ENZYMATIC FORMATION OF HOMOCITRIC ACID, AN INTERMEDIATE IN LYSINE BIOSYNTHESIS[1]

Murray Strassman[2] and Louis N. Ceci

Department of Microbiology, Research Laboratories
Albert Einstein Medical Center, Philadelphia 41, Pennsylvania

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The pathway for lysine biosynthesis in Escherichia coli and other bacteria via diaminopimelic acid has been thoroughly explained during the past several years. The mechanism for lysine biosynthesis in yeast and fungi is known, however, to be different from the E. coli pathway but is not as completely understood. The work of Mitchell and Houlahan (1948), Windsor (1951), Kuo (1962), and others, elucidated the details for the conversion of α-ketoadipic and α-aminoadipic acids to lysine. In contrast the individual steps in the formation of the 6 carbon chain of lysine from smaller molecule precursors have only been suggested from experiments with C^{14} labeled intermediates.

In experiments with acetate-1-or-2-C^{14} added to yeast growth medium we found several years ago (Strassman and Weinhouse, 1953), that the lysine samples isolated from the yeast protein were highly labeled. The patterns of acetate labeling obtained by degrading these lysine samples led us to propose as a mechanism the condensation of acetate and α-ketoglutarate to yield a homolog of citric acid, β-carboxy, β-hydroxyadipic acid, also called homocitric acid. By a series of reactions analogous to citrate in the citric acid cycle homocitric

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acid could yield homoisocitrate, oxalglutarate, α-ketoadipate and ultimately α-aminoadipic acid and lysine.

In an attempt to establish this pathway for lysine synthesis in yeast, we have tested the occurrence of the first step, the formation of homocitric acid from acetate and α-ketoglutarate, in yeast cell-free preparations. A preliminary report on this work has been presented (Ceci and Strassman, 1963). The yeast extracts were prepared by shaking Baker's yeast in the presence of small glass beads in a high speed refrigerated centrifuge shaker and dialyzing the supernatant against phosphate buffer. Such enzyme preparations have previously been used successfully in studies on the biosynthesis of valine and leucine (Strassman et al., 1960; Strassman and Ceci, 1963).

Although homocitric acid has been suggested as a product of the condensation of propionyl CoA and oxalacetate its actual production in biological systems has never been demonstrated. For these studies homocitric acid was prepared synthetically by reacting diethyl β-ketoadipate with hydrogen cyanide. The cyanhydrin formed was treated with concentrated hydrochloric acid and the product of the reaction was extracted with ether and recrystallized from ethyl acetate to yield crystals melting at 161-162°. Details of the synthetic procedure will be given in a future publication.

The structure of homocitric acid suggests the probable formation of a 5 membered lactone ring involving the hydroxyl group and the ε- or 6-carboxyl group. Lactone formation was indicated experimentally by potentiometric titration which showed that two carboxyls are readily neutralized with NaOH, while the third carboxyl required heating in the presence of excess NaOH. As shown on the next page, carbon-hydrogen analysis as well as the neutralization equivalent corresponded well to the calculated values for homocitric acid lactone.

HOMOCITRIC ACID LACTONE, $C_7H_8O_6$, mol. wt.- 188

calculated: C- 44.70, H- 4.26 neut. eq.- 62.7

found: C- 44.71, H- 4.31 neut. eq.- 62.7

Enzymatic formation of homocitric acid was tested by incubating carboxyl-C^{14}- labeled acetate with unlabeled α-ketoglutarate in the presence of Mg^{++}ions, ATP, CoA and the dialyzed yeast extract as described in Table I. Ether extraction of the deproteinized reaction mixture and paper chromatography of the extract (Strassman and Ceci, 1963) gave a highly radioactive spot which appeared only when both C^{14}- labeled acetate and α-ketoglutarate were incubated with yeast extract. The radioactive substance had the same Rf values as synthetic homocitric acid in 7 different solvent systems (Strassman et al., 1963) when spotted separately or together.

TABLE I

REQUIREMENTS FOR INCORPORATION OF C^{14}- LABELED ACETATE INTO ENZYMATICALLY FORMED HOMOCITRIC ACID

Complete system contained: 50 μmoles K phosphate (pH 7.2); 50 μmoles K acetate (10 μCuries of 1-or 2-C^{14}- labeled); 10 μmoles $MgCl_2$; 50 μmoles K α-ketoglutarate; 10 μmoles ATP, 0.8 μmoles CoA; 20 μmoles cysteine; 0.2 ml dialyzed yeast extract; 2.0 ml total volume. Incubated in N_2 for 1 hour at 37° C.

Omitted	C^{14}- Homocitrate spot
Acetate-2-C^{14}	
	c.p.m.*
----	24,280
$MgCl_2$	22,880
CoA	488
α-ketoglutarate	1,688
ATP	544
Cysteine	19,880
Enzyme	680
Acetate-1-C^{14}	
----	23,420

* Measured by placing eluted material on 7.5 sq. cm. plate and counting in Nuclear Chicago gas flow counter.

The requirements for the reaction were determined by measuring the incorporation of C^{14}- acetate into homocitric acid formed after 1 hour incubation at 37° and isolated by ether extraction and paper chromatography. As shown in Table I, good incorporation of acetate-2-C^{14} was obtained when both substrates and dialyzed yeast extracts were incubated under N_2 in the presence of potassium phosphate, magnesium ions, ATP, CoA and cysteine. If α-ketoglutarate or enzyme was omitted, low C^{14} incorporation into homocitrate occurred. The omission of cysteine or Mg ions had little effect on the activity of the homocitrate produced; however, if ATP or CoA was omitted, poor incorporation of radioactivity occurred. As shown in Table I, an equivalent amount of carboxyl labeled acetate gave the same amount of labeling as methyl labeled acetate, indicating that both carbons were incorporated to the same extent.

Enzymatically formed radioactive material was eluted from paper chromatograms in the presence of unlabeled homocitric acid. The diluted acid did not lose activity on three subsequent recrystallizations as shown in Table II, further indicating identity between labeled and unlabeled acids.

TABLE II

C^{14}- HOMOCITRIC ACID FORMED ENZYMATICALLY FROM α-KETOGLUTARATE AND ACETATE-1-C^{14}

Times Recrystallized	Specific Activity
	c.p.m.
1	680
2	666
3	660

Figure 1 shows the effect of α-ketoglutarate concentration on acetate-1-C^{14} incorporation into homocitrate. The activity observed in the homocitric acid spot in the absence of added α-ketoglutarate

is probably due to the formation of small amounts of α-ketoglutarate in the crude enzyme system. The direct proportionality between C^{14}-homocitrate formed and the amount of keto acid added, up to 40 μmoles, is evident. This further implicates the condensation of acetate and α-ketoglutarate to yield homocitric acid as the first step in the proposed lysine biosynthetic pathway. A subsequent step in this pathway, the oxidative decarboxylation of homoisocitric acid to α-ketoadipic acid, is documented in the following paper (Strassman et al., 1963).

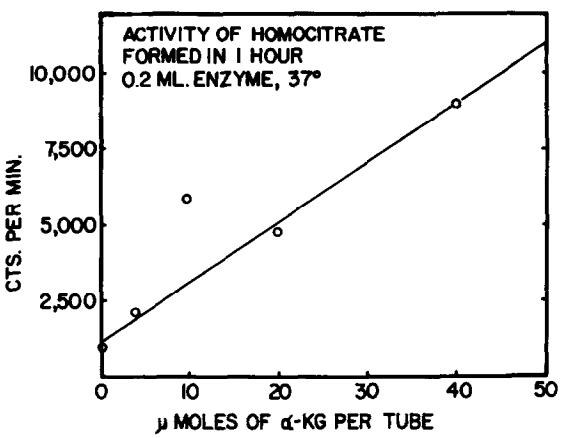

Figure 1. Effect of α-ketoglutarate concentration on acetate-1-C^{14} incorporation into homocitrate.

On the basis of the results reported here and the recently reported information concerning leucine biosynthesis (Strassman and Ceci, 1963; Jungwirth et al., 1963), it now appears fairly certain that the biosynthetic pathways of both leucine and lysine involve similar citric acid cycle type reactions. Such a generalized scheme for the biosynthesis of the keto acid precursors of these two amino acids, based on the classical formation of α-ketoglutaric acid, has been discussed in our previous publication on leucine biosynthesis (Strassman and Ceci, 1963).

REFERENCES

Ceci, L.N. and Strassman, M., Fed. Proc., 22, 243 (1963).
Jungwirth, C., Gross, S.R., Margolin, P. and Umbarger, H.E., Biochemistry, 2, 1 (1963).
Kuo, M.H., Saunders, P.P. and Broquist, H.P., Biochem. and Biophys. Res. Comm. 8, 227 (1962).
Mitchell, H.K. and Houlahan, M.B., J. Biol. Chem., 174, 883 (1948).
Strassman, M. and Weinhouse, S., J. Am. Chem. Soc., 75, 1680 (1953).
Strassman, M., Shatton, J.B. and Weinhouse, S., J. Biol. Chem., 235, 700 (1960).
Strassman, M. and Ceci, L., J. Biol. Chem., 238, 2445 (1963).
Strassman, M., Ceci, L., Silverman, B.E., Biochem. and Biophys. Res. Comm., 14, 268 (1964)
Windsor, E., J. Biol. Chem., 192, 595 (1951).