for the D (−) form apparently exists.

Studies with crystalline lactic dehydrogenase

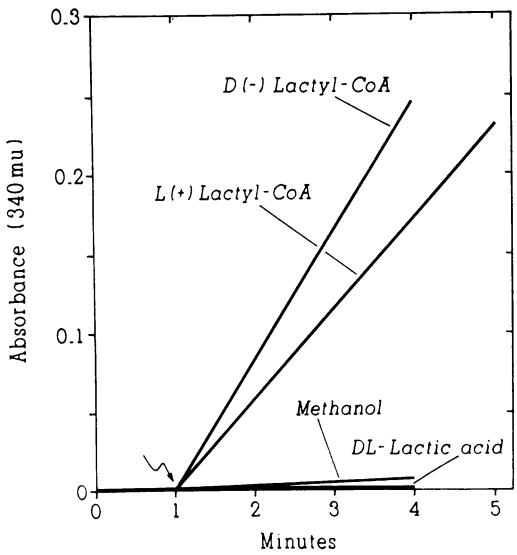


FIG. 1. *Oxidation of lactyl-CoA by a 60 to 80% ammonium sulfate fraction of Escherichia coli extract. The reaction mixture contained tris(hydroxymethyl)aminomethane buffer (pH 8.0), 250 μmoles; NAD, 0.3 μmole; extract, 0.1 ml; and water to give a total volume of 1.2 ml. The reaction was started by the addition of 0.1 μmole of lactyl-CoA at the point indicated by the arrow. When methanol was used as substrate, 200 μmoles were added, along with lactic acid, 10 μmoles. The reference cuvette contained no substrate. Assays were performed with a Cary model 14 recording spectrophotometer.*

under the assay conditions described in Fig. 1 showed that no NAD reduction occurred with lactyl-CoA as substrate. Further, we noted previously that this extract fraction is devoid of lactic dehydrogenase activity (Reeves and Ajl, 1965*a*).

Enzymatic synthesis of pyruvyl-CoA. Having shown the NAD-dependent oxidation of lactyl-CoA, we next attempted to demonstrate the product of this oxidation. For the biological synthesis and subsequent demonstration of pyruvyl-CoA, the following reaction mixture was used: phosphate buffer (*p*H 6.0), 50 μmoles; DL-lactate-*1*-C^{14}, sodium salt, 6.0 μc; ATP, 5 μmoles; CoA, 2 μmoles; reduced glutathione, 8 μmoles; $MgCl_2$, 5 μmoles; and cell extract, 14 mg of protein in a total volume of 1.11 ml. Controls were the same as described for the enzymatic synthesis of lactyl-CoA. After incubation for 1 hr at 37 C, 10 μmoles of NAD were added and the reaction mixture was incubated for an additional 15 min. The reaction was then terminated and, after ether extraction, the residues were taken up in water, to which authentic unlabeled pyruvate hydroxamate had been added, and placed on a Dowex-1 (Cl^-) column. The column was washed with water, and the presence of hydroxamates in eluates was tested for by spotting drops from each tube on filter paper and spraying with 5% $FeCl_3$ in 0.1 N HCl-methanol. Those tubes showing a positive test for hydroxamate were pooled and lyophilized. The spot, subsequently identified as pyruvate hydroxamate, usually came through the column in tubes no. 27 to 33. This treatment with Dowex-1 (Cl^-) removed most unreacted lactate-*1*-C^{14} remaining in the reaction mixture. The dried material was reconstituted with 0.1 ml of water, and samples were taken for Lipmann-Tuttle assays (Lipmann and Tuttle, 1945) and spotting chromatograms. Amounts of 10 μliters were spotted on filter paper strips. The chromatograms were developed and sprayed, and the resulting pyruvate hydroxamate spots were cut out and counted. The results showed that pyruvyl-CoA was produced from labeled lactic acid (Table 2).

To authenticate further the identity of the pyruvic acid moiety, the material used in the Lipmann-Tuttle assay was treated with an equal volume of saturated 2,4-dinitrophenylhydrazine in 2 N HCl. This mixture was incubated for 24 hr at 37 C to hydrolyze the hydroxamic acid and form the hydrazone derivative. The hydrazone derivative was then purified by the extraction procedure of Juni and Heym (1962). Material from a boiled enzyme control was carried through the same procedure. The final ethyl acetate solution was evaporated to dryness with a stream of N_2, and the residues were taken up in 0.1 ml of ethyl acetate and cochromatographed with authentic unlabeled pyruvic-2,4-dinitrophenylhydrazone in three different solvent systems. Spots were located by spraying with 4% alcoholic KOH, cut out, and counted. In each solvent system the pyruvic-2,4-dinitrophenylhydrazone spots were radioactive, thereby confirming the identity of the pyruvic acid.

In early attempts to isolate the hydroxamates

TABLE 2. *Chromatographic identification of pyruvyl-CoA**

Solvent system	R_F	Hydroxamate (count per min per μmole)	
		Experimental	Control
n-Amyl alcohol–acetic acid–water (4:1:5)	0.56	30,000	8,050
Octanol–formic acid–water (3:1:3)	0.34	33,600	5,800

* As hydroxamic acid derivative. Reaction mixture and experimental techniques given in text.

of C^{14}-lactyl- and C^{14}-pyruvyl-CoA chromatographically, spots were obscured by exogenous radioactivity. By cutting the chromatograms in 1-cm segments from spotting line to solvent front and counting each segment, it was found that residual, free lactate-*1*-C^{14} was smearing on the chromatograms. This lactic acid readily came over with the hydroxamate when ether-extracted at acid *p*H. This smearing was prevalent in all three alcohol solvent systems. To circumvent this problem, the treatment with Dowex-1 (Cl^-) was employed.

Unlike many hydroxamate derivatives, that of pyruvate is not stable for long periods of time. Authentic preparations stored at −20 C were seen to markedly decline over a period of 1 to 2 weeks. Consequently, all experiments were carried through to completion as promptly as possible. No loss of pyruvate hydroxamate was incurred by lyophilization, however. In experiments with authentic material, pyruvate hydroxamate could be recovered quantatively after lyophilization.

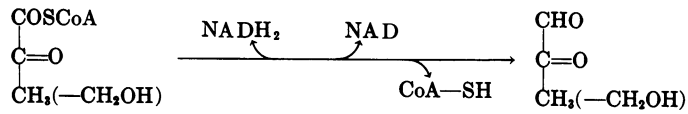
Discussion

In studying the enzymatic formation of hydroxypyruvic aldehyde from lactyl-CoA (Reeves and Ajl, 1965*a*), it was noted that this conversion required NAD for maximal activity. The most likely initial product in the further metabolism of lactyl-CoA is pyruvyl-CoA. This reaction had been sought in *Pseudomonas* sp., *Clostridium propionicum*, *C. kluyveri*, and pigeon-heart muscle, but without success (Vagelos, Earl, and Stadtman, 1959).

Research on this metabolic pathway began with the finding that propionyl-CoA would condense with glyoxylate to yield α-hydroxyglutarate (Reeves and Ajl, 1962). It has been shown that these same extracts from propionate-grown *E. coli* will catalyze the propionyl-CoA-dependent formation of citramalate from glyoxylate (Reeves and Ajl, 1965*b*). The fact that citramalate was formed in these experiments suggests that it may be formed via an isomerization of α-hydroxyglutarate. The further metabolism of α-hydroxyglutarate (or citramalate) is an enzymatic cleavage which yields acetate and lactate (Reeves and Ajl, 1963). From theoretical considerations, this cleavage should yield pyruvate rather than lactate, although the former could never be isolated. It is our contention that pyruvate is indeed one of the cleavage products, but that it is rapidly converted to lactate by an active lactic dehydrogenase present in crude extracts of propionate-grown cells. The data presented in this communication show that a lactyl-CoA synthetase catalyzes the formation of lactyl-CoA, and that this product is oxidized to pyruvyl-CoA.

The relation of this curious biochemical redundancy to the total economy of the cell is not known at this point. The fact that pyruvyl-CoA can be demonstrated only by using C^{14}-lactate of high specific activity indicates that this intermediate is very reactive and does not accumulate in appreciable amounts. In future experiments attempts will be made to obtain greater yields of pyruvyl-CoA by the use of purified enzymes from *E. coli* extracts.

The role of pyruvyl-CoA can only be speculated upon at this time. If this compound is indeed in the metabolic pathway leading to hydroxypyruvic aldehyde as we postulate, then it will ultimately have to be reduced at the thiolester bond and oxidized at the methyl group. There is precedent for the former, since Burton and Stadtman (1953) found an enzyme in *C. kluyveri* which catalyzes the reversible oxidative of acetaldehyde to acetyl-CoA. This would be analogous to the reaction:



We cannot speculate at this point whether the reduction of the thiolester bond or the oxidation of the methyl group takes place first. In consideration of the latter, the pyruvyl-CoA could undergo an enolization with subsequent hydration of the β-carbon. This would necessitate a reoxidation of the α-carbon to regenerate the keto group, and, finally, hydroxypyruvic aldehyde. A more concerted mechanism would be a direct oxidation of the methyl group to an alcohol group, as has been demonstrated in a pseudomonad which will oxidize octanoate to 8-hydroxyoctanoate (Kusunose, Kusunose, and Coon, 1964).

Acknowledgments

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