Glutamate Biosynthesis in Anaerobic Bacteria. II. Stereospecificity of Aconitase and Citrate Synthetase of *Clostridium kluyveri**

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ABSTRACT: The stereospecificity of aconitase and citrate synthetase in *Clostridium kluyveri* has been studied by determining the entry of various labeled precursors into carbon atoms 1 and 5 of L-glutamate. The label of [1-^{14}C]citrate, prepared with crystalline heart citrate synthetase, appeared almost exclusively (95%) in C-5 with only 5% in C-1. With [1-^{14}C]acetyl phosphate, 94% of the label was found in C-5, the remainder in C-1. With $^{14}CO_2$, 96% of the total radioactivity incorporated in L-glutamate was found in C-1 and 4% in C-5. With [1,5-^{14}C]citrate, the label was equally distributed in C-1 and C-5. [1-^{14}C]Pyruvate did not contribute label to C-1 or C-5, whereas [3-^{14}C]pyruvate entered only the γ-aminobutyrate moiety of L-glutamate.

It was concluded that both the aconitase and the citrate synthetase in these extracts had the usual stereospecificity described for these citric acid cycle enzymes in heart. The small entry of label into the opposite carboxyl carbon of L-glutamate in these experiments could be explained if one assumed that citrate synthetase of heart or *C. kluyveri* was only 90–95% stereospecific.

In the previous paper (Stern and Bambers, 1966) it was shown that *Clostridium kluyveri* synthesized L-glutamic acid by a citrate pathway involving the enzymes of the upper half of the citric acid cycle and L-glutamate dehydrogenase. However, the pioneering experiments of Tomlinson (1954) demonstrated that the origin of the carbon skeleton of glutamate (isolated from the acid hydrolysate of cells of *C. kluyveri* grown in a synthetic medium with alcohol, acetate, and CO_2 as carbon source) was inconsistent with the operation of the usual citric acid cycle reactions. Thus with $^{14}CO_2$ as labeled substrate, 7% of the ^{14}C in glutamic acid was located in the α-carboxyl group (C-1), 90% in the γ-carboxyl group (C-5), and the rest in C-2. With [1-^{14}C]-acetate as labeled substrate, 47% of the ^{14}C was found in C-1, 45% in C-3, 4.5% in C-5, and 2.3% in C-2 (*cf.* Figure 1).

Tomlinson hypothesized that, were a citrate pathway operative, then an unusual stereospecificity of aconitase, whereby dehydration occurred at carbon atoms 2 and 3 rather than at carbons atoms 3 and 4, could explain the observed reversal of the expected labeling pattern. Pig heart aconitase has been shown to exchange stereospecifically the hydrogen attached to C-4 of citrate in the posterior position (in terms of the anterior and posterior convention of Hirschmann, 1960), with the hydrogen of the medium (Englard, 1960; Gawron *et al.*, 1961; Hanson and Rose, 1963).

Another explanation of Tomlinson's data is that the stereochemical course of citrate synthesis catalyzed by the condensing enzyme is unusual. Figure 1 illustrates the origin of the carbon skeleton of oxalacetate and α-ketoglutarate from acetate and CO_2 through proven enzymatic reactions in *C. kluyveri* (Stern, 1965; Stern and Bambers, 1966). The stereochemical course of citrate biosynthesis (in heart) was proven by the elegant isotope experiments of Hanson and Rose (1963) to occur according to reaction A. In the Fischer projection the citrate formed may be written with the –COOH group on top and the oxalacetate-derived aconitase-active $-CH_2COOH$ group on the bottom, and both projecting behind the plane of the paper. The $-CH_2$-COOH on the left is derived from acetyl-CoA and is the aconitase-inactive group. In the condensation reaction the side of the carbonyl group of oxalacetate attacked by acetyl-CoA is opposite to that attacked by DPNH[1] in the L-malate dehydrogenase reaction. Route A then leads to the usual labeling of α-ketoglutarate predicted by the citric acid cycle. Reaction B illustrates the opposite (or unusual) stereochemical reaction of acetyl-CoA and oxalacetate. The usual stereospecific action of aconitase on this citrate (whose carbons 4 and 5 now derive from acetyl-CoA) results in a reversal of the carbon skeleton of α-ketoglutarate (and L-glutamate) as shown in route B.

It will be noted that Tomlinson's data (Figure 1) are consistent with about 10% of the glutamate being derived *via* route A. However, the largest fraction—*ca.*

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[1] Abbreviations: DPNH, reduced diphosphopyridine nucleotide; OAA, oxalacetate; GABA, γ-aminobutyrate; TPN, triphosphopyridine nucleotide, DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate.

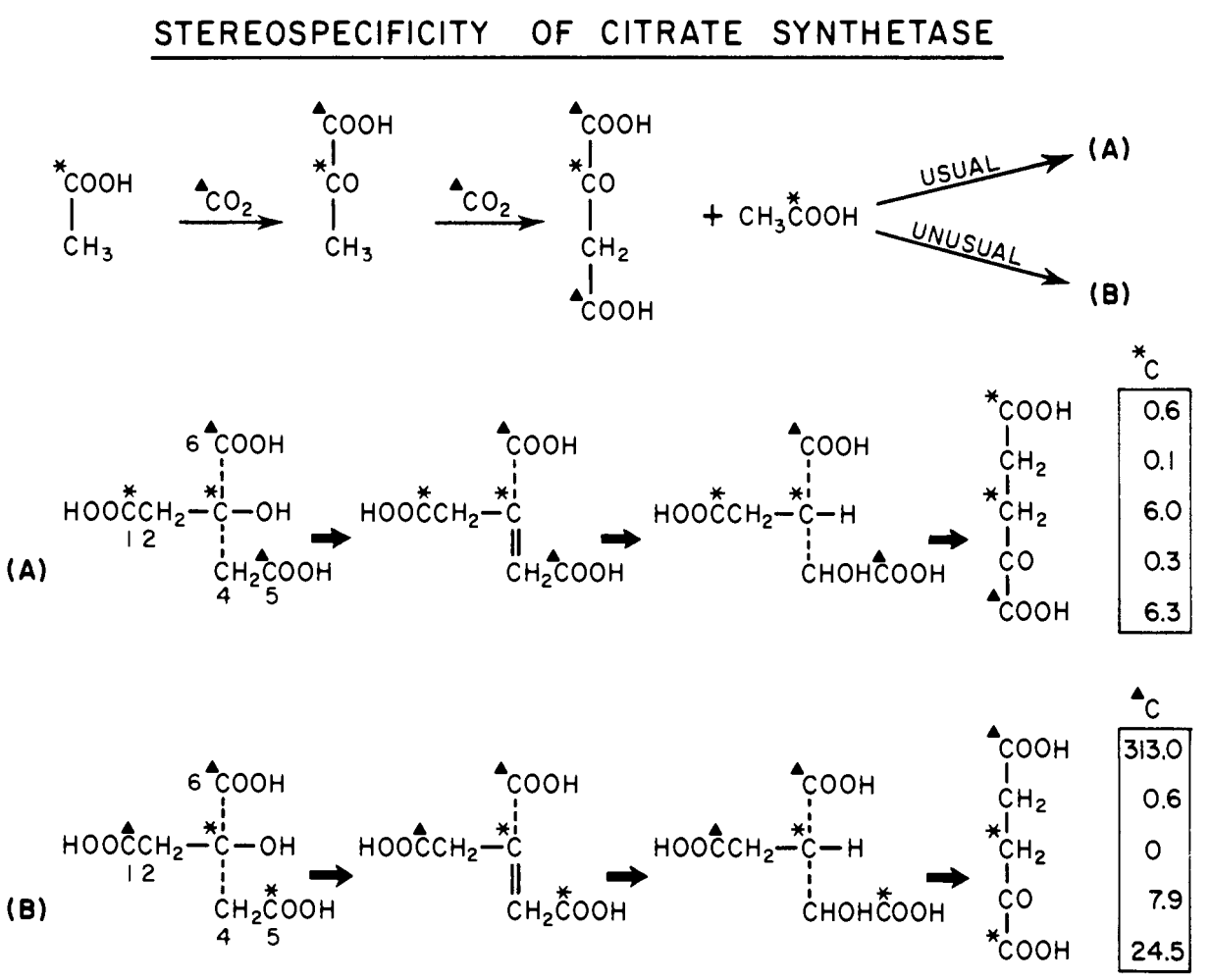

FIGURE 1: Pathway of carbon in conversion of acetate and CO_2 *via* citrate to L-glutamate. Recorded within enclosures is the radioactivity (counts per minute per mg) Tomlinson found in each glutamate carbon atom with $^{14}CO_2$ (▲C) or [1-^{14}C]acetate (*C).

90%—arose by a different pathway or an altered stereospecificity of route A. In this paper the stereospecificity of aconitase and citrate synthetase was investigated with the use of labeled citrate, acetyl phosphate, and carbon dioxide.

Experimental Section

Materials. C. kluyveri cells (Worthington Biochemical Corp.) were extracted before each experiment in the manner already described (Stern and Bambers, 1966). Isotopes were purchased as follows: $NaH^{14}CO_3$, Calbiochem; [1-^{14}C]acetic anhydride and [1,5-^{14}C]citric acid, Nuclear-Chicago Corp.; [1-^{14}C]acetyl-CoA, [1-^{14}C]pyruvate, and [3-^{14}C]pyruvate, New England Nuclear Corp. The [1,5-^{14}C]citrate had been prepared with symmetrical reagents so labeled molecules contained ^{14}C in either the C-1 or C-5 position. [1-^{14}C]-Citrate was prepared from L-malate, DPN, and [1-^{14}C]acetyl-CoA using crystalline L-malate dehydrogenase (Boehringer) and crystalline pig heart citrate synthetase (Boehringer). It was eluted from a Dowex 1-Cl^- resin column, 200–400 mesh, as a single symmetrical radioactive peak, according to the procedure of Spenser and Lowenstein (1962). The combined citrate fractions were concentrated by freeze-drying and the residue was dissolved in water.

Dilithium [1-^{14}C]acetyl phosphate was prepared from [1-^{14}C]acetic anhydride and inorganic phosphate by the procedure of Kornberg *et al.* (1956). By fractional precipitation with ethanol, a 60% pure preparation was obtained as assayed with hydroxylamine (Lipmann and Tuttle, 1945). The specific activity of the acetyl phosphate was obtained by taking a sample to dryness in 0.01 N HCl under a stream of nitrogen to distil off residual radioactive acetate, and then counting.

Highly purified chicken liver pyruvate carboxylase was kindly provided by Dr. M. F. Utter. It was free of lactate dehydrogenase and citrate synthetase.

Methods. All experiments were terminated by addition of 1.0 ml of 0.2 M acetate buffer, pH 4.5, and placing the suspension in a boiling water bath for 2 min. The pre-

cipitate was removed by centrifugation and samples of the supernatant used for determination of L-glutamate and for paper chromatographic analysis. L-Glutamate was determined manometrically at 37° and pH 4.5 with the specific *Escherichia coli* L-glutamate α-decarboxylase (Worthington Biochemical Corp.) and by using a flask with two side arms. After CO_2 evolution had ceased, 0.20 ml of Hyamine solution was introduced through a capillary stopper into the unused side arm and the flask shaken for 50 min to absorb all evolved CO_2. The Hyamine salts were removed quantitatively for counting. Samples of the supernatant from the decarboxylated residue were used for counting and paper chromatographic analysis.

The various radioactive materials were counted in a Nuclear-Chicago scintillation spectrometer either as aqueous solutions in Bray (1960) scintillation mixture or as Hyamine salts dissolved in toluene that contained 0.4% 2,5-diphenyloxazole. Samples were counted long enough to ensure statistical errors of 3% or less. Efficiency of counting was measured by the channels-ratio technique. The recovery of evolved radioactive CO_2 by the above Hyamine technique was 70%.

Descending chromatograms were run for 12 hr on Whatman No. 3MM paper with butanol–acetic acid–water (4:1:1) as solvent. Amino acids were located with ninhydrin spray and radioactivity measured in a Vanguard automatic strip scanner.

Nomenclature. The carbon atoms of citrate (Figure 1) are numbered according to the convention proposed by Hirschmann (1960). C-1 and C-2 arise from acetyl-CoA and carbons 3–6 from oxalacetate.

Stereospecificity of Aconitase. Synthetic [1-^{14}C]citrate was quantitatively converted to L-glutamate by prolonged incubation with *C. kluyveri* extract, TPN, and NH_4Cl (Table I). A control mixture with cold citrate showed complete disappearance of the citrate and its complete recovery as L-glutamate. The amount of radioactivity found in the α-carboxyl (C-1) of L-glutamate by specific enzymatic decarboxylation was 51,600 dpm, 3.6% of the total radioactivity in the molecule. If one corrects for the 70% recovery of radioactivity obtained with the Hyamine trapping procedure, then a maximum of 5.2% of the radioactivity in L-glutamate is present in the α-carboxyl carbon. Chromatography followed by radio-scanning and ninhydrin-spray analysis showed that before decarboxylation essentially all the radioactivity was present as a single symmetrical peak (R_F 0.18, ninhydrin positive). This material cochromatographed with authentic L-glutamate, R_F 0.18. After decarboxylation, this radioactive spot had disappeared and was replaced by a new ninhydrin-positive radioactive spot (R_F 0.39). This material cochromatographed with authentic γ-aminobutyrate, R_F 0.40. Both chromatograms contained a minor radioactive ninhydrin-negative peak of R_F 0.62, which was not identified.

Thus 95% of the [1-^{14}C]citrate had been converted to L-glutamate *via* route A (Figure 1) demonstrating that the aconitase had the usual stereospecificity. The origin of the radioactivity found in C-1 of L-glutamate is unclear. It could have resulted from unknown interfering reactions in the crude extract or it could possibly reflect that aconitase is 95% stereospecific and that 5% of citrate molecules were dehydrated at carbon atoms 2 and 3.

TABLE I: Conversion of [1-^{14}C]Citrate to [5-^{14}C]Glutamate.

Additions[a]	Glutamate Formed (μmoles)	Total Radioactivity (dpm) Before Decarboxylase	After Decarboxylase	CO_2 after Decarboxylase
[1-^{14}C]Citrate	7.48	1,378,000	1,368,000	51,600
[^{12}C]Citrate[b]	7.66	...	...	...
None	2.50	...	...	...

[a] The basic incubation mixture contained: Tris–HCl buffer, pH 7.0, 100 μmoles; $MgCl_2$, 4 μmoles; TPN, 0.8 mg; NH_4Cl, 15 μmoles; and *C. kluyveri* extract, 50 mg. Potassium [1-^{14}C]citrate, 4.95 μmoles, and potassium [^{12}C]citrate, 5.25 μmoles, were added as indicated; volume, 3.0 ml; incubation, 2 hr at 37° under hydrogen gas. [b] No residual citrate was detectable at end of incubation.

Stereospecificity of Citrate Synthetase. To test the stereospecificity of the citrate synthetase, citrate was generated *in situ* from either [1-^{14}C]acetyl phosphate or from [4-^{14}C]oxalacetate, and was then converted to L-glutamate.

In the experiments shown in Table II, citrate was generated from oxalacetate, [1-^{14}C]acetyl phosphate, and CoA (expt 1) or from L-malate, DPN, [1-^{14}C]acetyl phosphate, and CoA (expt 3), and then converted to L-glutamate. The large dependence of this over-all conversion on added TPN and NH_4Cl is shown in expt 2. The capacity of the extract to convert citrate to L-glutamate was measured in expt 4. In expt 5, L-glutamate biosynthesis from endogenous sources was determined and corrected for in the following calculations. It is seen (expt 1) that 4.24 μmoles of [1-^{14}C]acetyl phosphate was converted *via* citrate to L-glutamate. The remainder underwent enzymatic hydrolysis to acetate and phosphate. From the specific activity of the [1-^{14}C]acetyl phosphate (388,000 dpm) the radioactivity of the accumulated L-glutamate was 4.24 × 388,000 = 1,650,000 dpm. Of this, 99,100 dpm (141,600 dpm corrected) was found in the α-carboxyl carbon, or a maximum of 8.6% of the total radioactivity. In expt 3, L-malate dehydrogenase activity was limiting and only 1.57 μmoles of [1-^{14}C]acetyl phosphate was converted to L-glutamate. Total radioactivity in this L-glutamate was 1.57 × 388,000 = 609,000 dpm, of which 3.1% [13,000/(0.7 × 609,000)] was in the α-carboxyl carbon. Again after enzymatic decarboxylation, radiochromatography

TABLE II: Conversion of [1-^{14}C]Acetyl Phosphate to [5-^{14}C]Glutamate.

Expt No.	Additions[a]	Citrate Found	Glutamate Formed	Total Radioactivity (dpm × 10^{-6}) Before Decarboxylase	After Decarboxylase	CO_2 after Decarboxylase
1	OAA + [1-^{14}C]acetyl-P	0	5.40	5.40	5.39	0.099
2	OAA + [^{12}C]AcP	1.73	1.84	...	...	...
3	L-Malate + [1-^{14}C]AcP	0	2.73	5.44	5.36	0.013
4	Citrate	0	7.84	...	...	...
5	None	...	1.16	...	...	...

[a] The basic reaction mixture contained (expt 5): Tris–HCl buffer, pH 7.0, 100 μmoles; $MgCl_2$, 4 μmoles; TPN, 0.2 mg; NH_4Cl, 20 μmoles; CoA, 0.5 mg; and *C. kluyveri* extract, 29 mg. To it the following additions were made: expt 1, potassium oxalacetate, 10 μmoles, and dilithium [1-^{14}C]acetyl phosphate, 10 μmoles, containing 5,400,000 total dpm of which 3,880,000 dpm was in the acetyl phosphate; expt 2: potassium oxalacetate, 10 μmoles, and [^{12}C]-acetyl phosphate, 10 μmoles, but with TPN and NH_4Cl omitted; expt 3, potassium L-malate, 25 μmoles; DPN, 0.4 mg, and [1-^{14}C]acetyl phosphate, 10 μmoles; expt 4, potassium citrate, 9.0 μmoles; volume, 3.0 ml; incubation, 2 hr at 37° under hydrogen gas.

showed the presence of one major radioactive peak corresponding to γ-aminobutyrate.

It is not known how L-malate decreased both the radioactivity and specific activity of the C-1 of glutamate derived from [1-^{14}C]acetyl phosphate when compared to oxalacetate as the precursor of citrate. However, it is apparent that the proportion of radioactivity entering C-1 of glutamate from [1-^{14}C]acetyl phosphate is not significantly greater than that observed (5.2%) when pure [1-^{14}C]citrate was the precursor (Table I). Thus it can be concluded that in this experiment citrate synthesis proceeded practically exclusively by reaction A and glutamate biosynthesis by route A.

[4-^{14}C]Oxalacetate was generated *in situ* from pyruvate, $^{14}CO_2$, and ATP with highly purified pyruvate carboxylase, in the presence of acetyl-CoA as cofactor (Table III). This oxalacetate was then converted successively to citrate and L-glutamate with *C. kluyveri* extract. Of the radioactivity fixed into the organic fraction, 76% was present in the α-carboxyl carbon of L-glutamate. Radiochromatography of the solution before enzymatic decarboxylation showed essentially all the radioactivity in L-glutamate (R_F 0.17), with minor radioactive peaks at R_F 0.25 (alanine) and R_F 0.32 (probably L-malate, R_F 0.34). After decarboxylase treatment, the L-glutamate radioactive peak disappeared and the γ-aminobutyrate (R_F 0.43) which appeared had a coincident minor radioactive peak of about the same intensity as the alanine or L-malate radioactive peaks. The γ-aminobutyrate area was eluted and counted. The total radioactivity recovered in γ-aminobutyrate, *i.e.*, the α-carboxyl carbon, was 12,500 dpm, corresponding to 3.8% [12,500/(314,600 + 12,500)] of the total radioactivity in L-glutamate. Here again citrate synthesis proceeded almost exclusively by reaction A and L-glutamate biosynthesis by route A. It is possible that reaction B and route B accounted for some 4% (maximum 8%, based on approximately equal residual radioactivity—after decarboxylase action—in γ-aminobutyrate, alanine, and L-malate) of the glutamate synthesized.

The specific radioactivity of the C-1 of L-glutamate synthesized from [4-^{14}C]oxalacetate, 314,600/3.15 = 100,000, appears significantly greater than the specific radioactivity of the original $^{14}CO_2$ (70,500). However, if one assumes that endogenous L-glutamate formation is suppressed under the experimental conditions, then the C-1 specific radioactivity is 76,700, which is closer to the theoretical.

Two further experiments were carried out which, in effect, served to validate the methodology underlying the experiments described above and whose results gave further support to the conclusions drawn therefrom.

First, the position of the label in oxalacetate was varied by using differently labeled pyruvate compounds as substrates for pyruvate carboxylase. The particular labeled oxalacetate was then converted to glutamate. As shown in Table IV, [1-^{14}C]oxalacetate (from [1-^{14}C]pyruvate) did not enter L-glutamate in any significant degree. Routes A and B both predict the label would appear in C-6 of citrate and would be lost on conversion of citrate to α-ketoglutarate. [3-^{14}C]Oxalacetate (from [3-^{14}C]pyruvate) was found exclusively in the γ-aminobutyrate moiety of L-glutamate, consistent with its predicted appearance in C-2 of L-glutamate (route A) or C-4 of L-glutamate (route B). At the same time, [4-^{14}C]oxalacetate again appeared almost exclusively in C-1 of glutamate as before, demonstrating the predominant involvement of route A.

Second, [1,5-^{14}C]citrate was converted to L-glutamate by *C. kluyveri* extract (Table V). As expected, equal radioactivity appeared in the two carboxyl groups of L-glutamate. When compared with the almost complete conversion of [1-^{14}C]citrate to [5-^{14}C]-L-glutamate

TABLE III: Conversion of [4-^{14}C]Oxalacetate Formed from $^{14}CO_2$ to [1-^{14}C]Glutamate.

Substrate[a]	Glutamate Formed (μmoles)	Total Radioactivity (dpm) Before Decarboxylase	After Decarboxylase	CO_2 from Decarboxylase
[4-^{14}C]OAA	4.10	411,000	96,400	223,000
None	0.95	...	...	...

[a] This experiment was carried out in three stages: stage 1 (oxalacetate synthesis), [4-^{14}C]oxalacetate was accumulated by incubating the following compounds for 20 min at 30° in air; Tris–HCl buffer pH 8.0, 100 μmoles; $MgCl_2$, 4 μmoles; potassium pyruvate, 6.8 μmoles; ATP, 7 μmoles; $KH^{14}CO_3$, 30 μmoles (70,500 dpm/μmole); acetyl-CoA, 0.69 μmole; and purified pyruvate carboxylase (specific activity 20 μmoles/min per mg), 0.3 mg; volume, 1.0 ml. By assay with malic dehydrogenase, 5.1 μmole of [4-^{14}C]oxalacetate had accumulated; stage 2 (citrate formation), 15 μmoles of acetyl phosphate and *C. kluyveri* extract, 22 mg, and water to final volume of 2.77 ml were added and the mixture was incubated for 10 min at 37° under hydrogen gas; stage 3 (conversion of citrate to glutamate), TPN, 0.8 mg; NH_4Cl, 15 μmoles, and Tris–HCl buffer 7.0, 100 μmoles were added (final volume 3.0 ml) and the incubation was continued at 37° for 2 hr under hydrogen gas. In experiment with no substrate, pyruvate, ATP, and pyruvate carboxylase were omitted from stage 1 and cold $KHCO_3$ was used.

(Table I), it is evident that carbon 5 of [1,5-^{14}C]citrate entered C-1 of L-glutamate.

Discussion

The above experiments demonstrate that the aconitase and citrate synthetase present in these *C. kluyveri* extracts have the usual stereospecificity established for pig heart aconitase and citrate synthetase and illustrated by route A, Figure 1. However, 5–8% of added [1-^{14}C]-citrate or [1-^{14}C]acetate does appear in C-1 of L-glutamate, whereas 4–8% of $^{14}CO_2$ ([4-^{14}C]OAA) may appear in C-5 of L-glutamate. Since no recycling of acetate can occur *via* the abbreviated citric acid cycle present in *C. kluyveri* (Stern and Bambers, 1966), this process cannot explain the incorporation of acetate carboxyl carbon into C-1 of glutamate. It is possible to attribute these small and unusual distributions of labeled carbon to unknown interfering reactions. On the other hand, they could all be explained by a single assumption, namely, that citrate synthetase (of heart and *C. kluyveri*) is not a completely stereospecific enzyme. As a result, 92–95% of reactions between oxalacetate and acetyl-CoA result in reaction A, whereas 5–8% occur *via* reaction B. This interpretation is preferred to postulating two discrete citrate synthetases of opposite stereo-

TABLE V: Conversion of [1,5-^{14}C]Citrate to [1,5-^{14}C]-Glutamate.

Substrate[a]	Glutamate Formed (μmoles)	Glutamate Radioactivity (dpm) 1-Carbon[b]	GABA[c]
[1,5-^{14}C]Citrate	7.19	530,000	546,000
None	1.35	...	...

[a] The reaction mixture contained: Tris–HCl buffer pH 7.0, 100 μmoles; $MgCl_2$, 4 μmoles; TPN, 0.8 mg; NH_4Cl, 15 μmoles; *C. kluyveri* extract, 23 mg; and, as indicated, [1,5-^{14}C]citrate, 6.0 μmoles, specific activity 242,700 dpm; volume, 3.0 ml; incubation, 2 hr at 37° under hydrogen gas. [b] Determined with L-glutamate decarboxylase. [c] Isolated from decarboxylated mixture by paper chromatography.

TABLE IV: Incorporation of Oxalacetate Carbon into Carbon-1 and GABA Moieties of Glutamate.

Labeled[a] Precursor	Specific Activity[b]	Labeled OAA	L-Glutamate Found (μmole)	Radioactivity in α-COOH (dpm)	Radioactivity in GABA[c]
$^{14}CO_2$	106,000	[4-^{14}C]OAA	1.68	86,800	None
[1-^{14}C]pyruvate	140,000	[1-^{14}C]OAA	1.55	914	None
[3-^{14}C]pyruvate	200,000	[3-^{14}C]OAA	...	484	Intense

[a] This experiment was carried out with the same concentrations of reactants as used in Table III except for the following modifications: stage 1, 30 μmoles of $KH^{14}CO_3$ plus 6 μmoles of [^{12}C]pyruvate; or 6 μmoles of [1-^{14}C]-pyruvate plus 30 μmoles of $KH^{12}CO_3$; or 6 μmoles of [3-^{14}C]pyruvate + 30 μmoles of $KH^{12}CO_3$ served as substrates for the pyruvate carboxylase. [^{14}C]Oxalacetate (4.4 μmoles) was accumulated. Stage 2, CoA, 1 mg, was also added and *C. kluyveri* extract contained 40 mg of protein. Stage 3: termination occurred as in Table III. [b] Disintegrations per minute per micromole of precursor. [c] Determined by paper chromatography and radioscanning.

specificity, for it is unlikely that the proportions of two such enzymes would be the same in a crystalline heart preparation and a crude extract of *C. kluyveri*. It is considered unlikely that a lack of complete stereospecificity of aconitase contributed to the above results, since aconitase in pig heart (Englard and Colowick, 1957) and in rat liver (Wilcox *et al.*, 1950) appears to be absolutely stereospecific.

The occurrence of route A can account for only 10% of L-glutamate synthesized by growing *C. kluyveri* cells (Tomlinson, 1954). Hypothetical enzyme pathways (other than route B) which could explain Tomlinson's data have not been found in *C. kluyveri* (Stern and Bambers, 1966). Gottschalk and Barker (1966) have made an independent study of glutamate biosynthesis in *C. kluyveri* and presented equally convincing evidence that *C. kluyveri* citrate synthetase has an opposite stereospecificity to the pig heart enzyme and catalyzes reaction B so that route B can account for Tomlinson's data. The opposite results obtained in the two laboratories can be explained by assuming that a mutation involving the enzyme has occurred in the original Barker strain during continuous subculture by Worthington Laboratories. The possibility of contamination by another organism with normal citrate synthetase is very remote. Although the activity of citrate synthetase sometimes varied from batch to batch, the pattern of $^{14}CO_2$ incorporation into L-glutamate was the same in extracts with high or low activity. The cells produced large quantities of hydrogen during growth and the extracts possessed the enzymes alcohol dehydrogenase, CoA-linked aldehyde-DPN dehydrogenase, and phosphotransacetylase characteristic of *C. kluyveri* (Barker, 1957) as well as pyruvate synthetase and pyruvate carboxylase. Moreover, we have found that the citrate pathway of L-glutamate biosynthesis is present in the anaerobe *C. thermoaceticum* (Stern and Bambers, unpublished experiments) and determined that its citrate synthetase has the usual stereospecificity (reaction A). The *C. kluyveri* situation could well be unique.

The results of Gottschalk and Barker show that route B can account for 90% of glutamate biosynthesis under Tomlinson's conditions. To account for the remaining 10%, one has to assume either that two different citrate synthetases of opposite stereospecificity are present in the ratio of 9:1, or that the unusual synthetase is not completely stereospecific, a question we have raised above concerning the enzyme in heart and our extracts of *C. kluyveri*. For these reasons, a study to determine whether the crystalline heart synthetase is absolutely stereospecific is being undertaken.

Finally, it may be noted that the small but significant incorporation of $^{14}CO_2$ into C-2 of L-glutamate observed by Tomlinson remains unexplained, for *C. kluyveri* contains no α-ketoglutarate dehydrogenase. Therefore, reversal of this reaction cannot explain labeling of C-2 or C-1 by $^{14}CO_2$.

Addendum

The [1-^{14}C]citrate synthesized by pig heart citrate synthetase and used in the above experiments has been shown to have not less than 99.9% of its radioactivity in C-1 by three separate tests: (a) enzymatic cleavage (reverse reaction) with crystalline pig heart condensing enzyme yielding [1-^{14}C]acetyl-CoA and [^{12}C]oxalacetate, (b) enzymatic cleavage by citritase from *Aerobacter aerogenes* to [1-^{14}C]acetate and [^{12}C]oxalacetate, and (c) enzymatic conversion to [5-^{14}C]glutamate exclusively by extracts of *Clostridium thermoaceticum*. Thus pig heart citrate synthetase and citritase from *A. aerogenes* are each absolutely stereospecific (reaction A, Figure 1). They attack the same side of the carbonyl group of oxalacetate and the same carbon–carbon bond of citrate.

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