Synthesis of Glutamate and Citrate by *Clostridium kluyveri*. A New Type of Citrate Synthase*

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ABSTRACT: The reactions involved in the synthesis of glutamate from acetyl phosphate and oxalacetate or its precursors (acetyl phosphate, pyruvate, and carbon dioxide) have been investigated by tracer experiments with cell-free extracts of *Clostridium kluyveri*. Tomlinson's [Tomlinson (1954b)] observation, made with intact cells, that the α-carboxyl carbon of glutamate is derived from C-1 of acetate (or acetyl phosphate) and the γ-carboxyl from carbon dioxide has been confirmed with extracts. *C. kluyveri* has been shown to contain the enzymes citrate synthase, aconitase, isocitric dehydrogenase, and glutamate dehydrogenase, required for the conversion of oxalacetate and acetyl coenzyme A to glutamate. The accumulation of labeled citrate, α-ketoglutarate, and glutamate has been demonstrated using [^{14}C]carbon dioxide, [4-^{14}C]oxalacetate, or [1-^{14}C]acetyl phosphate as substrates, and the products were isolated. By converting specifically labeled [^{14}C]citrate to isocitrate by means of either yeast aconitase or *C. kluyveri* aconitase and then converting the [^{14}C]isocitrate to [^{14}C]glutamate with purified enzymes and determining the isotope distribution in the glutamate, the aconitase of *C. kluyveri* has been shown to have the usual stereospecificity. The citrate synthase of *C. kluyveri* has been found to be atypical in that it forms the isotopic antipode of the usual citrate. The stereospecificity of the synthase accounts for the unusual origin of the glutamate carbon atoms.

Tomlinson (1954b) demonstrated that the origin of the carbon atoms in glutamate synthesized by growing cultures of *Clostridium kluyveri* is unusual. Whereas most aerobic and facultatively anaerobic organisms synthesize glutamate by some reactions of the tricarboxylic acid cycle and incorporate the carboxyl carbon of acetate into C-5 of glutamate and carbon dioxide carbon into C-1 of glutamate, *C. kluyveri* incorporates the carboxyl carbon of acetate into C-1 and C-3 of glutamate and carbon dioxide carbon mainly into C-5 of glutamate. Tomlinson pointed out that this unusual origin of the carbon skeleton of glutamate could be the result of the presence in *C. kluyveri* of an atypical aconitase that formed the double bond of *cis*-aconitate adjacent to the carbon atoms derived from acetate. No evidence for or against this hypothesis has been published. We have investigated the path of glutamate synthesis in extracts of *C. kluyveri* and obtained evidence that the unusual labeling of glutamate derived from labeled acetate or carbon dioxide is not caused by an atypical aconitase, but is caused by the action of an atypical citrate synthase which forms [^{14}C]citrate that is the isotopic antipode of the [^{14}C]citrate formed by the usual synthase.

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Methods

Culture Methods. C. kluyveri was grown in the synthetic medium of Stadtman and Barker (1949) except that sodium thioglycolate (750 mg/l.) was used instead of Na_2S for small (<1 l.) cultures and $Na_2S_2O_4$ (35 mg/l.) was used for large cultures. Using a 5–10% (v/v) inoculum, cells were harvested after 24 hr of growth at 37°; the estimated final absorbance at 540 mμ was 0.7–0.9 in the Zeiss spectrophotometer Model M4Q III. Samples were diluted so the absorbance was <0.3 before measurement.

Preparation of Cell-Free Extracts. Frozen cells of *C. kluyveri* were thawed in 50 mM buffer (potassium phosphate or Tris–HCl, pH 7.4) containing 25 mM 2-mercaptoethanol. Cell suspension (2–3 ml) was sonicated with a 60W MSE ultrasonic disintegrator for 30–60 sec. Cell debris was removed by centrifugation at 20,000*g* for 20 min at 0–2°. Protein was determined by the method of Lowry *et al.* (1951). Extracts usually contained 15–25 mg of protein/ml. Sephadex-treated extracts were prepared by adding 1–2 ml of crude extract to a 1.5 × 14 cm column of Sephadex G-25 (medium) and eluting the protein with water. Buffer and 2-mercaptoethanol were added to the final concentrations indicated above. The final protein concentration was 5–10 mg of protein/ml.

Cell-free extract of bakers' yeast (Red Star) was prepared by grinding 2 g of fresh yeast in a cold mortar with 2 g of alumina (A-301, Alcoa Chemicals) and extracting the protein with 5 ml of 50 mM potassium phosphate or Tris–chloride buffer, pH 7.4. The extract was centrifuged and the clear solution was used.

Anaerobic Preincubation of C. kluyveri Extracts.

Preliminary experiments on aconitase and the pyruvate synthesizing enzyme system of *C. kluyveri* had shown that a preincubation of the extracts under H_2 was necessary to overcome a lag period in the enzyme activities. Unless otherwise indicated, extracts containing buffer and 2-mercaptoethanol (see above) were routinely incubated under H_2 for 30 min at 30° before use, although it was not demonstrated that this treatment is beneficial for all the enzymes studied.

Paper Chromatography. The solvent systems used for descending chromatography on Whatman No. 3MM paper were 2-butanol–formic acid–water (67:11:22) and 1-propanol–concentrated ammonia–water (6:3:1) (Hirsch, 1963). Radioactive spots were located by means of an automatic chromatogram scanner (Vanguard Instrument Co., Model 880) or by cutting the chromatograms in narrow strips and counting them in a liquid scintillation spectrometer (Packard Tri-Carb Model 3214). The scintillation fluid of Bray (1960) was used.

Chemicals and Enzymes. $Ba^{14}CO_3$ and [1-^{14}C]acetate were purchased from Calbiochem, Los Angeles, [1,5-^{14}C]citrate from New England Nuclear Corp., Boston, and DL-[^{14}C]aspartate from Volk Radiochemical Co., Chicago. Glutamic dehydrogenase (3 units/mg) and isocitric dehydrogenase (*ca.* 1.8 units/mg) were preparations of Boehringer and Soehne, Mannheim, Germany. Phosphotransacetylase (8 units/mg) was prepared by the method of Stadtman (1955). Glutamate–aspartate transaminase (170 Jenkins units/mg) was prepared (Jenkins, 1962) and generously donated by Dr. W. T. Jenkins.

Determination of ^{14}C in the Carboxyl Groups of [^{14}C]-Glutamate. α-CARBOXYL GROUP (Kemble and McPherson, 1954; Hoare, 1963a). Warburg vessels contained 0.8 ml of 0.5 M acetate buffer, pH 4.0, 1.5 ml of 10% (w/v) chloramine-T, and 0.1 ml of 20% (w/v) formaldehyde in the main compartment; 0.1 ml of [^{14}C]glutamate (about 5 μmoles) in the side arm; and 0.1 ml of 1 M hyamine hydroxide in methanol on filter paper in the center well. Vessels were shaken for 1 hr at 30° after the amino acid was added to the chloramine-T solution. Radioactivity of carbon dioxide trapped by hyamine was determined in the scintillation counter.

γ-CARBOXYL GROUP. Wachsman and Barker (1955) demonstrated and Hoare (1963a) confirmed that the carbon dioxide formed in the fermentation of glutamate by cell suspensions of *Clostridium tetanomorphum* is derived exclusively from the γ-carboxyl group. *C. tetanomorphum* was grown in the medium of Barker *et al.* (1959) and harvested after 15 hr of incubation at 37°. Cells from a 1-l. culture were washed twice with 10 volumes of 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% (w/v) $Na_2S \cdot 9H_2O$, suspended in 10 ml of the same buffer, and kept in ice in an evacuated Thunberg tube. Warburg vessels contained 1 ml of cell suspension in the main compartment, 0.1 ml of [^{14}C]-glutamate (about 5 μmoles) in one side arm, and 0.1 ml of 5 N H_2SO_4 in the other. Hyamine solution (0.1 ml) was placed in the center well on filter paper. The reaction was run for 60 min at 37° under helium. After tipping in the sulfuric acid, vessels were shaken for another 30 min to trap evolved CO_2.

Enzymatic Conversion of [^{14}C]Citrate to [^{14}C]Glutamate. A Sephadex G-25 treated extract of *C. kluyveri* was preincubated as described above except that the protein solution contained 0.5 mM ferrous ammonium sulfate. Warburg vessels contained 0.40 ml of extract (*ca.* 3 mg of protein); Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 1 mM; 2-mercaptoethanol, 25 mM; NADP,[1] 1 mM; [^{14}C]citrate, *ca.* 0.1 mM; and isocitric dehydrogenase (100 μg); in a final volume of 2.0 ml. The reaction was run under helium for 40–60 min at 30°, and was stopped by heating the reaction mixture at 100° for 3 min. Protein was removed by centrifugation. The reduction of α-ketoglutarate to glutamate was performed by adding 10 μmoles of NH_4Cl, 2 μmoles of $NADH_2$, and 50 μg of glutamic dehydrogenase to the supernatant solution. The resulting [^{14}C]glutamate and any remaining [^{14}C]citrate were adsorbed on a Dowex-1-formate column (0.8 × 3.5 cm). The [^{14}C]glutamate was eluted from the column with 1 N HCOOH, and the [^{14}C]-citrate with 6 N HCOOH (Busch *et al.*, 1952). The conversion of citrate to glutamate was always 90–95% complete. The [^{14}C]glutamate-containing fractions were concentrated *in vacuo* and purified by paper chromatography using 2-butanol–formic acid. The purity of the isolated [^{14}C]glutamate was checked by electrophoresis (0.2 M HCOOH, 45 v/cm, 30 min; and 50 mM ammonium formate, pH 3.85, 30 v/cm, 40 min). The procedure using a Sephadex G-25 treated extract of bakers' yeast (Red Star) as a source for aconitase was essentially the same, except that 0.5 μmole of α-ketoglutarate was added to the reaction mixture. Controls were run with isocitric dehydrogenase alone to make sure that the enzyme preparation did not contain aconitase.

Synthesis of [^{14}C]Citrate from [4-^{14}C]Aspartate and Acetyl Phosphate + CoA by Extracts of C. kluyveri. The complete system contained 0.5 ml of Sephadex G-25 treated extract (2.5 mg of protein) and the following in μmoles: potassium phosphate buffer, pH 7.4, 100; $MgCl_2$, 2; 2-mercaptoethanol, 25; lithium acetyl phosphate, 50; CoA, 1; DL-[4-^{14}C]aspartate, 2.5 (2.5 μc); α-ketoglutarate, 5; $NaHCO_3$, 10; avidin (2.5 units/mg), 0.1 mg; and glutamate–aspartate transaminase, 120 μg; in a final volume of 1.2 ml. The extract was incubated with avidin, buffer, and 2-mercaptoethanol for 30 min at 30° under hydrogen before adding the other components. The complete reaction mixture was incubated for 40 min at 30° under helium. The ^{14}C-organic acids were separated from residual [^{14}C]aspartate by chromatography on Dowex-1-formate (Busch *et al.*, 1952). The separated products were further purified by paper chromatography in 2-butanol–formic acid. They were located by radiochromatography, and each compound was eluted with 0.25 ml of water. The yields of acids in mμmoles were: citrate, 59 (113,900 cpm); α-ketoglutarate, 34 (65,600 cpm); succinate, 80 (154,500 cpm); and fumarate, 102 (197,000 cpm).

[1] Abbreviations used: NADP, nicotinamide-adenine phosphate; $NADH_2$, reduced nicotinamide-adenine phosphate; PTA, phosphotransacetylase.

Conditions for [^{14}C]citrate synthesis by an extract of bakers' yeast (Red Star) were the same, except that 50 μg of purified phosphotransacetylase was added. The reaction mixture was incubated for 30 min at 30° under helium. L-[^{14}C]Aspartate was completely converted to [^{14}C]citrate under these conditions.

Synthesis of [^{14}C]Citrate from [1-^{14}C]Acetate and Oxalacetate. The reaction mixture (2.0 ml), containing KPO_4 buffer, pH 7.4, 50 mM; $MgCl_2$, 1 mM; CoA, 0.25 mM; acetyl phosphate, 1 mM; and [1-^{14}C]acetate, 2.5 mM (2 μc/μmole), was placed in the main compartment of a Warburg vessel and a Sephadex G-25 treated extract of *C. kluyveri* (0.40 ml; 3.2 mg of protein) was placed in the side bulb. After 20 min of preincubation under hydrogen at 30° to activate the enzyme system, the hydrogen atmosphere was replaced by helium and the extract was tipped in. The reaction mixture was incubated for 30 min to permit the synthesis of [1-^{14}C]-acetyl CoA by the action of phosphotransacetylase and CoA–transphorase present in the *C. kluyveri* extract (Stadtman, 1955; Barker *et al.*, 1955). The 0.1 ml of 0.1 M potassium oxalacetate and 0.25 ml (5.8 mg of protein) of an extract of bakers' yeast were added. The incubation was continued for 15 min under helium and was stopped by heating the solution at 100° for 3 min. The acetate and citrate were separated on a Dowex-1-formate column as described above. The yield of [^{14}C]-citrate, further purified by paper chromatography in 2-butanol–formic acid, was 255,000 cpm (*ca.* 200 mμ-moles).

Synthesis of [^{14}C]citrate from oxalacetate and [^{14}C]-acetate by a crude extract of *C. kluyveri* was carried out in the same way. The reaction mixture contained 0.5 ml of extract (8.7 mg of protein) and the reaction time was 60 min. The isolated products were citrate, 2440 cpm; α-ketoglutarate, 80,900 cpm; and glutamate, 36,800 cpm.

Enzyme Assays

Glutamic Dehydrogenase (NADP). Sephadex G-25 treated extract of *C. kluyveri* containing 50 mM KPO_4 buffer, pH 7.6, and 25 mM 2-mercaptoethanol was activated by incubation for 30 min at 30° under hydrogen, and kept in ice in a completely filled syringe until needed. Spectrophotometric assays (340 mμ, 25°) were carried out in anaerobic silica cuvets (3 ml, 1-cm light path) in a helium atmosphere. The assay system contained KPO_4 buffer, pH 7.6, 50 mM; NH_4Cl, 10 mM; α-ketoglutarate, 10 mM; $NADPH_2$, 0.1 mM; and cell-free extract (0.6 to 1.8 mg of protein); in a final volume of 2.0 ml.

Isocitric Dehydrogenase (NADP). Cell-free extract was prepared and activated as described for the glutamic dehydrogenase assay. The assay system contained Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 3 mM; 2-mercaptoethanol, 25 mM; DL-isocitrate, 4 mM; NADP, 0.33 mM; and cell-free extract (1.5 mg of protein); in a final volume of 6.0 ml. The reaction mixture was incubated under helium at 30°. Aliquots (0.5 ml) were removed for analysis at 5-min intervals and the reaction was stopped by heating at 100°. The amount of α-ketoglutarate formed was determined spectrophotometrically by the reduction to glutamate with an excess of purified glutamic dehydrogenase, ammonium chloride, and $NADH_2$.

Aconitase. The assay was done by coupling the aconitase reaction with the isocitric dehydrogenase reaction. The activation of the enzyme was carried out as for isocitric dehydrogenase except that 0.5 μmole of ferrous ammonium sulfate was added/ml of extract. The assay system contained Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 3 mM; 2-mercaptoethanol, 25 mM; citrate, 4 mM; NADP, 0.33 mM; isocitric dehydrogenase (Boehringer), 100 μg; and cell-free extract (2.0 mg of protein); in a final volume of 6.0 ml. The determination of the formed α-ketoglutarate was done as described for the isocitric dehydrogenase assay. Controls were run without citrate and without *C. kluyveri* extract. The reaction rate was 1.6 times faster with *cis*-aconitate than with citrate.

Citrate Synthase. The assay system contained KPO_4 buffer, pH 7.4, 50 mM; $MgCl_2$, 1 mM; 2-mercaptoethanol, 25 mM; lithium acetyl phosphate, 20 mM; CoA, 0.5 mM; DL-[4-^{14}C]aspartate, 5 mM (0.25 μc/μmole); citrate, 2.5 mM; α-ketoglutarate, 15 mM; avidin, 0.25 unit; purified glutamate–aspartate transaminase, 120 μg; and 0.25 ml of cell-free extract (7.4 mg of protein); in a final volume of 2.0 ml. The reaction mixture was incubated under helium at 30°. Thecon centrations of acetyl phosphate, [4-^{14}C]oxalacetate, and [^{14}C]-citrate were measured after 30 and 60 min. Acetyl phosphate was determined by the method of Lipmann and Tuttle (1945); [4-^{14}C]oxalacetate by counting the evolved $^{14}CO_2$ after decarboxylation with 4-amino-antipyrine (MacDonald and Stanier, 1957); and [^{14}C]-

TABLE I: Compounds Synthesized from Acetyl Phosphate and [^{14}C]Carbon Dioxide.[a]

Compound	Radioactivity (cpm × 10^{-3})	CO_2 Incorpd (mμmoles)
Alanine	296	7.8
Aspartate	292	7.7
Glutamate	163	4.3
Pyruvate	539	14.2
Citrate	1660	43.7
α-Ketoglutarate	562	14.8
Phosphate esters (unidentified)	3929	103.4
$^{14}CO_2$ fixed	7425	195.0

[a] The system contained 0.40 ml of Sephadex G-25 treated cell-free extract of *C. kluyveri* (3.7 mg of protein); KPO_4 buffer, pH 7.4, 50 mM; $MgCl_2$, 1 mM; 2-mercaptoethanol, 25 mM; lithium acetyl phosphate, 25 mM; CoA, 0.5 mM; ATP, 1 mM; and $NaH^{14}CO_3$, 5 mM (22 μc/μmole) in a final volume of 2.0 ml. The system was incubated 90 min at 30° under hydrogen. Radioactive products were isolated and identified by paper chromatography and electrophoresis.

TABLE II: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Prepared from Acetyl Phosphate and $^{14}CO_2$ by Extracts of *C. kluyveri*.

Labeled Compd	^{14}C Added (cpm)	α-Carboxyl (cpm)	Carbon (%)	γ-Carboxyl (cpm)	Carbon (%)
L-[U-^{14}C]Glutamate	28,040	5,819	20.7	5,259	18.7
DL-[1-^{14}C]Glutamate	28,890	25,689	88.9	574	1.9
[^{14}C]Glutamate from *C. kluyveri*	12,000	1,448	12.1	8,614	71.8

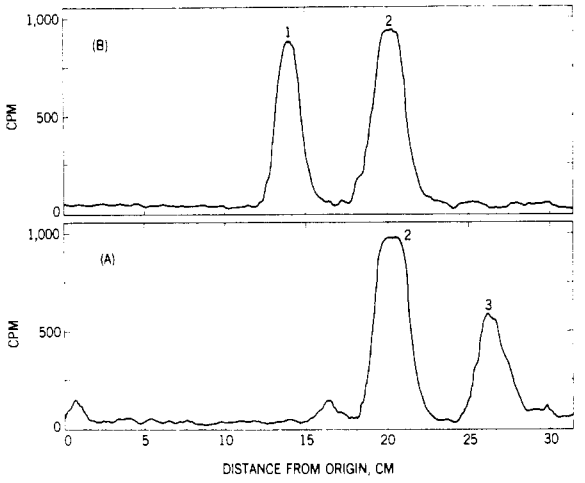


FIGURE 1: Formation of [^{14}C]-α-ketoglutarate and [^{14}C]glutamate from [1,5-^{14}C]citrate. Sephadex G-25 treated extract of *C. kluyveri* was preincubated as described for the aconitase assay. The complete system contained 0.20 ml of extract (1.9 mg of protein); Tris–HCl buffer, pH 7.4, 100 mM; $MgCl_2$, 2 mM; 2-mercaptoethanol, 25 mM; [1,5-^{14}C]citrate, 1 mM (0.3 μc/μmole); and NADP, 2 mM; in a final volume of 1.0 ml. Reaction was run for 45 min at 30° under helium, and was stopped by heating at 100° for 3 min. NH_4Cl (10 μmoles), 2 μmoles of $NADH_2$, and 50 μg of glutamic dehydrogenase were added to an aliquot (0.5 ml). After 5 min protein was precipitated as described. Samples were chromatographed in 2-butanol–formic acid and ^{14}C distribution was determined with a chromatogram scanner; A, original reaction mixture; B, after treatment with glutamic dehydrogenase; 1, glutamate; 2, citrate; 3, α-ketoglutarate.

citrate by isolation by Dowex-1 and paper chromatographic methods and estimation of its radioactivity.

Results

Citrate and Glutamate Formation by Cell-Free Extracts of C. kluyveri. The labeled compounds formed by a Sephadex G-25 treated extract of *C. kluyveri* from acetyl phosphate and radioactive carbon dioxide under hydrogen are shown in Table I. Besides a large phosphate ester fraction, which was not further investigated, a considerable percentage of the fixed radioactivity was present in citrate. Smaller amounts of ^{14}C were present in pyruvate, alanine, aspartate, and glutamate. The distribution of ^{14}C in the labeled glutamate was determined in order to see whether it agreed with Tomlinson's results (Tomlinson, 1954b) obtained with whole cells. Table II shows that most of the ^{14}C was incorporated into the γ-carboxyl group of glutamate. About 12% of the radioactivity was present in the α-carboxyl group, whereas Tomlinson found 7% in that position in experiments with whole cells. Control experiments carried out with uniformly and C-1-labeled glutamate demonstrated the reliability of the degradation procedures.

Aconitase and Isocitric Dehydrogenase Reactions in C. kluyveri Extracts. Tomlinson suggested that the

TABLE III: $^{14}CO_2$ Fixation with α-Ketoglutarate as Substrate by Extracts of *C. kluyveri*.[a]

Component Added or Omitted	$^{14}CO_2$ Fixed (mμmoles)	[^{14}C]Isocitrate (%)	[^{14}C]-Citrate (%)
None	47.4	3.3	71.0
+ Citrate	12.2	37.1	50.8
+ *cis*-Aconitate	6.7	64.1	12.9
+ DL-*threo*-Isocitrate	2.0	100.0	...
− α-Ketoglutarate	1.0	...	...

[a] The complete system contained 0.20 ml of cell-free extract (5.7 mg of protein); Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 5 mM; 2-mercaptoethanol, 25 mM; $NaH^{14}CO_3$, 5 mM (5.5 μc/μmole); α-ketoglutarate, 12.5 mM; and $NADPH_2$, 0.25 mM; in a final volume of 2.0 ml. Citrate, *cis*-aconitate or DL-*threo*-isocitrate (12.5 mM) were added as indicated. The reaction mixtures were incubated under hydrogen for 20 min at 30°. Isocitrate was determined by oxidative decarboxylation of isolated citrate–isocitrate mixtures with isocitric dehydrogenase and counting the $^{14}CO_2$ trapped by hyamine hydroxide; citrate and isocitrate were determined together by the same method, after addition of an extract of bakers' yeast as a source for aconitase.

TABLE IV: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Prepared from [^{14}C]Citrate by the Use of Yeast or *C. kluyveri* Aconitase.

Source of Aconitase	Radioactivity in [^{14}C]Glutamate				
	Total (cpm)	α-Carboxyl (cpm)	Carbon (%)	γ-Carboxyl (cpm)	Carbon (%)
C. kluyveri	10,150	1,211	11.9	7,425	73.1
Bakers' yeast	7,180	1,093	15.2	5,388	75.0

unusual labeling of glutamate derived from $^{14}CO_2$ may be explained by assuming that an atypical aconitase is present in *C. kluyveri* which forms the double bond between the central carbon atom of citrate and the carbon atom originating from the methyl group of acetate. Evidence that aconitase is present in *C. kluyveri* is presented in Figure 1. The action of Sephadex G-25 treated extract on [1,5-^{14}C]citrate under helium gave rise to the formation of [^{14}C]-α-ketoglutarate and [^{14}C]glutamate. The reverse reaction could be demonstrated by incubating α-ketoglutarate with $NADPH_2$ and $^{14}CO_2$ under hydrogen; [^{14}C]citrate was the main reaction product (Table III). The $^{14}CO_2$ fixation rate and the percentage of the fixed ^{14}C in citrate decreased when citrate, *cis*-aconitate, or isocitrate was added to the reaction mixture. Paper chromatography of aliquots of the reaction mixtures in 2-butanol–formic acid solvent showed that [^{14}C]-*cis*-aconitate accumulated only in very low concentrations even when the reaction was carried out in the presence of *cis*-aconitate. Probably the enzyme-bound intermediate in the interconversion of isocitrate and citrate does not exchange readily with free *cis*-aconitate (Speyer and Dickman, 1956; Rose *et al.*, 1962).

Stereospecificity of the Aconitase Reaction. In order to study the stereochemistry of the aconitase reaction [^{14}C]citrate prepared from acetyl phosphate and $^{14}CO_2$ (Table I) was converted with Sephadex G-25 treated *C. kluyveri* extract (as a source of aconitase) and purified isocitric dehydrogenase to [^{14}C]-α-ketoglutarate, which was subsequently reduced by the glutamic dehydrogenase reaction to [^{14}C]glutamate. The same experiment was carried out with an extract of bakers' yeast as a source for aconitase. The ^{14}C distribution in the carboxyl groups of both [^{14}C]glutamate samples was determined. Table IV demonstrates that both [^{14}C]-glutamate samples contained radioactivity mainly (73–75%) in the γ-carboxyl carbon. Therefore, the aconitases from yeast and *C. kluyveri* must dehydrate citrate in the same stereospecific way. This experiment disproved the hypothesis that the aconitase in *C. kluyveri* is responsible for the incorporation of $^{14}CO_2$ into the γ-carboxyl group of glutamate. It also revealed that the [^{14}C]citrate synthesized by *C. kluyveri* is the isotopic antipode of the compound formed from [1,4-^{14}C]-oxalacetate and acetyl-CoA by the usual citrate synthase, since the latter compound would be converted to [1-^{14}C]glutamate.

Citrate Synthase in Extracts of C. kluyveri. The specific activities of four enzymes involved in glutamate synthesis, namely, citrate synthase, aconitase, isocitric dehydrogenase, and glutamic dehydrogenase, are given in Table V. The isocitric and glutamic dehydrogenases

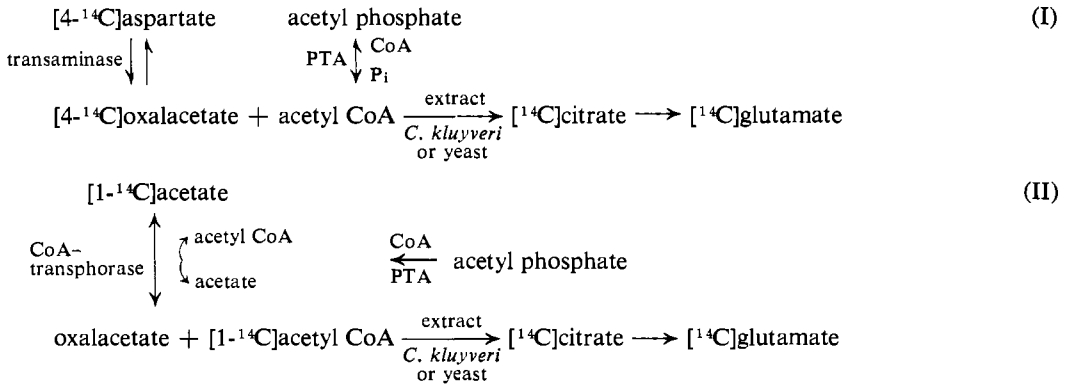
TABLE V: Enzymes Connected with Glutamate Synthesis in *C. kluyveri.*

Enzyme	Specific Activity (units[a]/g of protein)
Citrate synthase	0.38
Aconitase (aconitate hydratase)	4.9
Isocitric dehydrogenase (NADP)	12.0
Glutamic dehydrogenase (NADP)	6.7

[a] 1 unit = 1 μmole/min at 30°.

of *C. kluyveri* are specific with respect to NADP. Citrate synthase was determined by incubating [4-^{14}C]asparate with α-ketoglutarate, glutamate–aspartate transaminase, acetyl phosphate, coenzyme A, bicarbonate, and avidin in the presence of a Sephadex G-25 treated extract of *C. kluyveri* (see Methods). The glutamate–aspartate transaminase caused a rapid formation of [4-^{14}C]oxalacetate. The enzymatic decarboxylation of oxalacetate was shown by a separate experiment to be strongly inhibited by the added avidin. The bicarbonate was added to dilute any $^{14}CO_2$ that was formed and thus prevent any significant incorporation of ^{14}C from carbon dioxide into citrate by other possible routes. Acetyl coenzyme A was rapidly formed from acetyl phosphate and coenzyme A by the action of phosphotransacetylase which is present in *C. kluyveri* extracts (Stadtman, 1955). [^{14}C]Citrate accumulated because of the favorable equilibrium of the citrate synthase and the aconitase reaction, the absence of NADP required for the isocitric dehydrogenase reaction, and the presence of carrier citrate. The amount of citrate formed after 30 and 60 min was determined by isolating the acid by chromatographic methods and determining its radioactivity. Control experiments showed that a substantial fraction of the substrates remained throughout the incubation period; the final

SCHEME I



concentrations of acetyl phosphate and oxalacetate were 13.3 and 1.69 mM, respectively.

Besides [^{14}C]citrate, radioactive fumarate, succinate, and α-ketoglutarate (in thea bsence of carrier citrate) were formed from [4-^{14}C]aspartate by a *C. kluyveri* extract under the conditions used for citrate synthesis (see Methods). To detect a possible participation of fumarate or succinate in citrate synthesis, the radioactivity in the C-6-carboxyl group of the isolated [^{14}C]-citrate was determined by the successive actions of aconitase and isocitric dehydrogenase and by measuring the radioactivity of the evolved CO_2. Table VI shows that [^{14}C]citrate prepared from [4-^{14}C]aspartate and acetyl phosphate contained very little radioactivity in the C-6-carboxyl group, whereas in [^{14}C]citrate prepared from $^{14}CO_2$ and acetyl phosphate (Table I) 47% of the ^{14}C was located in that carbon atom. This result excludes the participation of a symmetrical compound like fumarate or succinate in citrate synthesis.

TABLE VI: Radioactivity in the C_6-Carboxyl Group of [^{14}C]Citrate Prepared from [4-^{14}C]Aspartate.[a]

Substrates for Citrate Synthesis	Radioactivity in [^{14}C]Citrate		
	Total (cpm)	C_6-Carboxyl Group (cpm)	(%)
$^{14}CO_2$ + acetyl phosphate	5600	2637	47.1
[4-^{14}C]Aspartate + acetyl phosphate	2500	56	2.2

[a] The complete system contained 0.40 ml of Sephadex G-25 treated extract of *C. kluyveri* (4.1 mg of protein); Tris–HCl buffer, pH 7.4, 50 mM; $MgCl_2$, 2 mM; 2-mercaptoethanol, 25 mM; NADP, 1 mM; isocitric dehydrogenase, 50 μg; and [^{14}C]citrate; in a final volume of 2.0 ml. The reaction mixture was incubated in a Warburg vessel 60 min at 30° under helium and the reaction was stopped by the addition of 0.1 ml of 5 N H_2SO_4 from the side arm. CO_2 was trapped by hyamine hydroxide placed in the center well of the vessel. The nonvolatile radioactive compounds were concentrated by chromatography on Dowex-1-formate. Paper chromatography in 2-butanol–formic acid showed that this fraction contained ^{14}C-labeled α-ketoglutarate and glutamate.

In separate experiments it was shown that the addition of malic dehydrogenase or the omission of α-ketoglutarate as reactant in the transamination reaction prevented citrate formation from [4-^{14}C]aspartate, acetyl phosphate, and coenzyme A.

Stereospecificity of the Citrate Synthase of C. kluyveri. The stereochemistry of the citrate synthesis by extracts of *C. kluyveri* was further studied by two experiments schematically described by the reaction sequences of Scheme I.

The citrate synthesis from [4-^{14}C]aspartate and acetyl phosphate (sequence I) by *C. kluyveri* extract was already described (see Methods). When using an extract of bakers' yeast as a source for citrate synthase, purified phosphotransacetylase was added to allow acetyl coenzyme A synthesis from acetyl phosphate and coenzyme A. [^{14}C]Citrate prepared either with *C. kluyveri* or bakers' yeast citrate synthase was isolated and converted to [^{14}C]glutamate by the successive actions of an extract of *C. kluyveri* as source for aconitase, purified isocitric dehydrogenase, and glutamic dehydrogenase. The ^{14}C distribution in the carboxyl groups of the two resulting ^{14}C-labeled glutamate samples is given in Table VII. Using bakers' yeast citrate synthase the C-1 carbon atom of glutamate is derived from the C-4 carbon atom of oxalacetate, in agreement with the stereochemical course of the usual tricarboxylic acid cycle. But with the *C. kluyveri* system the radioactivity is mainly located in the γ-carboxyl carbon of glutamate. Since bakers' yeast and *C. kluyveri* aconitase have the same stereospecificity and, in addition, only *C. kluyveri* aconitase was used for the conversion of both [^{14}C]-citrate samples to glutamate, the citrate synthase of *C. kluyveri* must synthesize a [^{14}C]citrate different from that formed by the bakers' yeast system.

The experiment described by sequence II was car-

TABLE VII: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Derived from [4-^{14}C]Aspartate and Acetyl Phosphate.

Source of Citrate Synthase	Radioactivity in [^{14}C]Glutamate				
	Total (cpm)	α-Carboxyl (cpm)	Carbon (%)	γ-Carboxyl (cpm)	Carbon (%)
C. kluyveri	2700	91	3.4	2195	81.3
Bakers' yeast	2850	2675	93.9	77	2.7

TABLE VIII: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Derived from [1-^{14}C]Acetate and Oxalacetate.

Source of Citrate Synthase	^{14}C-Labeled Product of the First Incubation	Radioactivity in [^{14}C]Glutamate				
		Total (cpm)	α-Carboxyl (cpm)	Carbon (%)	γ-Carboxyl (cpm)	Carbon (%)
Bakers' yeast	Citrate	4810	48	1.0	4431	92.1
C. kluyveri	Citrate	750	716	95.4	50	6.6
C. kluyveri	α-Ketoglutarate	2880	2763	95.9	104	3.6
C. kluyveri	Glutamate	1760	1631	92.6	95	5.4

ried out in a similar way. [1-^{14}C]Acetyl coenzyme A was synthesized from [1-^{14}C]acetate, acetyl phosphate, and coenzyme A by the actions of phosphotransacetylase and coenzyme A transphorase which are present in *C. kluyveri* extracts (Stadtman, 1955; Barker *et al.*, 1955). [^{14}C]Citrate synthesis was started by the addition of oxalacetate. Because a high concentration of crude extract was used in this experiment, a great part of the citrate formed was further converted to α-ketoglutarate and glutamate during the first incubation with *C. kluyveri* extract. The three-labeled products were isolated. Citrate and α-ketoglutarate were converted separately to glutamate. The distribution of ^{14}C was determined in the three [^{14}C]glutamate samples.

[1-^{14}C]Acetyl coenzyme A needed for [^{14}C]citrate synthesis by bakers' yeast citrate synthase was also prepared by the enzymes phosphotransacetylase and coenzyme A–transphorase present in *C. kluyveri* extracts. The amount of [^{14}C]citrate synthesized by *C. kluyveri* citrate synthase during the subsequent incubation with oxalacetate and yeast extract was negligible because the synthase activity of the added yeast extract was about 1000 times higher.

The data of Table VIII show that when bakers' yeast citrate synthase is used the carboxyl carbon of acetate is incorporated into the C-5 carbon atom of glutamate, whereas with the *C. kluyveri* system the radioactivity is mainly incorporated into C-1 of glutamate. These results confirm the conclusion drawn from the previous experiments.

Discussion

About 25% of the cell carbon of *C. kluyveri* is derived from dioxide when *C. kluyveri* grows on a synthetic medium containing ethanol, acetate, and bicarbonate as carbon sources (Tomlinson and Barker, 1954). Tracer experiments have shown that carbon dioxide is incorporated into most of the amino acids and that the carboxyl group of alanine and both carboxyl groups of aspartate originate exclusively from CO_2 (Tomlinson, 1954a). These results indicate that a net synthesis of a C_3 unit from carbon dioxide and acetate must occur in *C. kluyveri*.

Stern (1963, 1965) showed that cell-free extracts of *C. kluyveri* are able to synthesize pyruvate from acetyl coenzyme A and carbon dioxide under hydrogen. Recently Andrew and Morris (1965) reported the formation of radioactive alanine when extracts of *C. kluyveri* were incubated with acetyl coenzyme A and [^{14}C]carbon dioxide in a hydrogen atmosphere. We have observed that the appearance of [^{14}C]alanine as a reaction product is dependent upon the presence of free amino acids and an active transamination system in crude extracts which favor alanine formation from pyruvate and largely prevent other reactions of pyruvate. The reaction products are quite different when Sephadex-treated extracts, lacking free amino acids, are used. Under the latter conditions, 22% of the fixed ^{14}C was present in citrate, 7% in α-ketoglutarate, 2% in glutamate, and 53% in unidentified phosphate esters. The formation of labeled citrate and α-ketoglutarate from acetyl phosphate and [^{14}C]carbon dioxide indicates the occurrence in *C. kluyveri* of a portion of the tricarboxylic acid cycle. This has been supported by the demonstration of citrate synthase, aconitase, and isocitric dehydrogenase activities in extracts.[2]

[2] J. R. Stern (private communication) has also observed these activities in *C. kluyveri* extracts.

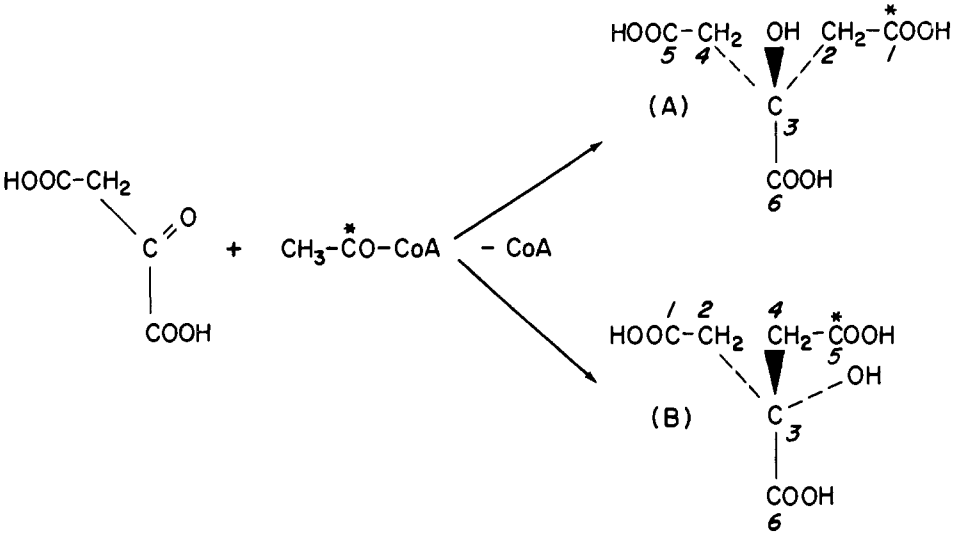


FIGURE 2: Formation of the isotopic isomers of citrate by the citrate synthase reactions.

The labeling pattern in the [^{14}C]glutamate formed enzymatically from acetyl phosphate and [^{14}C]carbon dioxide confirms the fact, first demonstrated by Tomlinson (1954b) in experiments with growing cells of *C. kluyveri*, that the γ-carboxyl carbon atom of glutamate is derived from carbon dioxide. If it is accepted that glutamate is formed *via* citrate, isocitrate, and α-ketoglutarate, the unusual type of glutamate synthesis could result from an unusual stereospecificity of either citrate synthase or aconitase. The existence of an atypical aconitase in *C. kluyveri*, postulated by Tomlinson (1954b), has been disproved by showing that the labeling pattern in [^{14}C]glutamate, formed from specifically labeled citrate by the action of aconitase, isocitric dehydrogenase, and glutamate dehydrogenase, is the same whether the aconitase is derived from bakers' yeast or *C. kluyveri*. Consequently the distinctive origin of glutamate in *C. kluyveri* must result from the atypical stereospecificity of the citrate synthase. Direct evidence has been presented that [^{14}C]citrate formed from either [4-^{14}C]oxalacetate and acetyl coenzyme A, or unlabeled oxalacetate and [1-^{14}C]acetyl coenzyme A, is the isotopic antipode of [^{14}C]citrate prepared with yeast enzymes under the same conditions.

The stereospecific condensation of oxalacetate and acetyl coenzyme A by the usual citrate synthase gives a product which has the configuration of citrate (A) (Hanson and Rose, 1963) (Figure 2). Aconitase forms the double bond between C-3 and C-4. This finally results in the formation of glutamate in which the α-carboxyl carbon is derived from C-4 of oxalacetate and the γ-carboxyl carbon from C-1 of acetate. The isotopic antipode (B, Figure 2) of the usual citrate, formed by the *C. kluyveri* synthase, is acted upon by aconitase so that the double bond is formed between the central carbon atom and the methylene group derived from the methyl group of acetyl coenzyme A. Consequently C-4 of oxalacetate, derived from carbon dioxide in *C. kluyveri* (Stern, 1963, 1965), becomes the γ-carboxyl carbon, and C-1 of acetate the α-carboxyl carbon of glutamate.

When glutamate is formed from acetyl phosphate, [^{14}C]carbon dioxide, and suitable cofactors by *C. kluyveri* extracts incubated under hydrogen, most of the ^{14}C in glutamate is found in the γ-carboxyl group. However, a small fraction of the isotope, usually 10–15%, is present in the α-carboxyl group. Tomlinson (1954b) observed a similar ^{14}C distribution in glutamate formed by growing bacteria. The reaction responsible for the secondary labeling in the α-carboxyl group has not been identified. It is probably not caused by a second citrate synthase of opposite stereospecificity, since the degree of α-carboxyl labeling is considerably less when [4-^{14}C]oxalacetate is substituted for [^{14}C]carbon dioxide as a substrate. A slow exchange between carbon dioxide and the α-carboxyl group of α-ketoglutarate or oxalosuccinate could account for the observed labeling pattern.

It has been established that glutamate synthesis in animals, plants, and some aerobic microorganisms proceeds *via* the reactions of the tricarboxylic acid cycle (Davis, 1955; Wiame, 1957). Where the ^{14}C distribution of glutamate formed from specifically labeled substrates was determined, it was found to be in agreement with the stereochemical course of the tricarboxylic acid cycle [for references, see Hoare, 1963b]. Glutamate synthesis *via* oxalacetate, citrate, and α-ketoglutarate may be also the predominant reaction sequence for glutamate formation in many anaerobic bacteria. So far amino acid biosynthesis in other fermentative bacteria has not been investigated. Some information was reported about glutamate synthesis in photosynthetic sulfur bacteria. Fuller *et al.* (1961) and Trüper (1964) found that the enzymes necessary for glutamate synthesis from pyruvate *via* the tricarboxylic acid cycle are present in *Chromatium strain D* and *Chromatium okenii*. Smillie

and Evans (1963) reported the presence of citrate synthase and isocitric dehydrogenase in *Chlorobium thiosulfatophilum*. The path of glutamate synthesis by *Rhodospirillium rubrum* (Hoare, 1963a) is still unknown, but it cannot involve reactions of the tricarboxylic acid cycle. No results have been published which indicate the presence of a *C. kluyveri* type citrate synthase in other organisms.

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