

The Citrate Cleavage Enzyme

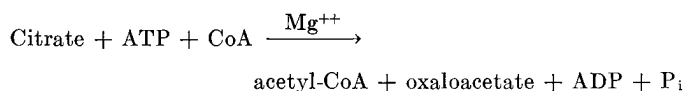
III. CITRYL COENZYME A AS A SUBSTRATE AND THE STEREOSPECIFICITY OF THE ENZYME*

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The citrate cleavage enzyme catalyzes the reaction (2)



The enzyme from chicken liver has been purified approximately 100-fold, and there is no indication that the over-all reaction is catalyzed by separable enzymes. No intermediates have been detected by a variety of methods. It has been shown, however, that an impure preparation of citryl coenzyme A could be cleaved by the enzyme (3, 4). It is the purpose of this paper to report on the synthesis and purification of citryl coenzyme A and to present additional information concerning its cleavage by the citrate cleavage enzyme.

A number of years ago, Topper and Bandurski¹ related that the oxaloacetate arising on cleavage of citrate by the action of citrate cleavage enzyme comes from the carbon 4 portion of the citrate molecule formed from oxaloacetate by the action of citrate-condensing enzyme on oxaloacetate and acetyl-CoA. Recent experiments on the synthesis of fatty acids from citrate preparations isotopically labeled in various positions in the pigeon liver system (5) and in the rat mammary gland (6) confirmed the unpublished experiments of Topper and Bandurski. It was thought advisable, however, to demonstrate this in a more rigorous manner, and data in this paper show that citrate cleavage enzyme purified from chicken liver has the same stereospecificity as the citrate-condensing enzyme.

EXPERIMENTAL PROCEDURE

Citrate cleavage enzyme was prepared and assayed with citrate as substrate according to Srere (2). Citrate-condensing enzyme was prepared from pig heart and assayed according to Srere and Kosicki (7). Fumarase and malate dehydrogenase, CoA, ATP, NADH, NAD, 5,5'-dithiobis-(2-nitrobenzoic acid), ethyl chloroformate, and diethylaminoethyl cellulose were obtained from commercial sources.

The aldol cleavage of citryl-CoA was measured in 1-cm cuvettes containing: Tris-HCl buffer, pH 7.4, 100 μ moles; NADH,

0.15 μ mole; malate dehydrogenase, 0.2 unit;² citryl-CoA, approximately 0.1 μ mole; citrate cleavage enzyme; and water in a final volume of 1 ml. The production of oxaloacetate in the presence of malate dehydrogenase caused an oxidation of NADH and a decrease in absorption at 340 m μ . Absorbance readings were taken every $\frac{1}{2}$ minute, and the rate of NADH oxidation from 1 to 3 minutes was used to calculate the rate of the cleavage reaction. Total amounts of citryl-CoA were measured in a similar system except that only 0.01 to 0.03 μ mole of citryl-CoA was added to an excess of citrate cleavage enzyme, and the total decrease in absorbance at 340 m μ was determined.

Sulfhydryl was measured according to the method of Ellman (8). Acetyl-CoA was determined enzymatically with citrate-condensing enzyme (9). Hydroxamate was measured by the method of Lipmann and Tuttle (10). Oxaloacetate was determined enzymatically by using malate dehydrogenase and NADH and by measuring the total decrease in absorbance at 340 m μ .

Citryl-CoA was prepared by adding small aliquots of a tetrahydrofuran solution of 0.25 M citryl anhydride (3) to a solution of 0.006 M CoA in 0.1 M KHCO₃ until the test for free —SH was negative. A yield of 5 to 10% of enzymatically active citryl-CoA was obtained based on initial CoA. This material (after adding H₂O to reduce the ionic strength to that of 0.003 M HCl) was chromatographed on DEAE-cellulose-chloride with a linear LiCl gradient in 0.003 N HCl according to the method of Moffatt and Khorana (11). A typical chromatogram is shown in Fig. 1. Not all the peaks were completely characterized but of the adenine-containing peaks, *Peak II* seemed to be CoA-SH (citrate cleavage assay), *Peak III* was citryl-CoA (see below), and *Peak IV* seemed to be CoA-SSCoA (spectral data and release of —SH upon reduction with NaBH₄). Over 90% of the citryl-CoA added to the column was recovered in *Peak III*. The citryl-CoA solution (*Peak III*) was concentrated in a rotary evaporator at room temperature. In some preparations, LiCl was removed according to the procedure of Moffatt and Khorana (11). The presence of LiCl did not effect V_{max} or K_m for citryl-CoA cleavage.

Citrate-1-¹⁴C and citrate-3,4-¹⁴C were prepared enzymatically with acetate-1-¹⁴C and fumarate-2-¹⁴C, respectively, with crystalline condensing enzyme from pig heart (5, 7). Citrate-condensing enzyme has been shown to catalyze the synthesis of asymmetrically labeled citrate by Potter and Heidelberger (12) and Lorber *et al.* (13). The radioactive purity of these asymmetri-

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¹ Y. Topper and R. Bandurski, private communication to P. A. Srere.

² For all enzyme activities given in this paper, 1 unit of activity corresponds to 1 μ mole of substrate utilized (or 1 μ mole of product formed) per minute. Specific activities of enzymes are expressed as enzyme units per mg of protein.

cally labeled citrates was established by cochromatography with an authentic sample of citrate (14).

Citrate cleavage was allowed to take place in the presence of NADH and malate dehydrogenase so that the oxaloacetate formed was converted to malate. The incubation mixture contained 100 μ moles of Tris-HCl buffer (pH 7.4), 10 μ moles of Mg^{++} , 3 μ moles of NADH, 10 μ moles of mercaptoethanol, 1.4 μ moles of CoA, 1 unit of malate dehydrogenase, 0.95 unit of citrate cleavage enzyme, and 5 μ moles of citrate-1- ^{14}C (1.96×10^6 c.p.m.) in a total volume of 1.0 ml. The reaction was started by addition of 7 μ moles of K_2ATP , and the progress of the reaction was followed by measuring the decrease in absorption at 340 $m\mu$ due to the oxidation of NADH by oxaloacetate. The reaction had slowed appreciably at 30 minutes, but was allowed to proceed for another 60 minutes. The total amount of citrate cleaved in terms of NADH oxidation was 0.7 μ mole.

The incubation mixture was deproteinized by heating to 90° in a water bath for 6 minutes, and the denatured protein was removed by centrifugation. To the supernatant solution were added as carriers 30 μ moles each of sodium acetate, sodium malate, and sodium citrate. To hydrolyze the acetyl-CoA which had been formed, the reaction mixture was adjusted to pH 11 by the addition of 3 N KOH, and the solution was incubated at 37° for 15 minutes and at room temperature for 1 hour. The hydrolysate was adjusted to pH 1 with 4 N H_2SO_4 and then placed on a silicic acid column for separation of the acids by the method of Varner (15).

RESULTS

Citryl-CoA—Characterization of the citryl-CoA is shown in Table I. The ratio of absorbance for 280 $m\mu$:260 $m\mu$ is 0.23 and that for 250 $m\mu$:260 $m\mu$ is 0.89. These ratios correspond to those for many adenine nucleotides, whereas the absorbance

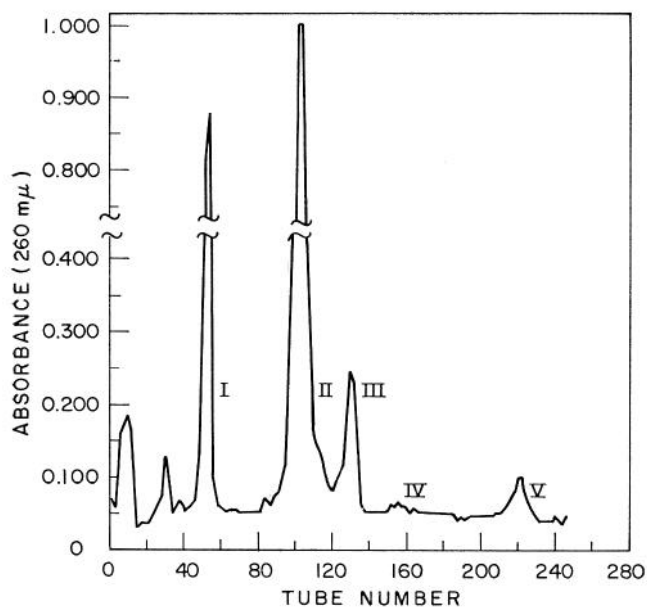


FIG. 1. Isolation of citryl-CoA by DEAE-cellulose-chloride chromatography. The cellulose column was 2 \times 20 cm. Fractions were approximately 15 ml. A linear gradient of LiCl from 0 to 0.15 M in 0.003 N HCl was used as eluting material. All conditions for this chromatography were as described by Moffatt and Khorana (11). For description of materials in Peaks I to V, see text.

TABLE I
Characterization of citryl-CoA

Assay procedure*	Amount of citryl-CoA
	μ moles/ml
Absorbance at 260 $m\mu$	1.25
Hydroxamate.....	1.23
Acetyl-CoA produced by treatment with cleavage enzyme.....	0.47
Oxaloacetate produced by treatment with cleavage enzyme.....	0.51
Free sulfhydryl.....	0.0
Sulfhydryl after treatment with condensing enzyme.....	0.60

* See text for methods used.

TABLE II
Comparison of citrate cleavage and citryl-CoA cleavage enzyme activities in protein fractions obtained during purification of citrate cleavage enzyme

Protein fraction*	Specific activity	
	Citrate cleavage enzyme	Citryl-CoA cleavage enzyme
	$\times 10^6$	
Enzyme extract of chicken liver, (medium, 0.4 M KCl in 20% ethanol).....	0.65	0.65
Acid precipitation.....	0.87	0.87
Zinc precipitation.....	2.4	2.4
Ethanol.....	3.6	3.9
Ammonium sulfate.....	13	18

* The purification procedure of citrate cleavage enzyme from chicken liver described earlier (2) was used in this experiment. The names of the protein fractions correspond to those fractions obtained in our earlier work (2).

ratio 260 $m\mu$:232 $m\mu$ is 1.88 and is thus similar to other thioesters of CoA (17). The material forms an ethanol-insoluble hydroxamic acid, which we have found to be typical of citryl-hydroxamate prepared with citryl anhydride and hydroxylamine. There is no free sulfhydryl group, but treatment with excess citrate-condensing enzyme causes the liberation of free $-SH$, and cleavage in the presence of citrate cleavage enzyme leads to the formation of equal amounts of acetyl-CoA and oxaloacetate. Since only 50% of the nucleotide is cleaved by either the cleavage enzyme or the condensing enzyme, and since the stereospecificity of both these enzymes is the same (see below), it seems likely that the purified preparation of citryl-CoA is a mixture of diastereomers. Hydrolysis of citryl-CoA with 0.1 N KOH for 30 minutes at room temperature causes an increase in the 260 $m\mu$:232 $m\mu$ ratio and the appearance of free sulfhydryl groups.

When the citrate cleavage enzyme is purified according to earlier procedures (2), the ratio of activity in the citrate cleavage assay to the activity in the citryl-CoA cleavage assay remains essentially constant (Table II). Although there is a tendency for increasing activity toward citryl-CoA with purer enzyme preparations, further chromatography of such purified enzyme on DEAE-cellulose with a number of different elution systems always yields enzyme fractions in the eluate that show the same cleavage activity against citrate and citryl-CoA. An example of such a chromatographic analysis is shown in Fig. 2.

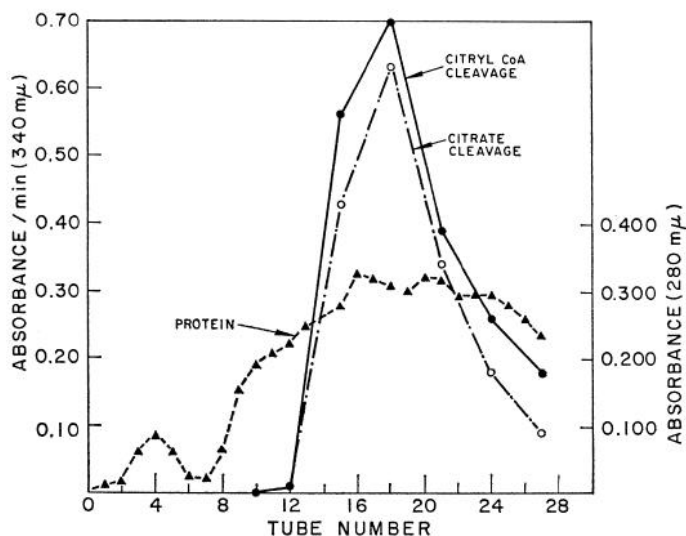


FIG. 2. Chromatography of citrate cleavage enzyme on DEAE-cellulose. An ammonium sulfate fraction of cleavage enzyme was freed of salt by passage through a Sephadex G-25 column. The salt-free eluate was placed on a DEAE-cellulose column previously equilibrated with 0.002 M potassium phosphate, pH 7.4. The protein was eluted with a gradient of potassium phosphate, pH 7.4, from 0.002 M to 0.125 M. Fractions were approximately 15 ml each. Protein (\blacktriangle — \blacktriangle) was determined spectrophotometrically at 280 m μ . Citrate cleavage (\circ — \circ) and citryl-CoA cleavage (\bullet — \bullet) activities were determined spectrophotometrically at 340 m μ as described in the text.

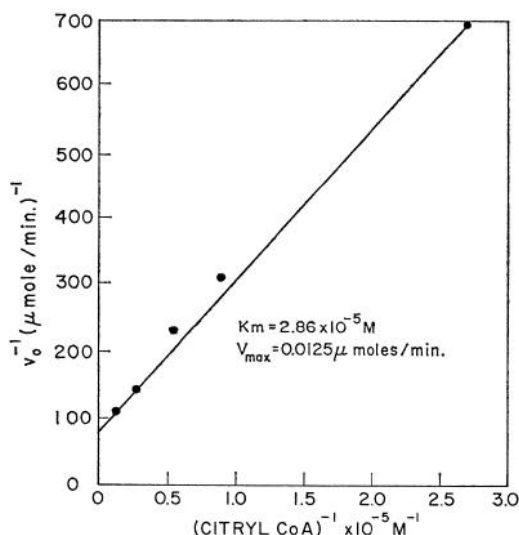


FIG. 3. Determination of apparent K_m and V_{max} of citryl-CoA cleavage.

The dependence of the rate of cleavage of citryl-CoA on the concentration of citryl-CoA is illustrated in Fig. 3. In the coupled assay system used (see "Experimental Procedure"), the apparent K_m for citryl-CoA is approximately 3×10^{-5} M. No differences in K_m or V_{max} have been observed in the use of citryl-CoA before or after its chromatography on DEAE-cellulose-chloride.

Where the cleavage of citrate requires ATP, CoA, and Mg^{++} and is slightly stimulated by mercaptoethanol (2), none of these components is required for the cleavage of citryl-CoA. We have observed an effect of pH on the cleavage of citrate in the coupled assay system, but no such dependency has been observed with

the cleavage of citryl-CoA. The rate of cleavage is constant from pH 6.5 to pH 8.1.

During the purification of the enzyme the activity against citryl-CoA appeared at times to be lost at the zinc precipitation step. Subsequently, zinc was found to have significantly different inhibitory effects on the activity of the enzyme toward the two substrates (Table III). Citryl glutathione was not cleaved by the enzyme.

Stereospecificity—Fig. 4 shows that the isolated acetate is radioactive, and that no radioactivity is found in the major malate fractions when citrate-1- ^{14}C is cleaved. The agreement between the radioactivity values and NaOH titration values for acetate and citrate indicates that good separation of these acids has taken place.

All the radioactivity recovered from the cleavage products of citrate-1- ^{14}C is found in the acetate, *i.e.* acetyl-CoA. It appears, therefore, that the citrate cleavage enzyme has the same stereospecificity for citrate as the citrate-condensing enzyme.

Confirmation of this conclusion was obtained when citrate-3,4- ^{14}C was subjected to the action of the cleavage enzyme in a manner similar to that described above. In this case, acetate contained very little radioactivity, and the bulk of the radioactivity appeared in the malate fractions (Fig. 5). The aqueous radioactive malate solution was separated from butanol-chloroform, and the indicator (phenol red) was absorbed on charcoal (70° for 5 minutes). The charcoal was removed by filtration, and the clear solution was concentrated in a flash evaporator to give the sodium salt. Cochromatography (18) with authentic malic acid showed a single spot containing all the radioactivity. In experiments with citrate-3,4- ^{14}C a small but significant amount of radioactivity appears with acetate. It did not seem likely that this represented a nonspecific cleavage of citrate, since no radioactivity is associated with the main malate fraction formed by the cleavage of citrate-1- ^{14}C . When experiments with ^{14}C -pyruvate or ^{14}C -fumarate replacing the labeled citrate in the incubation mixture were performed, a small amount of radioactivity appeared as acetate. It is thus likely that the ^{14}C -acetate was formed by decarboxylation of pyruvate, and the pyruvate from decarboxylation of oxaloacetate that arises during the cleavage of citrate.

DISCUSSION

It has seemed logical (3) to assume that the citrate cleavage activity could be described by two segmental reactions,

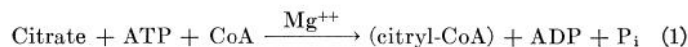


TABLE III

Effect of zinc on citrate and citryl-CoA cleavage

Zn ⁺⁺	Citrate cleavage	Citryl-CoA cleavage
M	%	
0	100*	118
5.0×10^{-6}	118	110
2.5×10^{-5}		55
5.0×10^{-5}	93	22
1.5×10^{-4}	67	0

* All rates were referred to citrate cleavage in the absence of zinc as 100%. The enzyme used here was a fraction eluted from DEAE-cellulose as described in Fig. 2.

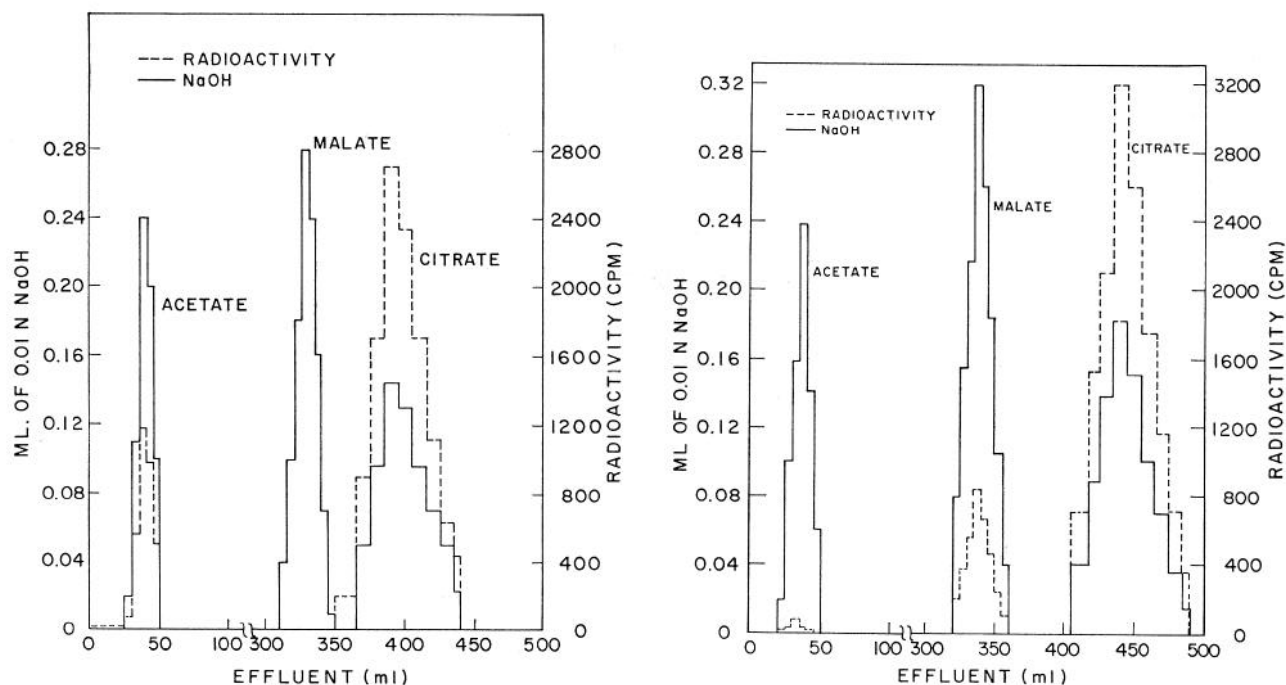


FIG. 4 (left). Chromatographic separation and radioactivity of products formed by the enzymatic cleavage of citrate-1-¹⁴C

FIG. 5 (right). Chromatographic separation and radioactivity of products formed by the enzymatic cleavage of citrate-3,4-¹⁴C

Step 1, although unique as a CoA activation reaction, can be considered to be similar to synthetase reactions best illustrated by the glutamine synthetase reaction (19). It is interesting to note that activation of malate, succinate, itaconate, glutarate, and citrate, all polycarboxylate anions, leads to the production of ADP and P_i , rather than of AMP and PP_i , which are observed with the activation of monocarboxylate anions. Our earlier results (3) gave no indication that citryl-CoA was being formed and trapped since the total hydroxamate formed always equaled the oxaloacetate formed and lower recoveries of oxaloacetate would be expected if citryl-CoA was trapped as citryl-hydroxamate. Chromatography of the hydroxamate fraction obtained from the reaction mixture showed the presence of only acetylhydroxamate. Recently, however, we have found that under the conditions initially used for isolation of the hydroxamate fraction, the citrylhydroxamate would not be extracted from the reaction mixture. Although the stoichiometric data would rule against formation of citrylhydroxamate, we reinvestigated this aspect of the problem. Small quantities of hydroxamate were found in the ethanol-insoluble fractions of the reaction mixtures, but chromatography proved it to be acetylhydroxamate. Since zinc has an inhibitory effect on citryl-CoA cleavage (see Table III), an attempt was made to trap citryl-CoA by enzyme cleavage of citrate in the presence of zinc. The results obtained also did not indicate the presence of citryl-CoA. These data are in contrast to the observations of other workers on the cleavage of malate and itaconate (20, 21) where, in the presence of hydroxylamine, malyhydroxamate and itaconylhydroxamate could be identified.

Several enzymes are known (hydroxymethylglutaryl-CoA cleavage (22), malyl-CoA cleavage (21), and itaconyl-CoA cleavage (23)) which catalyze reactions similar to Reaction 2. Our early data (3, 4) indicated that citryl-CoA could be cleaved by our enzyme preparations, but the preparations of citryl-CoA then used were relatively impure and the rates of cleavage were

slow, so that it was necessary to interpret those results cautiously. The results in the present paper, with the use of purified citryl-CoA, would indicate that citryl-CoA is an intermediate, probably enzyme-bound in the cleavage reaction. Because the purification procedure and cellulose chromatography of the enzyme lead to preparations with similar activities in the citrate cleavage and citryl-CoA cleavage assays, it would seem that either the activation and cleavage take place on one enzyme, or the two enzymes (an "activating enzyme" and a "cleavage enzyme") form a tightly bound complex.

The results of our studies on stereospecificity are not unexpected. Cleavage enzyme is present in most tissues and, if it were not stereospecific, the early experiments with cruder enzyme systems would have yielded results indicating a lack of specificity in citrate formation and utilization.

The chemical synthesis of citryl-CoA should lead to diastereomers. The absorbance data and the amount of hydroxamate formed from the purified preparation of citryl-CoA indicate that a thioester of a reasonable purity (90%) has been isolated. Data obtained on treatment of this material with citrate cleavage enzyme or with citrate-condensing enzyme lead to a figure of purity of only 50%. Since these enzymes have the same stereospecificity, it is likely that only one diastereomer is being acted upon by the enzymes. The unnatural diastereomer seems to inhibit the condensing enzyme (4) but is without effect on either citryl-CoA or citrate cleavage by the cleavage enzyme. It is interesting to note that the cleavage of hydroxymethylglutaryl-CoA to acetyl-CoA and acetoacetate also is stereospecific (22).

Eggerer and Remberger (24) have recently shown that citryl-CoA prepared by a different method (25) is a substrate for the citrate-condensing enzyme. They mention in their papers that their preparations of citryl-CoA are also cleaved by preparations of the cleavage enzyme. Although the results make it seem likely that citryl-CoA is an intermediate in the reactions catalyzed by the citrate cleavage and condensing enzyme, final proof

must await the isolation of the CoA derivative from enzyme reaction mixtures.

SUMMARY

Citryl coenzyme A synthesized from a mixed anhydride of citric acid and coenzyme A was purified by chromatography on diethylaminoethyl cellulose-chloride. Analysis of the synthetic preparation indicated it contained a nucleotide thioester of greater than 90% purity. When this citryl coenzyme A was incubated with citrate cleavage enzyme from chicken liver, half of the nucleotide thioester was cleaved to acetyl coenzyme A and oxaloacetate. During purification and column chromatography of the citrate cleavage enzyme, its activity in the cleavage of citryl coenzyme A remained essentially equal to its activity in the cleavage of citrate. The latter activity, however, requires the presence of coenzyme A, adenosine triphosphate, and Mg^{++} .

The stereospecificity of citrate cleavage enzyme was shown to be the same as that for the citrate-condensing enzyme with citrate-1- ^{14}C and citrate-3,4- ^{14}C prepared enzymatically with citrate-condensing enzyme.

Acknowledgment—We would like to thank Mrs. Britt Torp for her excellent assistance during the course of this work.

Addendum—A recent paper by Eggerer and Remberger (26) presents additional evidence that makes it seem likely that citryl-CoA is an intermediate in the reaction catalyzed by the citrate cleavage enzyme. These authors report the isolation of citrylhydroxamate from a reaction mixture containing citrate, ATP, CoA, Mg^{++} , NH_2OH , and the enzyme. Our inability to demonstrate the formation of citrylhydroxamate in similar systems is explained by Eggerer and Remberger as being due to the lability of citrylhydroxamate. Other data reported by them concerning citryl-CoA as a substrate are in substantial agreement with the findings reported here.

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