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Glutamate Biosynthesis in Anaerobic Bacteria. I. The Citrate Pathways of Glutamate Synthesis in *Clostridium kluyveri**

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ABSTRACT: Extracts of *Clostridium kluyveri* possess the enzymes of the upper half of the citric acid cycle—citrate synthetase, aconitase, *d*-isocitrate dehydrogenase—and L-glutamate dehydrogenase. They are, therefore, able to synthesize glutamate from citrate and precursors of citrate such as acetate, pyruvate, and oxalacetate. These extracts also contain pyruvate carboxylase, oxalacetate decarboxylase, an L-malate–diphosphopyridine nucleotide (DPN) dehydrogenase, and an L-malate–triphosphopyridine nucleotide (TPN) oxidodecarboxylase. The following citric acid cycle enzymes could not be detected in the extracts: α-ketoglutarate dehydrogenase, succinate dehydrogenase, and succinyl-CoA synthetase. Trace amounts of fumarase are present. Enzymes of the glutaconate and citramalate pathways of glutamate biosynthesis are absent.

Tomlinson (1954a) described an unusual distribution of ^{14}C in the glutamate isolated from cells of *Clostridium kluyveri* grown in a synthetic medium that contained $^{14}CO_2$ or [1-^{14}C]acetate. The label was found mostly but not exclusively in the opposite carboxyl of glutamate to that predicted by the operation of the Krebs citric acid cycle. Tomlinson hypothesized that either an unusual stereospecificity of aconitase or a C-4 + C-1 addition reaction could account for his results. Although nothing was known about the occurrence of a citric acid cycle in *C. kluyveri*, it had been reported (Ochoa *et al.*, 1951) that another strict anaerobe, *C. butylicum*, had no citrate synthetase. More recently a C-4 + C-1 addition reaction, namely, the carboxylation of crotonyl-CoA to glutaconyl-CoA, has been described in rat liver (Tustanoff and Stern, 1960), which could account for Tomlinson's data.

This paper describes the occurrence in *C. kluyveri* of the enzymes of the citrate pathway of glutamate biosynthesis—citrate synthetase, aconitase, isocitrate, and glutamate dehydrogenases—as well as certain other enzymes, and the absence of crotonyl-CoA carboxylase and the glutaconate pathway. The succeeding paper describes the stereospecificity of the citrate synthetase and aconitase enzymes.

Experimental Section

Materials. Washed, frozen cells of *C. kluyveri* were obtained from the Worthington Biochemical Corp. These cells were derived from an original Barker strain that Worthington has maintained on sterile solid medium over a period of 12 years. Large scale cultures were grown as described by Stadtman (1955) using sterile media for inocula up to 500 ml, and then nonsterile media of 12 and 208 l. All dehydrogenases were products of C. Boehringer and Co. L-Glutamate decarboxylase from *Escherichia coli* was purchased from Worthington Biochemical Corp. Chemicals used were DL-β-methylaspartic acid (Sigma Chemical Co.), glutaconic acid (K and K Laboratories), DL-β-hydroxyglutarate (Farchan Laboratories), DL-isocitric lactone (Calbiochem), and oxalacetic acid (Sigma Chemical Co.); sodium L-α-hydroxyglutarate was a gift of Dr. H. A. Krebs. The lactone was hydrolyzed prior to use.

Methods. C. kluyveri cells were suspended in 4 volumes (per gram frozen weight) of 0.2 M Tris–HCl buffer, pH 7.4, that contained 10^{-3} M potassium L-cysteine, and sonicated for 8 min in a Raytheon 10 KC sonifier at

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2–5°. Mercaptoethanol was added to a final concentration of 0.02 M and the suspension was spun for 45 min at 25,000*g*. The clear, green supernatant was used in all experiments. Citrate was determined chemically (Stern, 1957). *d*-Isocitrate (*threo*-D_s-isocitrate), oxalacetate, pyruvate, and α-ketoglutarate were determined spectrophotometrically with the appropriate dehydrogenase. In the presence of excess glutamate, α-ketoglutarate was determined chemically (Friedemann and Haugen, 1942). Glutamate was determined manometrically with the specific L-glutamate decarboxylase. L-Malate was determined with a highly purified L-malate–TPN[1] enzyme prepared in this laboratory.

Results

Citrate Synthesis. The capacity of *C. kluyveri* extracts to synthesize citrate was discovered when it was observed that, in the presence of CoA, extracts catalyzed a greater disappearance of oxalacetate than could be accounted for by simple enzymatic decarboxylation to pyruvate (Table I). Since these extracts had been shown to catalyze the oxidation of pyruvate to acetyl-CoA (Stern, 1963a, 1965), it was logical to ascertain whether the deficit in oxalacetate recovery was accounted for by citrate formation. This proved to be the case. Citrate synthesis from oxalacetate was increased by addition of pyruvate and especially by generating acetyl-CoA from acetyl phosphate and CoA *via* endogenous transacetylase (Stadtman, 1952).

TABLE I: Conversion of Oxalacetate to Pyruvate and Citrate.

Additions[a]	Oxalacetate	Pyruvate	Citrate[b]
Pyruvate	...	−3.50	0
Oxalacetate	−5.50	+2.14	+1.95
Oxalacetate + pyruvate	−6.10	+1.90	+2.82
Oxalacetate + acetyl phosphate + CoA	−10.6	+2.05	+5.65

[a] The reaction mixtures contained: Tris–HCl buffer, pH 7.0, 100 μmoles; $MgCl_2$, 4 μmoles; *C. kluyveri* extract, 6.5 mg; and, where indicated, potassium pyruvate, 9.7 μmoles; oxalacetate, 17.4 μmoles; acetyl phosphate, 25 μmoles; and CoA, 1 mg; final volume, 1.0 ml; incubation, 30 min at 30° in air. [b] All values are net change in micromoles.

[1] Abbreviations: TPN, triphosphopyridine nucleotide; TTZ and TTZH, oxidized and reduced triphenyltetrazolium chloride; SC, semicarbazide; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TPNH, reduced TPN; DPNH, reduced DPN.

TABLE II: Synthesis of Citrate from Malate or Oxalacetate or Pyruvate and Bicarbonate.

Additions[a]	Citrate (μmoles)	*d*-Isocitrate (μmole)
Oxalacetate, CoA	1.38	...
Oxalacetate, CoA, acetyl phosphate	7.25	0.86
Pyruvate, CoA, acetyl phosphate, ATP, and bicarbonate	1.97	...
L-Malate, CoA, DPN	0	...
L-Malate, CoA, DPN, acetyl phosphate	3.41	0.31
L-Malate, CoA, acetyl phosphate	0.92	...

[a] The reaction mixtures contained as indicated: Tris–HCl buffer, pH 8.0, 100 μmoles; $MgCl_2$, 4 μmoles; potassium oxalacetate, 20 μmoles; acetyl phosphate, 20 μmoles; potassium pyruvate, 20 μmoles; potassium L-malate, 25 μmoles; CoA, 0.5 mg; ATP, 5 μmoles; DPN, 0.2 mg; potassium bicarbonate, 20 μmoles; and *C. kluyveri* extract, 5.5 mg; volume, 1.0 ml; incubation, 30 min at 30° under hydrogen gas.

As shown in Table II, the extracts also synthesized citrate from L-malate, DPN, acetyl phosphate, and CoA demonstrating the presence of L-malate dehydrogenase and the coupling of reactions 1–3

$$\text{L-malate} + \text{DPN}^+ \rightleftharpoons \text{oxalacetate} + \text{DPNH} + \text{H}^+ \quad (1)$$

$$\text{acetyl phosphate} + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + \text{phosphate} \quad (2)$$

$$\text{oxalacetate} + \text{acetyl-CoA} + \text{H}_2\text{O} \rightleftharpoons \text{citrate} + \text{CoASH} \quad (3)$$

catalyzed by malate dehydrogenase, transacetylase, and citrate synthetase, respectively. *C. kluyveri* extracts have been shown (Stern, 1963a, 1965) to contain the pyruvate carboxylase first described by Keech and Utter (1963) which catalyzes reaction 4. Thus, as seen in Table II,

$$\text{pyruvate} + \text{ATP} + \text{CO}_2 \rightleftharpoons \text{oxalacetate} + \text{ADP} + \text{P}_i \quad (4)$$

the extracts also synthesize appreciable amounts of citrate from pyruvate, ATP, CO_2, acetyl phosphate, and CoA through coupling of reactions 4, 2, and 3.

The citrate synthetase activity of extracts was found to vary considerably from batch to batch of harvested

cells. Rates of citrate synthesis from oxalacetate, acetyl phosphate, and CoA ranged from 0.033 to 1.32 μmoles/mg of protein per 30 min at 30°. Most batches fell in the lower range of activity. In contrast, the activity of aconitase and the dehydrogenases mentioned below varied over a 2- to 4-fold range. The reason for this variation is not completely clear; but certainly it was essential to add a mercaptan either before or soon after sonication of the cells to preserve citrate synthetase activity of the extract. Maintaining the extract under reducing conditions (hydrogen gas) during frozen storage and experimental use also helped preserve the activity of this and other enzymes. It is possible that the time of harvesting may affect apparent citrate synthetase activity of the cells.

Aconitase. The presence of aconitase in *C. kluyveri* extract became evident when it was observed that *d*-isocitrate, as well as citrate, accumulated in the presence of added, or generated, oxalacetate, and acetyl-CoA (Table II). Table III demonstrates the interconversion of the three tricarboxylic acids, citrate, *cis*-aconitate, and *d*-isocitrate, by *C. kluyveri* extract. This experiment was performed in air before it was appreciated that reducing conditions had marked stabilizing and/or activating influences on aconitase and other enzymes in the extract. As a result, some enzyme inactivation occurred during the experiment. It is seen that the rate of conversion of *d*-isocitrate to citrate is greater than that of citrate to *d*-isocitrate, whereas the rates of conversion of *cis*-aconitate to citrate and to *d*-isocitrate are approximately equal. These kinetic parameters are the same as those found with pig heart aconitase (Dickman, 1961). Table IV shows the time course and stoichiometry of the conversion of *d*-isocitrate to citrate (and α-ketoglutarate) catalyzed by *C. kluyveri* extract under hydrogen gas. Although there is also a very slow oxidation of *d*-isocitrate by endogenous *d*-isocitrate dehydrogenase and TPN, it is seen that in 20 min an equilibrium is attained when citrate constitutes 87.4% of the total tricarboxylic acid. The equilibrium is then progressively and slowly displaced by the contaminating *d*-isocitrate dehydrogenase reaction. This equilibrium value is comparable to that of pig heart aconitase for which Krebs (1953) determined that citrate comprised 90.9% of the total tricarboxylic acid at pH 7.4 and 25°.

Isocitric Dehydrogenase. Table V shows that *C. kluyveri* extracts also catalyze the TPN-dependent oxidation of *d*-isocitrate to α-ketoglutarate. Mg^{2+} and the carbonyl trapping agent, semicarbazide, further diminished *d*-isocitrate conversion to citrate by the

TABLE III: Interconversion of Tricarboxylic Acids.

Additions[a]	Citrate[b]	*cis*-Aconitate	*d*-Isocitrate
Citrate, 17.7[b]	−3.50	+2.76	+0.74
cis-Aconitate, 5.0	+1.74	−3.26	+1.52
d-Isocitrate, 2.10	+1.02	+0.14	−1.16

[a] The reaction mixtures contained: Tris–HCl buffer, pH 8.0, 100 μmoles; $MgCl_2$ 4 μmoles; tricarboxylic acid as indicated and *C. kluyveri* extract, 5.0 mg; volume, 1.0 ml; incubation, 60 min at 30° in air; citrate determined chemically; isocitrate with *d*-isocitrate dehydrogenase; and *cis*-aconitate by difference. [b] All values are in micromoles.

TABLE IV: Stoichiometry of Conversion of Isocitrate to Citrate and α-Ketoglutarate.

Time (min)	*d*-Isocitrate[a] Recovered (μmoles)	*d*-Isocitrate[a] −Δ (μmoles)	Citrate (μmoles)	α-Ketoglutarate (μmole)
0	2.54	0	0	0
10	0.93	1.61	1.31	0.16
20	0.20	2.34	2.06	0.18
40	0.10	2.44	2.02	0.30
80	0.13	2.41	...	0.41
120	0.08	2.46	1.95	0.63

[a] The reaction mixture contained: Tris–HCl buffer, pH 8.0, 100 μmoles; potassium *dl*-isocitrate, 5.10 μmoles; and *C. kluyveri* extract, 8.8 mg; volume, 1.0 ml; incubated at 30° under hydrogen gas.

TABLE V: Synthesis of α-Ketoglutarate and Citrate from Isocitrate.

Additions[a]	Citrate (μmoles)	α-Ketoglutarate (μmoles)	*d*-Isocitrate[b] (μmole)
Isocitrate	5.28	0	...
Isocitrate + TPN	3.16	1.53	0.51
Isocitrate + TPN + Mg	2.15	0.95	0.20
Isocitrate + TPN + Mg + SC	1.96	1.08	0.12
TPN + Mg	0	0	0

[a] The complete reaction mixture contained: Tris–HCl buffer, pH 8.0, 100 μmoles; $MgCl_2$, 4 μmoles; *dl*-isocitrate, 10.2 μmoles; TPN, 0.8 mg; semicarbazide (SC), 50 μmoles; and *C. kluyveri* extract, 8.2 mg; volume, 1.0 ml; incubation, 30 min at 30° under hydrogen gas. [b] Amount recovered.

competing aconitase reaction and stimulated *d*-isocitrate metabolism presumably *via* the dehydrogenase reaction. However, less α-ketoglutarate accumulated in the presence of Mg^{2+} with or without semicarbazide. This could be accounted for by an increased dismutation of α-ketoglutarate to glutamate (see below). The *d*-isocitric dehydrogenase was specific for TPN and did not react with DPN. No cleavage of *d*-isocitrate to glyoxylate and succinate was detectable in these extracts. Andrew and Morris (1965) have also noted the absence of isocitratase in another strain of *C. kluyveri*.

Glutamate Dehydrogenase. The reductive amination of α-keto glutarate to L-glutamate is demonstrated in Table VI, when DPN is used as cofactor. In an atmosphere of helium, there is an endogenous source of DPN reduction and hence some glutamate is formed in the absence of an added hydrogen donor. Addition of ethanol alone has little effect. Addition of ethanol plus DPN increases glutamate synthesis through coupling of the DPN-linked alcohol dehydrogenase with L-glutamate dehydrogenase. Hydrogen gas has long been known to effect DPN reduction in *C. kluyveri* (Korkes, 1955) *via* a hydrogenase, and, as shown, hydrogen gas promotes the reductive amination of α-ketoglutarate. Under hydrogen, alcohol dehydrogenase is more active than hydrogenase in reducing DPN. Table VII shows that maximum rates of glutamate synthesis from α-ketoglutarate with hydrogen or ethanol as reductant require addition of ammonium ion and DPN or TPN. It is apparent that in the untreated extract ammonium ion is limiting and DPN (or TPN) less so. Thus *C. kluyveri* L-glutamate dehydrogenase reacts well with either DPNH or TPNH. Tests also showed that both DPN^+ and TPN^+ were active in catalyzing the oxidation of L-glutamate.

TABLE VI: Reductive Amination of α-Keto glutarate to L-Glutamate by Ethanol and Hydrogen.

Additions[a]	L-Glutamate	
	Hydrogen (μmoles)	Helium (μmoles)
α-Ketoglutarate, NH_4Cl, ethanol	1.45	0.39
α-Ketoglutarate, NH_4Cl, ethanol, DPN	3.92	1.06
α-Ketoglutarate, NH_4Cl, DPN	1.84	0.41

[a] The reaction mixtures contained: Tris–HCl buffer, pH 7.0, 100 μmoles; potassium α-ketoglutarate, 25 μmoles; NH_4Cl, 25 μmoles; DPN, 0.4 mg; ethanol, 25 μmoles; and *C. kluyveri* extract, 8.8 mg; volume, 1.0 ml; incubation, 60 min at 30° under hydrogen or helium gas.

TABLE VII: Requirements for Reductive Amination of α-Ketoglutarate to L-Glutamate by Ethanol and Hydrogen.

Additions[a]	L-Glutamate (μmoles)
NH_3, DPN	0.12
α-Ketoglutarate, NH_3	1.57
α-Ketoglutarate, NH_3, DPN	1.92
α-Ketoglutarate, NH_3, TPN	2.09
α-Ketoglutarate, NH_3, DPN, ethanol	3.74
α-Ketoglutarate, NH_3, ethanol	1.74
α-Ketoglutarate, DPN, ethanol	1.00

[a] The complete reaction mixture contained: Tris–HCl buffer, pH 7.0, 100 μmoles; potassium α-ketoglutarate, 25 μmoles; DPN or TPN, 0.4 mg; NH_4Cl, 25 μmoles; ethanol, 25 μmoles; and *C. kluyveri* extract, 5.5 mg; volume, 1.0 ml; incubation, 60 min at 30° under hydrogen gas.

Conversion of Tricarboxylic Acids to L-Glutamate. The conversion of citrate and *d*-isocitrate, in the presence of TPN and NH_4Cl, to L-glutamate is demonstrated in Table VIII. The pathway is obviously *via* aconitase, *d*-isocitrate, and L-glutamate dehydrogenases, as follows

TABLE VIII: Glutamate Synthesis from Citrate and Isocitrate.

Additions[a]	L-Glutamate (μmoles)
— TPN, NH_4Cl	0.12
Citrate, TPN, NH_4Cl	1.20
Isocitrate, TPN, NH_4Cl	2.44

[a] The complete system contained: Tris–HCl buffer, pH 7.0, 100 μmoles; $MgCl_2$, 4 μmoles; potassium citrate, 10 μmoles, or potassium *dl*-isocitrate, 10 μmoles; TPN, 0.4 mg; NH_4Cl, 10 μmoles; and *C. kluyveri* extract 8.2 mg; volume, 1.0 ml; incubation, 60 min at 30° under hydrogen gas.

$$\text{citrate} \rightleftharpoons \text{isocitrate} \quad (5)$$

$$\text{isocitrate} + TPN^+ \rightleftharpoons \alpha\text{-ketoglutarate} + TPNH + H^+ \quad (6)$$

$$\alpha\text{-ketoglutarate} + TPNH + H^+ + NH_4Cl \rightleftharpoons \text{glutamate} + TPN^+ \quad (7)$$

Activation of Enzymes by Hydrogen Gas. The various dehydrogenase reactions could be measured by coupling each to TTZ reduction in the presence of the appropriate pyridine nucleotide (Table IX). The extracts contained a flavoprotein enzyme(s) that catalyzed electron transport from DPNH or TPNH to TTZ. Using this test, the activity of aconitase, fumarase, L-glutamate, *d*-isocitrate, and L-malate dehydrogenases was found to be about 3-fold greater in hydrogen than in air. That this was largely the result of activation of these enzymes through reduction consequent to hydrogenase activity, rather than stabilization of initial activity, was suggested by the following observations: (a) the flavoproteins of the extract were rapidly bleached by hydrogen; (b) the activity of L-malate dehydrogenase, only slightly greater in nitrogen than in air, was twice as great in hydrogen as in nitrogen (Table IX); and (c) the activity of an enzyme after incubation in air could be enhanced by subsequent incubation under hydrogen.

TABLE IX: Activation of Various Enzymes by Hydrogen Gas.

Additions[a]	TTZH (μmoles) Air	Hydrogen
TPN	0.01	0
Isocitrate + TPN	0.44	1.40
cis-Aconitate + TPN	0.21	0.64
DPN	0	0.08
L-Glutamate + DPN	0.40	1.24
L-Malate + DPN	0.34[b]	0.91
Fumarate + DPN	0.01	0.19
Succinate + DPN	0.02	0.04
α-Keto glutarate + DPN + CoA	0.12	0.10
+ DPN + CoA	...	0.01

[a] The reaction mixtures contained: Tris–HCl buffer, pH 8.0, 100 μmoles; $MgCl_2$, 4 μmoles; TTZ, 15 μmoles; TPN or DPN, 0.2 mg; *C. kluyveri* extract, 5 mg; and as indicated the potassium salts of *dl*-isocitrate, 4.2 μmoles; *cis*-aconitate, 10 μmoles; succinate, 50 μmoles; and 25 μmoles of L-glutamate, or L-malate, or fumarate or α-ketoglutarate; CoA, 0.5 mg; incubation, 30 min at 30° in air or in hydrogen gas. [b] Under nitrogen, 0.46 μmole.

Other Citric Acid Cycle Enzymes. The presence of a DPN-linked L-malate dehydrogenase was demonstrated in the experiments on citrate synthesis (Table II) and TTZ reduction (Table IX). It was further shown that hydrogen gas caused the reduction of oxalacetate to L-malate, provided DPN was added. TPN, although reduced by hydrogen, could not substitute for DPN in this reaction. The extracts also catalyzed a reduction of TTZ by L-malate which was stimulated by TPN and resulted in the accumulation of some pyruvate and acetyl-CoA but not oxalacetate. This suggested the presence of a TPN-specific L-malate enzyme catalyzing the oxidative decarboxylation of L-malate to pyruvate. It is probable that the oxalacetate decarboxylase activity demonstrated in *C. kluyveri* extracts (Table I) is due in part to L-malate enzyme. Fumarate caused a small but significant reduction of TTZ (Table IX) indicating the presence of traces of fumarase. Neither succinate nor α-ketoglutarate caused a significant reduction of TTZ; nor did the extracts take up additional oxygen, in the presence of phenazine methosulfate, when succinate or α-ketoglutarate were added. Thus succinate and α-ketoglutarate dehydrogenases appear to be absent. A negative assay for succinyl-CoA synthetase further suggested the absence of an α-ketoglutarate oxidation system.

Possible Alternate Pathways of Glutamate Biosynthesis. Since crotonyl-CoA is known (Bartsch and Barker, 1961) to be an intermediate of fatty acid metabolism in *C. kluyveri*, the possibility was explored that carboxylation of crotonyl-CoA (Tustanoff and Stern, 1960) might result in glutamate biosynthesis *via* the reactions:

$$\text{crotonyl-CoA} + CO_2 + \text{ATP} \longrightarrow \text{glutaconyl-CoA} \longrightarrow \text{glutaconate} \xrightarrow{NH_3} \text{glutamate, or glutaconate} \longrightarrow \alpha\text{-hydroxyglutarate} \longrightarrow \alpha\text{-ketoglutarate} \longrightarrow \text{glutamate}$$

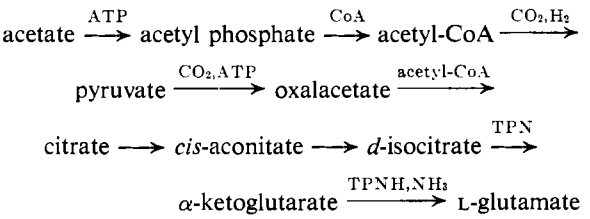
However, the extracts were devoid of both acetyl-CoA and crotonyl-CoA carboxylase activity; neither did they catalyze the hydration or amination of glutaconate, nor the reduction of TTZ by glutaconate or α-hydroxyglutarate, in the presence of DPN or TPN. Thus no enzymes of this pathway could be detected. DL-β-Methylaspartate was not converted to L-glutamate, indicating the absence of the isomerase first described in *C. tetanomorphum* (Barker *et al.*, 1958). Also tests for citramalyl-CoA synthesis from acetyl-CoA and pyruvate (Losada *et al.*, 1960) were negative. Thus essential enzymes of the citramalate pathway of glutamate biosynthesis were not present. This pathway could not account, in any case, for the pattern of labeling of carbon atoms 3 and 4 of glutamate as determined by Tomlinson.

Other Anaerobic Bacteria. The enzymes of the citrate pathway of glutamate synthesis have been found in extracts of *C. thermoaceticum* (Stern and Bambers, unpublished experiments) and will be the subject of a separate communication. Extracts of *C. pasteurianium* had small amounts of citrate synthetase, but contained no measurable aconitase, *d*-isocitrate dehydrogenase, or L-glutamate dehydrogenase activities.

Discussion

The above experiments, taken together with earlier ones showing the biosynthesis of oxalacetate from acetyl-CoA and CO_2 *via* pyruvate synthetase and pyruvate carboxylase (Stern, 1963a, 1965), demonstrate the occurrence in *C. kluyveri* of a pathway of glutamate synthesis from acetate and CO_2 which involves the

upper portion of the citric acid cycle, namely, the enzymes citrate synthetase, aconitase, and *d*-isocitrate dehydrogenase, plus L-glutamate dehydrogenase. The stepwise reaction sequence can be written as follows:



Of the remaining enzymes of the citric acid cycle, only L-malate–DPN dehydrogenase is present, as are two ancillary cycle enzymes, L-malate–TPN oxidodecarboxylase and oxalacetate decarboxylase. α-Ketoglutarate and succinate dehydrogenases, succinyl-CoA synthetase, and fumarase (except trace amounts) are absent.

Evidence for a biosynthetic role of the upper half of the citric acid cycle in glutamate synthesis was first derived by Roberts *et al.* (1953) in growing *E. coli*. When mutants of *E. coli* that lacked citrate synthetase were obtained and were shown to have an absolute requirement for α-ketoglutarate or glutamate for growth and an almost complete loss of the capacity to oxidize acetate (Gilvarg and Davis, 1956), it became clear that the citric acid cycle was the only significant pathway of acetate oxidation and glutamate biosynthesis in this species. This pathway can, of course, function under aerobic or anaerobic conditions in animal tissues, yeast, and facultative aerobic bacteria. Our experiments show that it also occurs in some strict anaerobes.

However, the operation of an anerobic citric acid cycle is not necessarily coupled to glutamate biosynthesis. Thus Gest *et al.* (1962) have presented evidence for the operation of an anaerobic light-dependent citric acid cycle in *R. rubrum* cells, which effects the conversion of organic acids to CO_2 and molecular hydrogen. Moreover, the enzymes of the citric acid cycle are present in extracts of *R. rubrum* (Eisenberg, 1953). However, the labeling pattern of glutamate derived from $^{14}CO_2$ and [1-^{14}C]acetate (Hoare, 1963; Elsden, 1962) is both unique and inconsistent with its origin *via* the citrate pathway.

The unusual labeling pattern of glutamate formed from $^{14}CO_2$ and [1-^{14}C]acetate in *C. kluyveri* cells (Tomlinson, 1954a) suggests that the operation of the citrate pathway demonstrated here must involve an unusual stereochemical course of one component enzymatic reaction. Therefore, a study of the stereospecificity of aconitase and citrate synthetase was undertaken and the results are presented in the accompanying paper (Stern *et al.*, 1966).

The metabolic significance, if any, of the finding of two enzyme pathways of L-malate synthesis, (1) the direct reductive carboxylation of pyruvate by L-malate enzyme in the presence of TPNH and (2) the reductive carboxylation of pyruvate to L-malate by coupling of pyruvate carboxylase and L-malate dehydrogenase, in presence of DPNH, is unknown. Tomlinson (1954b) found that the carboxyl carbon of glycine arose from CO_2 and the α-carbon from the carboxyl carbon of acetate. This suggests that glycine (or glyoxylate) is derived from carbons 1 and 2 of L-malate or oxalacetate. However, attempts to demonstrate enzymatic cleavage of L-malate (Stern; 1963b, Tuboi and Kikuchi, 1963) or of oxalacetate were unsuccessful.

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