Presence and Stereospecificity of Citrate Synthase in Anaerobic Bacteria*

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ABSTRACT: The citrate synthase of *Clostridium kluyveri* was shown previously to be different from the usual citrate synthase. When one of the substrates, acetyl-CoA or oxalacetate, is labeled with ^{14}C, the *C. kluyveri* enzyme forms the isotopic antipode of the [^{14}C]citrate synthesized by the usual synthase. Three other strictly anaerobic bacteria, *Clostridium acidi-urici*, *Clostridium cylindrosporum*, and *Desulfovibrio vulgaris*, have now been found to contain the *C. kluyveri* type of citrate synthase. This conclusion is based upon the following results. Cell-free extracts of these bacteria synthesize [1-^{14}C]glutamate from [1-^{14}C]acetyl phosphate, coenzyme A, and oxalacetate. [^{14}C]Citrate synthesized from acetyl-CoA and [4-^{14}C]oxalacetate and separately converted into [^{14}C]glutamate by the subsequent actions of aconitase, isocitric dehydrogenase, and glutamic dehydrogenase yields [5-^{14}C]glutamate, whereas the use of the usual citrate synthase leads to [1-^{14}C]-glutamate. [^{14}C]Citrate synthesized from acetyl-CoA and [4-^{14}C]oxalacetate is cleaved by citritase from *Streptococcus diacetilactis* into oxalacetate and [^{14}C]-acetate, whereas [^{14}C]citrate prepared by the usual citrate synthase is converted to [^{14}C]oxalacetate and acetate.

Clostridium pasteurianum, *Clostridium thermoaceticum*, *Methanobacillus omelianskii*, *Propionibacterium arabinosum*, and *Propionibacterium shermanii* probably contain the usual citrate synthase, since their cell-free extracts form [5-^{14}C]glutamate from [1-^{14}C]acetyl phosphate, coenzyme A, and oxalacetate.

The anaerobic bacterium *Clostridium kluyveri* has been shown to contain an atypical citrate synthase which forms [5-^{14}C]citrate from [1-^{14}C]acetyl-CoA[1] and oxalacetate, whereas the usual citrate synthase forms [1-^{14}C]citrate from these substrates (Gottschalk and Barker, 1966). This enzyme participates in the synthesis of glutamate by *C. kluyveri* and accounts for the unusual origin of the glutamate carbon atoms first observed by Tomlinson (1954) in experiments with whole cells. Glutamate is synthesized by a series of reactions starting with a reductive carboxylation of acetyl-CoA to pyruvate and a second carboxylation of pyruvate to oxalacetate; the latter reacts with a second molecule of acetyl-CoA to form citrate. Citrate is then converted into glutamate by the sequential action of aconitase, isocitric dehydrogenase, and glutamic dehydrogenase. This reaction sequence accounts for the observed origin of the glutamate carbon atoms. C-1 and C-3 are derived from the carboxyl carbon of acetate and C-5 originates from carbon dioxide.

We have extended this investigation by examining several other anaerobic bacteria for the presence of citrate synthase, aconitase and isocitric dehydrogenase and for the stereospecificity of the citrate-synthesizing enzyme. Three other anaerobic species have been found to contain the *C. kluyveri* type of citrate synthase.

Methods

Culture Methods. The following bacteria were grown in media described in the literature: *Butyribacterium limosus* ATCC 8486 and *B. rettgeri* ATCC 10825 (Kline and Barker, 1950); *Clostridium acidi-urici* 9a and *Clostridium cylindrosporum* HCl (Rabinowitz, 1963); *C. kluyveri* (Stadtman and Barker, 1949a); *Clostridium lactoacetophilum* (Bhat and Barker, 1947); *Clostridium pasteurianum* ATCC 6013 (Lovenberg *et al.*, 1963); *Clostridium* SB_4 (Costilow *et al.*, 1966); *Clostridium tetanomorphum* Hl (Barker *et al.*, 1959); *Clostridium thermoaceticum* (Lentz and Wood, 1955); *Desulfovibrio vulgaris* NCIB 8303 (medium N with citrate replaced by fumarate; Saunders *et al.*, 1964); *Methanobacillus omelianskii* Mb2 (Johns and Barker, 1960); *Propionibacterium arabinosum* ATCC 4965 and *Propionibacterium shermanii* ATCC 9614 (Perlman and Barrett, 1958); *Veillonella* O.B. (Johns, 1951); and *Streptococcus diacetilactis* DRCI (Harvey and Collins, 1961).

Preparation of Cell-Free Extracts. Frozen cells (0.2–0.5 g) were thawed in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 25 mM 2-mercaptoethanol. Cell suspensions of clostridia, *D. vulgaris*,

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[1] Abbreviations used: CoA, coenzyme A; NADP, nicotinamide–adenine dinucleotide phosphate; $NADH_2$, reduced nicotinamide–adenine dinucleotide; PTA, phosphotransacetylase; TCA cycle, tricarboxylic acid cycle.

TABLE I: Formation of [^{14}C]Glutamate from [1-^{14}C]Acetyl Phosphate, Coenzyme A, and Oxalacetate by Extracts of Anaerobic Bacteria, and ^{14}C Distribution in the Carboxyl Groups of the Isolated [^{14}C]Glutamate Samples.

Organism	Protein Added (mg)	Glutamate Isolated		Radioactivity in [^{14}C]Glutamate Degraded				
		Cpm × 10^{-2}	mμmoles	Total (cpm)	α-Carboxyl (cpm)	Carbon (%)	γ-Carboxyl (cpm)	Carbon (%)
C. acidi-urici	3.4	295	190	4220	4020	95	40	0.9
C. cylindrosporum	3.0	81	40	2540	2450	96	39	1.5
C. kluyveri	4.8	85	90	1510	1390	92	140	9.3
C. pasteurianum	4.8	583	200	5880	340	5.8	5200	88
C. tetanomorphum[a]	3.7	0	0	—	—	—	—	—
C. thermoaceticum	2.2	661	440	7850	69	8.8	6790	86
D. vulgaris	3.6	190	110	7180	6900	96	160	2.2
M. omelianskii	3.2	203	140	2020	170	8.4	1490	74
P. arabinosum	1.2	325	330	2370	20	0.8	2340	99
P. shermanii	1.0	460	460	5920	240	4.0	5420	92

[a] Extracts of *B. limosus*, *B. rettgeri*, *C. lactoacetophilum*, *Clostridium* SB_4, and *Veillonella* O.B. also formed no [^{14}C]glutamate.

M. omelianskii, and *Veillonella* O.B. were sonicated with a 60-w MSE (Measuring and Scientific Equipment, Ltd.) ultrasonic disintegrator for 30 sec. Cell suspensions of butyribacteria and of propionibacteria were sonicated for 2 min. Cell debris was removed by centrifugation at 20,000*g* for 20 min at 0–2°. The supernatant solution was passed through a column of Sephadex G-25 (1.5 × 15 cm) to remove low molecular weight compounds. Potassium phosphate buffer (pH 7.4) and 2-mercaptoethanol were added to final concentrations of 50 and 25 mM, respectively. The protein concentration determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard, was 3–8 mg of protein/ml.

Preparation of Citritase. A cell-free extract of *S. diacetilactis* was prepared by grinding 3 g of wet cells with 3 g of alumina (A-301, Alcoa Chemicals) in a cold mortar for 15 min. The protein was extracted with 5 ml of 50 mM Tris·HCl buffer (pH 7.3). The extract was treated with Dowex 1 (Cl) as described by Harvey and Collins (1963) and fractionated with protamine sulfate as described by Ward and Srere (1965). The resulting citritase preparation (6.9 mg) had a specific activity of 17.4 EU/mg of protein.

Paper Chromatography. The solvent systems 2-butanol–formic acid–water (67:11:22) and 1-propanol–concentrated ammonia–water (6:3:1) (Hirsch, 1963) were used for chromatography on Whatman No. 3MM paper. Radioactive spots were located by cutting the chromatograms in narrow strips, which were counted in Bray's (1960) scintillation fluid in a liquid scintillation spectrometer (Packard Tri-Carb Model 3214).

Enzymes and Chemicals. Glutamic dehydrogenase (3 units/mg), malic dehydrogenase (778 EU/mg), and phosphotransacetylase (2900 EU/mg) were preparations of Boehringer and Soehne, Mannheim (Germany). Glutamate–aspartate transaminase (170 Jenkins units/mg (Jenkins, 1962)) was generously donated by Dr. W. T. Jenkins. DL-[4-^{14}C]Aspartate was purchased from Volk Radiochemical Co., Chicago, and [1,5-^{14}C]citrate and [1-^{14}C]acetic anhydride from New England Nuclear Corp., Boston. Lithium [1-^{14}C]-acetyl phosphate was prepared by the method of Kornberg *et al.* (1956), except that an 1.6-fold excess of KH_2PO_4 over [1-^{14}C]acetic anhydride was used.

Determination of ^{14}C in the Carboxyl Groups of [^{14}C]Glutamate. The procedure employed was described previously (Gottschalk and Barker, 1966). The α-carboxyl group of glutamate was converted to CO_2 by the chloramine T method (Kemble and McPherson, 1954); the γ-carboxyl group was obtained as CO_2 by the fermentation of glutamate with a cell suspension of *C. tetanomorphum* (Wachsman and Barker, 1954).

Synthesis of [^{14}C]Glutamate from [1-^{14}C]Acetyl Phosphate, Coenzyme A, and Oxalacetate. Warburg vessels contained in a final volume of 2.0 ml: cell-free extract, 0.5–1.0 ml (protein concentrations are given in Table I); potassium phosphate buffer, pH 7.4, 50 mM; 2-mercaptoethanol, 25 mM; $MgCl_2$, 2 mM; ferrous ammonium sulfate, 0.5 mM; avidin (3.3 units/mg), 75 μg; phosphotransacetylase, 10 μg (main compartment); lithium [1-^{14}C]acetyl phosphate (1.0 × 2.9 × 10^5 cpm/μmole), 3 mM; CoA, 0.5 mM; NADP, 1 mM (side arm 1); and potassium oxalacetate, 5 mM (side arm 2). The vessels were shaken for 30 min under hydrogen. Then hydrogen was replaced by helium, and the reaction was started by tipping the contents of the side arms into the main compartment. After 60-min incubation at 30° (40° when extracts of *C. thermoaceticum* were used), the reaction was stopped by heating in a boiling water bath for 3 min. Protein was removed by centrifugation and 10 μmoles of NH_4Cl, 2 μmoles of $NADH_2$, and 50 μg of glutamic dehydrogenase were added to each sample to convert

any accumulated α-ketoglutarate to glutamate. After 5 min the glutamic dehydrogenase was inactivated by heat, 5 μmoles of carrier glutamate was added to each sample, and the solutions were applied to small columns (0.6 × 2.0 cm) of Dowex 50-12X (H^+), 200–400 mesh. The columns were washed with water and the glutamate was eluted with 0.2 M ammonia. When the glutamate-containing fractions were radioactive, they were concentrated and the glutamate was purified by paper chromatography in 2-butanol–formic acid and eluted. The ^{14}C distribution in the carboxyl groups of the isolated [^{14}C]glutamate was determined.

Synthesis of [^{14}C]*Citrate and* [α-^{14}C]*Ketoglutarate from* [*1-*^{14}C]*Acetyl Phosphate, Coenzyme A, and Oxalacetate.* The reaction mixture contained the same components as for [^{14}C]glutamate synthesis, except that NADP and ferrous ammonium sulfate were omitted. The specific activity of the [1-^{14}C]acetyl phosphate was 150,000 cpm/μmole for extracts of *C. acidi-urici* and *D. vulgaris* and 875,000 cpm/μmole for *C. cylindrosporum* extract. Citrate and α-ketoglutarate were isolated by chromatography of the samples on Dowex 1-8X formate, 200–400 mesh. After washing the column (0.6 × 3 cm) with 1 M HCOOH, both acids were eluted with 6 M HCOOH, concentrated, separated by paper chromatography in 2-butanol–formic acid, and eluted. α-Ketoglutarate (25,600 (21,300) cpm) and citrate (4000 (3400) cpm) were obtained with an extract of *C. acidi-urici* containing 6.8 mg of protein (*C. cylindrosporum*, 3.6 mg of protein). With *D. vulgaris* extract (3.6 mg of protein), citrate (69,000 cpm) was isolated. The [α-^{14}C]ketoglutarate samples were converted to glutamate as described above. The [^{14}C]citrate samples were converted into glutamate by an extract of *C. kluyveri* as reported previously (Gottschalk and Barker, 1966).

Synthesis of [^{14}C]*Citrate and* [α-^{14}C]*Ketoglutarate from* [*4-*^{14}C]*Aspartate, Acetyl Phosphate, and Coenzyme A.* The reaction mixture contained in a final volume of 2.0 ml: cell-free extract, 3.5–5 mg protein; potassium phosphate buffer, pH 7.4, 50 mM; 2-mercaptoethanol, 25 mM; $MgCl_2$, 2 mM; [4-^{14}C]aspartate (954,000 cpm/μmole), 2.5 mM; potassium α-ketoglutarate, 2.5 mM; lithium acetyl phosphate, 5 mM; CoA, 0.5 mM; avidin, 75 μg; phosphotransacetylase, 10 μg; and glutamate–aspartate transaminase, 50 μg. The experiments were carried out as described above. α-Ketoglutarate (116,000 (57,600) cpm) and citrate (18,000 (6300) cpm) were isolated using an extract of *C. acidi-urici* (*C. cylindrosporum*). With *D. vulgaris* extract, citrate (137,000 cpm) was obtained.

Cleavage of [^{14}C]*Citrate by Citritase.* The reaction mixture contained in a final volume of 1.0 ml: Tris·HCl buffer, pH 7.3, 50 mM; [^{14}C]citrate, approximately 0.2 mM; $MgCl_2$, 0.2 mM; $NADH_2$, 0.3 mM; malic dehydrogenase, 10 μg; and citritase, 200 μg. When the optical density at 340 mμ was constant, the sample was heated for 3 min at 100°. After centrifugation the supernatant solution was applied to a column, 8 mm in diameter, which contained 1.5 g of Dowex 1-8X formate, 200–400 mesh. Acetate, malate, and citrate were eluted with formic acid as indicated in Figure 1. Fractions of 1 ml were taken and 0.1 ml of each fraction was counted in a liquid scintillation spectrometer. Malate was identified by paper chromatography in two solvent systems, acetate by chromatography on Amberlite IRC 50 according to Seki (1958) and by gas chromatography.

Results

The formation of [^{14}C]glutamate from [1-^{14}C]acetyl phosphate and oxalacetate by extracts of a number of anaerobic bacteria was studied. Besides the substrates the reaction mixtures contained coenzyme A and phosphotransacetylase to synthesize [1-^{14}C]acetyl-CoA, NADP for the isocitric dehydrogenase reaction, and avidin to prevent the enzymatic decarboxylation of oxalacetate. Under the conditions used α-ketoglutarate can accumulate provided citrate synthase, aconitase and isocitric dehydrogenase are present and α-ketoglutarate dehydrogenase is absent in the bacterial extract. After stopping the reaction any accumulated α-ketoglutarate was converted to glutamate by adding purified glutamic dehydrogenase, $NADH_2$, and NH_4Cl. Data on the ^{14}C distribution in the carboxyl groups of the isolated glutamate are given in Table I.

With extracts of *Butyribacterium limosus*, *Butyribacterium rettgeri*, *C. lactoacetophilum*, *Clostridium* SB_4, *C. tetanomorphum*, and *Veillonella* O.B. no [^{14}C]-glutamate was formed, which indicates that these organisms lack one or more of the three enzymes necessary to synthesize α-ketoglutarate from oxalacetate and acetyl-CoA. Extracts of *C. acidi-urici*, *C. cylindrosporum*, *C. kluyveri*, *C. pasteurianum*, *C. thermoaceticum*, *D. vulgaris*, *M. omelianskii*, *P. arabinosum*, and *P. shermanii* formed [^{14}C]glutamate, indicating the presence of citrate synthase, aconitase, and isocitric dehydrogenase in these organisms. The ^{14}C distribution in the carboxyl groups of the isolated [^{14}C]glutamate samples showed that, like *C. kluyveri*, the two purine-fermenting clostridia, *C. acidi-urici* and *C. cylindrosporum*, and *D. vulgaris* formed [1-^{14}C]glutamate from [1-^{14}C]acetyl-CoA and oxalacetate. Extracts of the other bacteria studied incorporated the carboxyl group of acetyl-CoA largely into C-5 of glutamate, which is in agreement with the usual stereochemical course of the tricarboxylic acid cycle.

Citrate and α-ketoglutarate synthesis by extracts of *C. acidi-urici*, *C. cylindrosporum*, and *D. vulgaris* was further studied in order to determine whether the labeling pattern of these compounds is consistent with that of glutamate. Two ^{14}C-labeled substrates, [1-^{14}C]acetyl phosphate and [4-^{14}C]aspartate, were employed. In the first series of experiments [1-^{14}C]-acetyl phosphate, CoA, phosphotransacetylase, oxalacetate, and avidin were incubated with extracts of these organisms. The [^{14}C]citrate and [α-^{14}C]ketoglutarate that accumulated were isolated as described under Methods. Each compound was converted separately into glutamate. Citrate was first converted to α-keto-glutarate by the action of aconitase and isocitric dehy-

TABLE II: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Derived from [α-^{14}C]Ketoglutarate or [^{14}C]-Citrate Formed from [1-^{14}C]Acetyl Phosphate and Oxalacetate.

		α-Carboxyl Carbon			γ-Carboxyl Carbon		
		Glutamate Degraded	CO_2 Formed		Glutamate Degraded	CO_2 Formed	
Organism	Compd Isolated	(cpm)	Cpm	%	(cpm)	Cpm	%
C. acidi-urici	α-Ketoglutarate	3350	3200	96	2330	15	0.6
C. acidi-urici	Citrate	760	720	95	760	10	1.3
C. cylindrosporum	α-Ketoglutarate	2050	2030	99	1420	37	2.6
C. cylindrosporum	Citrate	1060	1040	98	620	14	2.3
D. vulgaris	Citrate	5340	5090	95	5340	100	1.8

TABLE III: ^{14}C Distribution in the Carboxyl Groups of [^{14}C]Glutamate Derived from [α-^{14}C]Ketoglutarate or [^{14}C]-Citrate Formed from [4-^{14}C]Aspartate and Acetyl Phosphate.

		α-Carboxyl Carbon			γ-Carboxyl Carbon		
		Glutamate Degraded	CO_2 Formed		Glutamate Degraded	CO_2 Formed	
Organism	Compd Isolated	(cpm)	Cpm	%	(cpm)	Cpm	%
C. acidi-urici	α-Ketoglutarate	7150	150	2.1	4010	3290	82
C. acidi-urici	Citrate	2440	20	0.8	1960	1600	82
C. cylindrosporum	α-Ketoglutarate	3420	580	17	3420	2700	79
C. cylindrosporum	Citrate	1310	290	22	780	600	77
D. vulgaris	Citrate	34700	150	0.4	34700	28450	82

drogenase present in extracts of *C. kluyveri;* α-ketoglutarate was converted to glutamate by means of glutamic dehydrogenase. In another series of experiments [4-^{14}C]oxalacetate generated from [4-^{14}C]aspartate and α-ketoglutarate by the action of glutamate–aspartate transaminase was allowed to react with acetyl-CoA in the presence of *C. acidi-urici, C. cylindrosporum,* or *D. vulgaris* extract to form [^{14}C]citrate and [α-^{14}C]ketoglutarate which were also isolated and converted into [^{14}C]glutamate. The ^{14}C distribution in the carboxyl groups of the resulting [^{14}C]glutamate samples is shown in Tables II and III. Using [1-^{14}C]-acetyl-CoA as substrate the [^{14}C]glutamate samples prepared from [^{14}C]citrate or [α-^{14}C]ketoglutarate were predominantly labeled in the α-carboxyl carbon. With [4-^{14}C]oxalacetate as labeled substrate the major part of the radioactivity was found in C-5 of the glutamate samples. However, with the *C. cylindrosporum* extract, a considerable percentage of the radioactivity, about 20%, was present in the α-carboxyl group. It is evident from these data that *C. acidi-urici, C. cylindrosporum,* and *D. vulgaris* contain the *C. kluyveri* type of citrate synthase. *C. cylindrosporum* may also contain the usual type of synthase. However, a more probable explanation of the greater randomization between the carboxyl groups in the experiment of Table III (and Table IV) as compared to those of Tables I and II is that the *C. cylindrosporum* cells used in the former experiments were slightly contaminated with an aerobic bacterium containing a high level of the normal synthase. Unfortunately, not every 20-l. culture of *C. cylindrosporum* was examined for contamination.

An independent method was employed to prove the configuration of [^{14}C]citrate synthesized from [4-^{14}C]-oxalacetate and acetyl-CoA by extracts containing the *C. kluyveri* type of citrate synthase. The citritase from *Escherichia coli* and *Streptococcus faecalis* has been shown to cleave citrate stereospecifically (Wheat and Ajl, 1955; Gillespie and Gunsalus, 1953). The action of this enzyme is such that [^{14}C]citrate synthesized from acetyl-CoA and [4-^{14}C]oxalacetate by the usual synthase is cleaved into acetate and [4-^{14}C]oxalacetate. Using the citrate synthase of the *C. kluyveri* type for [^{14}C]citrate synthesis from these substrates the cleavage products should be [1-^{14}C]acetate and oxalacetate. A partly purified citritase with a specific activity of 17.4 units/mg of protein was prepared from *S. diacetilactis* (Harvey and Collins, 1963). [1,5-^{14}C]Citrate and [^{14}C]-citrate samples prepared from [4-^{14}C]oxalacetate and acetyl-CoA by extracts of bakers' yeast, *C. acidi-urici, C. cylindrosporum, C. kluyveri, C. pasteurianum,* or *D. vulgaris* were cleaved by the citritase preparation

TABLE IV: Cleavage of Various [^{14}C]Citrate Samples by Citritase.[a]

Source of Citrate	Radioactivity in Reaction Products: Acetate Cpm × 10^{-2}	Acetate % [b]	Malate Cpm × 10^{-2}	Malate % [b]
[1,5-^{14}C]Citrate	148	50.7	145	49.3
Bakers' yeast	8	0.4	1942	99.6
C. acidi-urici	1190	99.0	12	1.0
C. cylindrosporum	98	92.4	8	7.6
C. kluyveri	1020	87.6	145	12.4
C. pasteurianum	8	1.0	817	99.0
D. vulgaris	1255	99.6	5	0.4

[a] The [^{14}C]citrate samples (except [1,5-^{14}C]citrate) were prepared from [4-^{14}C]oxalacetate and acetyl-CoA using extracts of the indicated organisms. Conditions for the conversion of citrate to acetate and malate and the isolation of these acids are described under Methods. [b] The percentages are based on the total radioactivity in the two products.

in the presence of malic dehydrogenase and $NADH_2$. The reaction products, acetate and malate, and the remaining citrate, which contains some isocitrate, were separated on a Dowex 1 column (formate).

Figure 1A–C shows the ^{14}C distribution in the reaction products for the cleavage of three [^{14}C]citrate samples. Using [1,5-^{14}C]citrate as substrate equal amounts of the radioactivity are present in acetate and malate (Figure 1B). [^{14}C]Citrate prepared from [4-^{14}C]oxalacetate and acetyl-CoA by bakers' yeast is cleaved into acetate and [^{14}C]malate (Figure 1A), whereas [^{14}C]citrate synthesized from the same substrates by an extract of *C. acidi-urici* is cleaved into [^{14}C]acetate and malate (Figure 1C). At least 99% of the radioactivity of the cleaved [^{14}C]citrate is present in acetate. Table IV presents quantitative data on the results of the cleavage of these and other [^{14}C]citrate samples by citritase. [^{14}C]Citrate prepared by extracts of *C. acidi-urici*, *C. cylindrosporum*, *C. kluyveri*, and *D. vulgaris* is converted mainly to [^{14}C]acetate and unlabeled malate, although with *C. kluyveri* extract about 12% and with *C. cylindrosporum* about 8% of the ^{14}C was present in malate. [^{14}C]Citrate prepared with an extract of *C. pasteurianum* or bakers' yeast is converted almost exclusively (99%) to [^{14}C]malate and unlabeled acetate. These results confirm the conclusions concerning the distribution of isotope in these samples of [^{14}C]citrate, reached by other methods.

Table V presents data which demonstrate that acetyl-CoA and oxalacetate are the substrates for citrate synthesis by *C. kluyveri*, *C. acidi-urici*, and *D. vulgaris*. The amount of ^{14}C found in nonvolatile organic acids decreases markedly when coenzyme A or oxalacetate is omitted from the complete system. When coenzyme A and phosphotransacetylase are omitted, the replacement of [^{14}C]acetyl phosphate by [^{14}C]acetate causes a further decline in activity with extracts of *C. acidi-urici* and *D. vulgaris*. In the complete systems the reaction

TABLE V: Requirements for Citrate Synthesis.[a]

Components Added or Omitted	Nonvolatile Radioactivity: *C. acidi-urici* Cpm × 10^{-3}	*C. acidi-urici* mμmoles	*C. kluyveri* Cpm × 10^{-3}	*C. kluyveri* mμmoles	*D. vulgaris* Cpm × 10^{-3}	*D. vulgaris* mμmoles
None	35	233	11	72	117	776
−CoA; −PTA	4.4	29	1.9	13	14	81
−Oxalacetate	0.7	5	2.3	16	2.6	17
−CoA; −PTA; −[1-^{14}C]Acetyl phosphate; +[1-^{14}C]Acetate	2.0	8	3.6	14	16	66

[a] The cell-free extracts contained 4.2 mg of protein/ml (*C. acidi-urici*), 6.4 mg/ml (*C. kluyveri*), and 3.1 mg/ml (*D. vulgaris*). The complete systems contained in a final volume of 1.0 ml: cell-free extract, 0.25 ml; potassium phosphate buffer, pH 7.4, 50 mM; $MgCl_2$, 2 mM; 2-mercaptoethanol, 25 mM; [1-^{14}C]acetyl phosphate (150,000 cpm/μmole), 5 mM; CoA, 0.5 mM; oxalacetate, 5 mM; PTA, 10 μg; and avidin, 75 μg. The [1-^{14}C]acetate (5 mM) had a specific activity of 242,000 cpm/μmole. The reaction mixture was incubated under helium for 60 min at 30°. The reaction was stopped by heating for 3 min at 100°. After centrifugation, the remaining acetyl-CoA and acetyl phosphate were hydrolyzed by the addition of 50 μmoles of potassium arsenate buffer, pH 7.5, and 10 μg of PTA. After incubation for 10 min at 30° 150 μmoles of HCl and 250 μmoles of acetic acid were added and the solution was evaporated to dryness twice to remove residual [^{14}C]acetate. The remaining material was dissolved in 2.0 ml of water and an aliquot was counted.

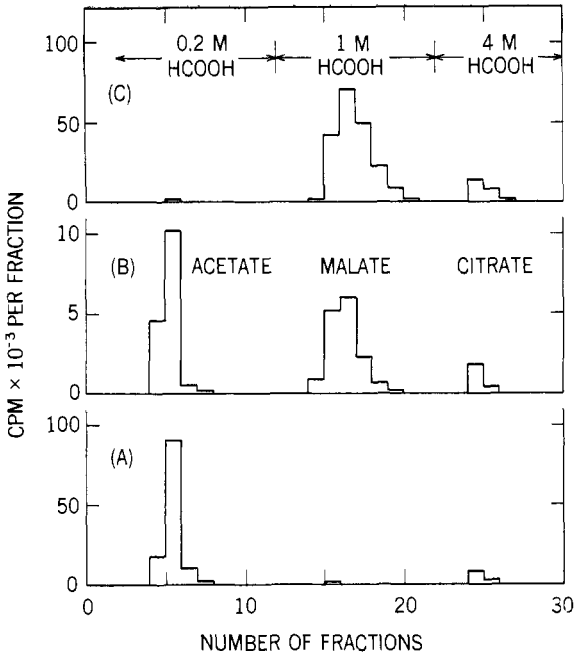

FIGURE 1: ^{14}C distribution in the reaction products of [^{14}C]citrate cleavage by citritase. (A) The [^{14}C]citrate used as substrate was prepared from [4-^{14}C]oxalacetate (derived from [4-^{14}C]aspartate, 1 $\mu c/\mu$mole) and acetyl-CoA by an extract of bakers' yeast (see Methods); 1 μ-mole of citrate was added to the reaction mixture to increase the accumulating [^{14}C]citrate. (B) [1,5-^{14}C]-Citrate was used as substrate. (C) [^{14}C]Citrate prepared from [4-^{14}C]aspartate and acetyl-CoA by an extract of of *C. acidi-urici* was cleaved.

products have been found to be mainly citrate and α-ketoglutarate by paper chromatography in 2-butanol–formic acid. The specific activity of the citrate synthase was calculated from this experiment to be 3.7, 0.7, and 16.1 μmoles/min per g of protein for extracts of *C. acidi-urici*, *C. kluyveri*, and *D. vulgaris*, respectively. The very low citrate synthase activity of *C. cylindrosporum* was not determined.

Discussion

It is well established for many strict and facultative aerobic organisms, that glutamate is formed from oxalacetate and acetyl-CoA by reactions of the tricarboxylic acid cycle (Umbarger and Davis, 1962). The results of this investigation show that several anaerobic bacteria use the same sequence of reactions for glutamate synthesis. Extracts of *C. pasteurianum*, *C. thermoaceticum*, *M. omelianskii*, *P. arabinosum*, and *P. shermanii* catalyze the formation of [5-^{14}C]-glutamate from [1-^{14}C]acetyl phosphate, coenzyme A, and oxalacetate. This is consistent with the synthesis of glutamate *via* the tricarboxylic acid cycle. Although the participating enzymes have not been demonstrated in this study, it is likely that these organisms synthesize at least part of their glutamate *via* citrate. The presence of citrate synthase, aconitase, isocitric dehydrogenase, and glutamic dehydrogenase in *C. thermoaceticum* has been shown recently by Stern (1966). He found that the citrate synthase of *C. thermoaceticum* acts in the same stereospecific way as the pig heart enzyme, which is confirmed by the presented data. That extracts of *C. pasteurianum* contain the same type of citrate synthase follows from the cleavage of [^{14}C]citrate, synthesized from [4-^{14}C]oxalacetate and acetyl-CoA by the action of citritase of *S. diacetylactis*, to ^{14}C-oxalacetate and acetate.

Glutamate synthesis by *M. omelianskii* was studied recently by Knight *et al.* (1966). When radioactive carbon dioxide was added to the growth medium and glutamate was isolated from the protein hydrolyzate of the cells, two-thirds of the glutamate radioactivity was found in the two carboxyl groups and one-third in C-2 and C-4. These findings suggest the existence of another path for glutamate synthesis in *M. omelianskii* in addition to the tricarboxylic acid cycle, since the incorporation of $^{14}CO_2$ into the carboxyl group of acetate is excluded (Stadtman and Barker, 1949b). In another strict anaerobe, *Peptostreptococcus elsdenii*, the presence of the necessary enzymes for glutamate synthesis from oxalacetate and acetyl-CoA by the reactions of the tricarboxylic acid cycle has been demonstrated (Somerville, 1965). The data obtained in tracer experiments are consistent with the presence of the usual type of citrate synthase in this organism (Somerville and Peel, 1964).

Besides the group of anaerobes which contains the usual type of citrate synthase there is a second group containing the atypical citrate synthase first observed in *C. kluyveri* (Gottschalk and Barker, 1966). The presence of this enzyme in *C. acidi-urici*, *C. cylindrosporum*, and *D. vulgaris* was suggested by the observation that extracts of these organisms form [1-^{14}C]glutamate from [1-^{14}C]acetyl phosphate, coenzyme A, and oxalacetate. In order to confirm this, [^{14}C]citrate was prepared from [4-^{14}C]oxalacetate and acetyl-CoA by cell-free extracts. The enzymic conversion of these [^{14}C]citrate samples into glutamate by the usual tricarboxylic acid cycle enzymes yielded [5-^{14}C]glutamate. The cleavage by citritase led to oxalacetate and [^{14}C]-acetate. The tracer experiments carried out with the two citrate synthases are summarized in Figure 2.

The action of citritase on [^{14}C]citrate provides a convenient method for determining the position of the radioactive carbon in citrate. Control experiments with an extract of bakers' yeast as the source of citrate synthase showed that both citrate synthesis and citrate cleavage by citritase is at least 99% stereospecific. The results with [^{14}C]citrate samples prepared by extracts of *C. acidi-urici* and *D. vulgaris* demonstrate that these organisms contain exclusively the atypical citrate synthase. [^{14}C]Citrate prepared with *C. cylindrosporum* and *C. kluyveri* extracts showed some cross-labeling in the cleavage products, ranging from 8 to 12%. With *C. cylindrosporum* it is probable and with *C. kluyveri* possible that this cross-labeling is due to

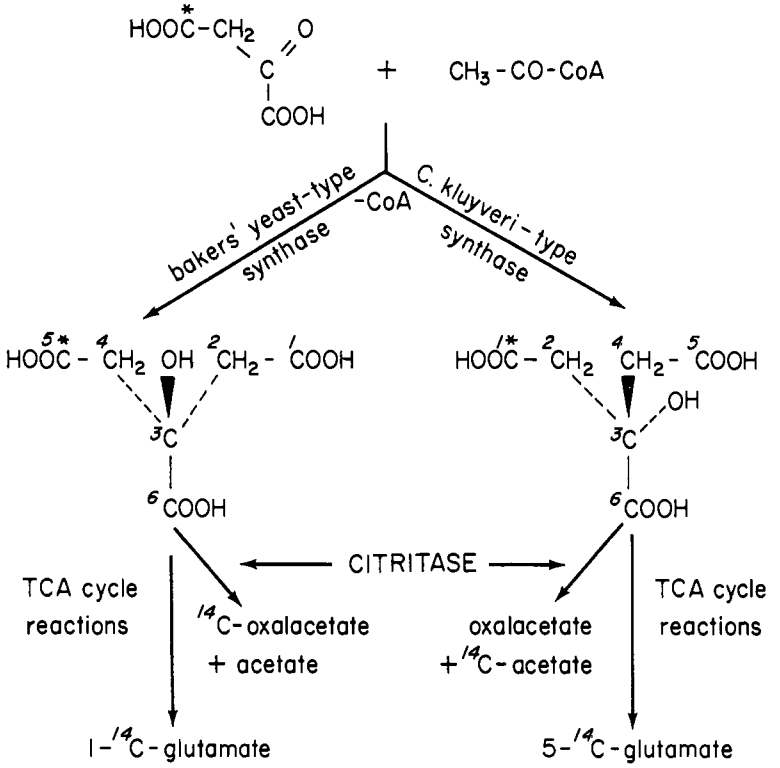

FIGURE 2: Reactions involved in the formation and degradation of [^{14}C]citrate.

contamination of the large (20 l.) cultures with small numbers of bacteria containing a high level of the usual citrate synthase activity. Another possibility with *C. kluyveri* is that the pure cultures contain a second strain which possesses the usual citrate synthase. The results of Stern *et al.* (1966), who found only the normal citrate synthase in commercially grown *C. kluyveri*, point to the latter possibility. This interpretation is also supported by the fact that Tomlinson (1954) observed some cross-labeling in the glutamate isolated from whole cells grown in pure culture.

Extracts of *B. limosus*, *B. rettgeri*, *C. lactoacetophilum*, *C.* SB_4, *C. tetanomorphum*, and *Veillonella* O.B. are apparently not able to form glutamate from acetyl-CoA and oxalacetate. The fact that some of these bacteria were grown on fairly complex media might be the reason for this result. It is possible that at least some of these species could form the necessary enzymes for glutamate synthesis if suitable synthetic media were available for their growth. These bacteria may also make use of other pathways for glutamate synthesis, for example, the reductive carboxylation of succinyl-CoA to α-ketoglutarate (Buchanan and Evans, 1965).

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Reversible Aggregation of Acetylcholinesterase. II. Interdependence of pH and Ionic Strength*

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ABSTRACT: The reversible aggregation of acetylcholinesterase, extracted from the electroplax of *Electrophorus electricus*, was investigated in regard to the effects of pH (4.0–10.0) and ionic strength (0.1–0.5) using the sucrose gradient centrifugation technique. In ionic strengths below 0.3, at neutral pH, the enzyme reversibly aggregates into a polydisperse, rapidly sedimenting molecular form (fast components) which is in equilibrium with the slower form (slow components). In 0.1 ionic strength, at pH 5.0 and below, the fast components disappear and very rapidly sedimenting enzymatic material (pellet material) is formed and recovered in the bottom of the gradient. A study of the interaction of pH and ionic strength on the aggregation process indicates a dominant ionic effect, a delicate balance between these agents at pH 5.0, and the electrostatic nature of the regulating forces.

The complexities inherent in the observed aggregation, particularly the question of heterogeneity, are discussed. A possible participation of acetylcholinesterase, commensurate with its observed physicochemical properties, in the macromolecular complex involved in the permeability cycle of the electrogenic membrane is suggested.

The kinetic and mechanistic aspects of acetylcholinesterase (acetylcholine acyl-hydrolase, EC 3.1.1.7) extracted from the electroplax of *Electrophorus electricus* have been investigated in great detail. Consequently, today, there is a wealth of data from which some of the details of the enzymatic active site have been deduced (Wilson, 1954; Friess and McCarville, 1954; Krupka and Laidler, 1961). Information describing its macromolecular properties is, by comparison, scarce, and in some cases contradictory. For instance, the sedimentation coefficient (estimated by schlieren optics) of the enzyme has been reported to be 4 (Hargreaves *et al.*, 1963), 109 and 10.8 (Lawler, 1963), and 10.8 S (Kremzner and Wilson, 1964). Since the specific activities (in millimoles of acetylcholine hydrolyzed per milligram of protein) of the enzyme preparations used were, respectively, 66, 425, and 660, part of the discrepancies might be attributed to interference by contaminating proteins in the detection of the

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