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Nonfunctional Tricarboxylic Acid Cycle and the Mechanism of Glutamate Biosynthesis in *Acetobacter suboxydans*

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Acetobacter suboxydans does not contain an active tricarboxylic acid cycle, yet two pathways have been suggested for glutamate synthesis from acetate catalyzed by cell extracts: a partial tricarboxylic acid cycle following an initial condensation of oxalacetate and acetyl coenzyme A, and the citramalate-mesaconate pathway following an initial condensation of pyruvate and acetyl coenzyme A. To determine which pathway functions in growing cells, acetate-*1*-^{14}C was added to a culture growing in minimal medium. After growth had ceased, cells were recovered and fractionated. Radioactive glutamate was isolated from the cellular protein fraction, and the position of the radioactive label was determined. Decarboxylation of the C5 carbon removed 100% of the radioactivity found in the purified glutamate fraction. These experiments establish that growing cells synthesize glutamate via a partial tricarboxylic acid cycle. Aspartate isolated from these hydrolysates was not radioactive, thus providing further evidence for the lack of a complete tricarboxylic acid cycle. When cell extracts were analyzed, activity of all tricarboxylic acid cycle enzymes, except succinate dehydrogenase, was demonstrated.

Previous nutritional studies demonstrate that addition of glutamic acid or its precursors is not essential for growth of *Acetobacter suboxydans* (5); therefore, one can assume that this bacterium can synthesize this amino acid. The pathway used by most biological systems for glutamate synthesis involves the tricarboxylic acid cycle, yet numerous studies support the absence of a functional tricarboxylic acid cycle in this bacterium (18). In an attempt to explain glutamate synthesis in this deficient aerobe, two pathways were proposed. The first pathway, proposed by Röhr (32) and embellished by Maragoudakis et al. (25), involves an initial condensation of pyruvate and acetyl coenzyme A (CoA) producing citramalate which is then converted to glutamate via mesaconate and β-methylaspartate intermediates (citramalate-mesaconate pathway). A second pathway, proposed by Sekizawa et al. (34), is reported to involve an initial condensation of glyoxylate and oxalacetate to form oxalomalate, and this is decarboxylated to γ-hydroxy-α-ketoglutarate and finally converted to α-ketoglutarate and glutamate (glyoxylate-oxalacetate pathway). This second pathway has never been confirmed in *A. suboxydans* nor reported in other organisms.

Although numerous studies have been published concerning the presence or lack of various tricarboxylic acid cycle enzymes, citrate synthase activity was only recently discovered in *A. suboxydans* (26, 38). In 1969, Greenfield and Claus (18) published the first detailed evidence for isocitrate dehydrogenase in cell extracts. They also reconfirmed the presence of aconitase and an α-ketoglutarate amino acid transaminase, and they were able to demonstrate the ability of extracts to synthesize glutamate from ^{14}C-citrate. This represented the third proposed pathway for glutamate biosynthesis catalyzed by *A. suboxydans* extracts (aconitase-isocitrate dehydrogenase pathway).

This report describes experiments designed to determine the importance of the aconitase-isocitrate dehydrogenase pathway and the citramalate-mesaconate pathway for glutamate synthesis in growing cells. A search for the remaining tricarboxylic acid cycle enzymes was also made to determine which enzyme(s)

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is responsible for the lack of a functional cycle in *A. suboxydans*.

MATERIALS AND METHODS

Organisms. The organisms used in this study were *A. suboxydans* ATCC 621 and *A. aceti* ATCC 15973, both obtained from the American Type Culture Collection.

Nonradioactive growth and extract preparation. These conditions were described previously (18).

Glutamate-free growth medium. A chemically defined medium containing the growth factors of Underkofler et al. (37) and formulated for nutritional studies in our laboratory (5) was used in all studies. This medium contained 4 g of glycerol, 20 μg of D-calcium pantothenate, 20 μg of *para*-aminobenzoate, 20 μg of nicotinate, 20 μg of $MgSO_4 \cdot 7H_2O$, 20 μg of $(NH_4)_2SO_4$, 20 mg of NaCl, 20 mg of $FeSO_4$, 2 mg of $MnSO_4 \cdot 1H_2O$, 50 mg of KH_2PO_4, 5 mg of K_2HPO_4, and distilled water (resistance > 0.85 megohms) to 100 ml. The *p*H was adjusted to 6.0 with 2 N KOH, and the final volume was adjusted prior to heat sterilization.

Cell growth on acetate-1-^{14}C. A 0.2-ml volume of the glycerol stock culture (18) was used to inoculate 50 ml of the liquid medium described by Kitos et al. (22) and grown for 24 hr at 28 C with shaking (200 reciprocations/min at 1.5 inch stroke amplitude). A 0.2-ml sample from this subculture was then used to inoculate 100 ml of glutamate-free synthetic medium containing 25 μmoles of sodium acetate-*1*-^{14}C (50 μCi) in a 2-liter baffled flask. The small amount of glutamate carried over with the inoculum served as an initial stimulation for growth in the synthetic medium (5). After 5 days of growth at 28 C on the shaker, the cells were harvested by centrifugation and washed once with a solution containing nonradioactive acetate at 10 times the original concentration of radioactive acetate and then twice with distilled water. The total cell yield was 290 mg (wet weight).

Glutamate-^{14}C isolation from acetate-1-^{14}C-grown cells. Protein was isolated from fractionated cells by the method of Roberts et al. (31). The protein fraction was suspended in 3 ml of 6 N HCl and hydrolyzed in a sealed tube at 105 C for 15 hr. Excess HCl was removed by repeated evaporation under reduced pressure over solid KOH. The solution was passed through a membrane filter (Millipore Corp., Bedford, Mass.) prior to the final evaporation. The residue remaining after evaporation was extracted with 20% isopropyl alcohol, and the extract was applied as a band to Whatman no. 3MM paper. Glutamate-^{14}C was separated by electrophoresis with a Savant flat-plate high-voltage electrophoresis system, model FP-22A (Savant Instruments Inc., Hicksville, N.Y.) by using a 2,000-v potential for 1 hr in pyridine-acetic acid-water (5:11:384) at *p*H 4.0. The radioactive area corresponding to known glutamate was eluted, chromatographed on Whatman no. 3MM paper in *t*-butyl alcohol-methyl ethyl ketone-formic acid-water (40:30:15:15), eluted again, and used in the experiments described below. To check the purity of the isolated radioactive material, samples were chromatographed by using the following solvent systems: *t*-butyl alcohol-methyl ethyl ketone-water-ammonium hydroxide (40:30:20:10; [13]), ethyl alcohol-ammonia-water (80:4:16; [14]), *n*-butyl alcohol-glacial acetic acid-water (50:25:25; [13]), *n*-butyl alcohol-pyridine-water (30:30:30; [14]), the upper phase from a mixture of ethyl acetate-water-formic acid (60:35:5; [13]), pyridine-acetic acid-water (50:35:15; [8]). In all cases, the isolated radioactive material migrated with known glutamate standards.

Analysis of C1 from isolated glutamate-^{14}C. The C1 carboxyl group was converted to CO_2 by commercially prepared L-glutamate decarboxylase (36). This reaction was carried out in Warburg flasks containing the isolated glutamate-^{14}C from cell hydrolysates, 300 μmoles of acetate buffer (*p*H 5.0), and 2 μmoles of nonradioactive glutamate in the main compartment; the center well contained 0.2 ml of hyamine hydroxide. After 2 hr of incubation at 37 C, the hyamine solution was removed quantitatively by rinsing the center well three times with scintillation fluid, and the amount of radioactivity was determined. Samples of authentic DL-glutamate-*1*-^{14}C added to reaction mixtures yielded 100% recovery (based upon the L-isomer) of the radioactivity as CO_2 trapped in hyamine hydroxide, whereas DL-glutamic acid-*5*-^{14}C, treated in the same manner, yielded no measurable radioactivity.

Analysis of C5 from isolated glutamate-^{14}C. Degradation of the C5 carboxyl group was accomplished by using a suspension of *Clostridium tetanomorphum* and the procedure described by Gottschalk and Barker (16). Glutamate-^{14}C from cell hydrolysates was placed directly in the main chamber of a Warburg flask along with phosphate buffer at *p*H 7.0 and 2 μmoles of nonradioactive glutamate. One side arm contained 0.5 ml of *C. tetanomorphum* cell suspension, the other side arm contained 0.1 ml of 5 N H_2SO_4, and the center well contained 0.2 ml of hyamine hydroxide. The vessels were gassed with helium for 10 min, and then the reaction was started by tipping the cell suspension into the main compartment. After 60 min at 37 C, H_2SO_4 was tipped in to stop the reaction and release dissolved CO_2. The flasks were continually shaken for an additional 60 min to absorb all evolved CO_2. The hyamine solution was then removed quantitatively, and its radioactivity was determined.

Samples of authentic DL-glutamic acid-*5*-^{14}C added to reaction mixtures yielded 100% recovery of the radioactivity as CO_2 trapped in hyamine. It should be noted that apparently both the D- and L-isomers of glutamate were decarboxylated by *C. tetanomorphum*. Samples of DL-glutamic acid-*1*-^{14}C yielded no significant radioactivity in hyamine hydroxide after similar treatment.

Radioactive measurements. A Packard Tri-Carb liquid scintillation spectrophotometer, model 2209 (Packard Instrument Co., Downers Grove, Ill.), was used for all radioactive determinations. Duplicate samples in hyamine hydroxide were counted in 10 ml of scintillator fluid of the following composition: 7.0 g of 2,5-diphenyloxazole, 0.6 g of 1,4-bis-2(4-

methyl-5-phenoxazolyl)-benzene, and 1,000 ml of toluene. Counting efficiency was determined to be 65% by using the internal standard method with benzoate-^{14}C.

Enzyme assays. All assays were performed spectrophotometrically at room temperature with a Beckman DU or DU-2 spectrophotometer.

Citrate synthase (EC 4.1.3.7) was measured by following the decrease in absorbancy at 232 nm dependent upon the breakage of the thioester bond of acetyl CoA in the presence of oxalacetate (10).

Aconitase (EC 4.2.1.3) was measured by observing the increase in absorbancy at 240 nm due to the formation of *cis*-aconitate in the presence of citrate (28).

Isocitrate dehydrogenase (EC 1.1.1.41) was assayed by following the reduction of nicotinamide adenine dinucleotide (NAD) at 340 nm in the presence of isocitrate (23).

α-Ketoglutarate dehydrogenase, NAD-linked, was assayed by measuring the reduction of NAD at 340 nm in the presence of α-ketoglutarate and CoA (20), or by a modification of this procedure using 3-acetyl pyridine-NAD and measuring its reduction at 365 nm (2).

α-Ketoglutarate dehydrogenase (EC 1.2.4.2), 2,6-dichlorophenol-indophenol (DCPIP)-linked, was determined by measuring the reduction of DCPIP at 600 nm in the presence of α-ketoglutarate and thiamine diphosphate (38).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by using phenazine methosulfate (PMS) as the immediate electron acceptor and DCPIP as the terminal electron acceptor and following the reduction of DCPIP at 600 nm in the presence of succinate (3).

Fumarase (EC 4.2.1.2) was measured by following the appearance of fumarate at 240 nm in the presence of malate (28).

Malate dehydrogenase, pyridine nucleotide specific (EC 1.1.1.37), was assayed by following the reduction of NAD at 340 nm in the presence of malate (38).

Malate dehydrogenase, DCPIP specific, was measured by following the reduction of DCPIP at 600 nm in the presence of malate (38).

Chemicals. Sources of reagents used in this study were the same as reported previously (18). In addition, acetyl-*1*-^{14}C-CoA, DL-glutamate-*1*-^{14}C, and DL-glutamate-*5*-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. Succinic acid, fumaric acid, and DCPIP were obtained from Fisher Scientific Co., Pittsburgh, Pa. Oxalacetic acid was purchased from Mann Research Laboratories, New York, N.Y.; PMS was obtained from Sigma Chemical Co., St. Louis, Mo.; and thiamine diphosphate was purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

RESULTS

Experimental rationale. Although manometric studies suggest that *A. suboxydans* cannot oxidize acetate, it can nevertheless incorporate this compound in the presence of an energy source. If one begins with acetate-*1*-^{14}C (acetyl-*1*-^{14}C-CoA) and isolates the glutamate from cellular protein, it should be possible to distinguish between the two major pathways proposed for glutamate biosynthesis, based upon the labeling pattern found in the newly synthesized glutamate (Fig. 1). If an acetate-pyruvate condensation occurs via the citramalate-mesaconate pathway, the labeling of the glutamate should be exclusively in the C1 carboxyl group. If an acetate-oxalacetate condensation occurs via the aconitase-isocitrate dehydrogenase pathway, labeling should be exclusively in the C5 carboxyl group, since an inoperative tricarboxylic acid cycle would allow no recycling and therefore no randomization of the labeling pattern. If recycling should occur, contrary to all previous reports in *A. suboxydans*, then C5 should contain twice as much radioactivity as C1, i.e., two-thirds of the total label should appear in C5 and one-third in C1, and it would still be possible to distinguish between the two proposed synthetic mechanisms.

Analysis of degradation and recovery techniques. Before any degradation experiments could be properly interpreted, it was necessary to examine whether radioactive glutamate could be selectively degraded and whether the position of its radioactive carbon atoms could be accurately determined. Citrate-*1(5)*-^{14}C was used as the substrate in order to form radioactive glutamate uniformly labeled in the C1 and C5 positions. When radioactive glutamate was so formed and degraded, analysis demonstrated an equal amount of radioactivity in the C1 and C5 carbonyl groups (Table 1).

Stereospecificity of citrate synthase and aconitase. Before attempting to analyze the labeling pattern of glutamate synthesized by whole cells, it was first necessary to determine whether the citrate synthase and aconitase of *A. suboxydans* exhibited the usual stereospecificity of that established for the pig heart enzymes (11, 19) and that assumed in the experimental rationale (Fig. 1). This analysis was important, since some bacteria contain a citrate synthase having a stereospecificity opposite to that assumed in our rationale (15–17), and this alone would reverse the labeling pattern obtained from a citrate-mediated pathway for glutamate synthesis. When acetyl-*1*-^{14}C-CoA plus oxalacetate was used in place of citrate as a precursor for cell extract-catalyzed glutamate synthesis, essentially all (97%) of the radioactivity found in glutamate was located in the C5 position, as predicted from

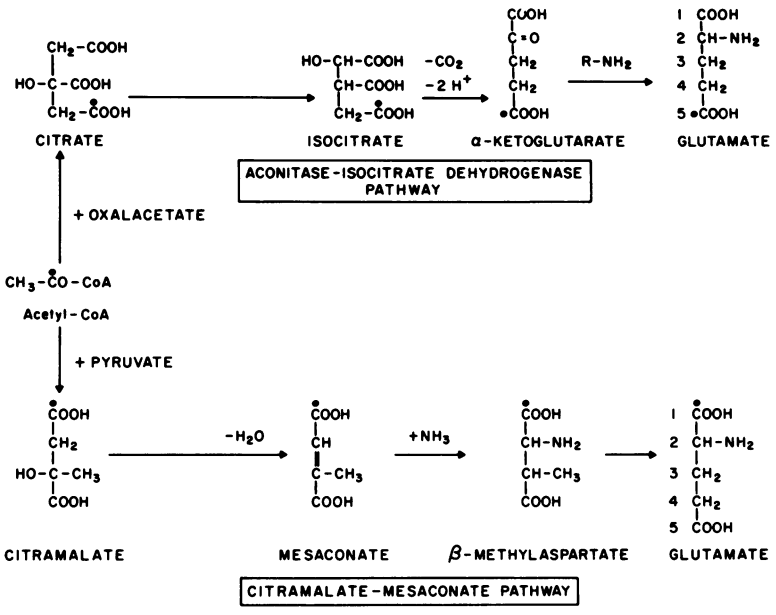

FIG. 1. *Comparison of expected labeling patterns of glutamate synthesized from acetyl-1-^{14}C-CoA via the aconitase-isocitrate dehydrogenase pathway and the citramalate-mesaconate pathway.*

aconitase and isocitrate dehydrogenase with the usual stereospecificity (Table 2).

Distribution of radioactivity in glutamate synthesized from acetate-1-^{14}C by whole cells. Growth of cells on acetate-*1*-^{14}*C*, isolation, and analysis of the radioactive glutamate were as described in the Materials and Methods. All radioactivity in the newly synthesized glutamate molecule was located in the C5 position, proving that glutamate synthesis in growing cells proceeds by way of the aconitase-isocitrate dehydrogenase pathway (Table 3). The total lack of label in the C1 position also indicates a lack of α-ketoglutarate recycling via the tricarboxylic acid cycle. This conclusion is strengthened by the fact that aspartate, isolated from these same hydrolysates, was not radioactive.

Tricarboxylic acid cycle enzymes. The lack of evidence for recycling of α-ketoglutarate in these experiments reconfirms the numerous reports by others that *A. suboxydans* is devoid of a total, functional tricarboxylic acid cycle. Isocitrate dehydrogenase was once thought to be absent in *A. suboxydans*. However, the recent demonstration of this enzyme (18) has stimulated our search for the remaining tricarboxylic acid cycle enzymes to determine the cause for this "break" in the cycle.

Table 4 compares the activity of these enzymes in extracts of *A. suboxydans* with that of *A. aceti*, a species with a completely operative tricarboxylic acid cycle (M. R. R. Rao, Ph.D. thesis, Univ. of Illinois, Urbana, 1955). It should be noted that in all cases where activities could be measured, the specific activities of the enzymes from *A. suboxydans* were many times less than the comparable enzymes found in *A. aceti*. All attempts to demonstrate succinate dehydrogenase activity

TABLE 1. *Distribution of radioactivity in C1 and C5 carbon atoms of glutamate-^{14}C synthesized from citrate-1(5)-^{14}C by cell-free extracts of A. suboxydans*[a]

Carbon atom	Radioactivity (counts/min)
C1	14,990
C5	14,960

[a] Reaction mixture contained 50 μmoles of citrate-*1(5)*-^{14}*C* (10,960 counts per min per μmole), 4.2 μmoles of nicotinamide adenine dinucleotide, 25 μmoles of L-aspartate, 0.6 μmole of pyridoxal phosphate, 4 μmoles of $MnCl_2$, 100 μmoles of potassium phosphate buffer (*p*H 7.3), dialyzed cell-free extract containing 22 mg of protein (18), and water in a total volume of 3.0 ml. After 2.5 hr at 30 C, the reaction mixture was deproteinized, and samples were removed and separately treated with either glutamic decarboxylase to remove specifically the C1 carbonyl group (36) or a suspension of *Clostridium tetanomorphum* to remove specifically the C5 carbonyl group (16).

TABLE 2. *Distribution of radioactivity in C1 and C5 carbon atoms of glutamate-^{14}C synthesized from acetyl-1-^{14}C-CoA and oxalacetate by cell-free extracts of A. suboxydans*[a]

Carbon atom	Radioactivity[b] (counts/min)	Per cent of total
C1	206	3.3
C5	6,300	96.7

[a] Conditions were the same as the complete system described in Table 1 with the substitution of 30 μmoles of oxalacetate plus 5 μmoles of acetyl-1-^{14}C-CoA (1 μCi) for citrate, and substitution of $MgCl_2$ for $MnCl_2$.

[b] Decarboxylation and determination of radioactivity as described in Table 1 and Materials and Methods. All values were corrected for control flasks containing heat-inactivated cell-free extract.

TABLE 3. *Distribution of radioactivity in glutamate-^{14}C synthesized from acetate-1-^{14}C by growing cells of A. suboxydans*[a]

Carbon atom	Radioactivity (counts/min)	Per cent of total
Total	46,710	100
C1	60	0.1
C5	47,220	101.1

[a] Conditions were as described in Materials and Methods.

TABLE 4. *Comparative activities of tricarboxylic acid cycle enzymes in extracts of A. aceti and A. suboxydans*

Enzyme assayed	Specific activity[a]	
	A. aceti	*A. suboxydans*
Citrate synthase	200	7.3
Aconitase	7,150	20.1
Isocitrate dehydrogenase	980	11.4
α-Ketoglutarate dehydrogenase (NAD)	287	0
α-Ketoglutarate dehydrogenase (DCPIP)	16	1.5
Succinate dehydrogenase	460	0
Fumarase	1,470	23.4
Malate dehydrogenase (NADP)	46	0
Malate dehydrogenase (DCPIP)	20	6.1

[a] Nanomoles of substrate converted per minute per milligram of protein.

in *A. suboxydans* were unsuccessful. These enzymatic data are in agreement with the Warburg respirometric studies (Table 5) where, besides acetate, the only tricarboxylic acid cycle intermediate not oxidized by the cell-free extracts was succinate. It is concluded that the lack of succinate dehydrogenase is responsible for the malfunctioning of a complete tricarboxylic acid cycle in this bacterium.

TABLE 5. *Oxidation of glucose and the tricarboxylic acid cycle intermediates by extracts of A. suboxydans*[a]

Substrate	Oxygen uptake (μmoles)
Glucose	73.5
Acetate	0.5
Citrate	13.6
Isocitrate	11.9
α-Ketoglutarate	2.6
Succinate	0.3
Fumarate	4.5
Malate	4.4
Oxalacetate	14.4

[a] Each Warburg flask contained 50 μmoles of substrate, 2 μmoles of $MgCl_2$, 2 μmoles of $MnCl_2$, 3 mg of NAD, 100 μmoles of phosphate buffer (*p*H 7.3), dialyzed cell-free extract containing 24.4 mg of protein (18), and water in a total volume of 1.8 ml. The center well contained 0.2 ml of 2 N KOH. The reaction was carried out in air at 30 C for 2.5 hr. All values were corrected for endogenous blanks.

DISCUSSION

The labeling pattern of glutamate isolated from cells grown in the presence of acetate-1-^{14}C unequivocally establishes that glutamate synthesis in growing cells proceeds by way of the aconitase-isocitrate dehydrogenase pathway. This labeling pattern demonstrates that growing cells do not utilize the citramalate-mesaconate pathway for glutamate synthesis.

Maragoudakis et al. (25) report that cell extracts synthesize radioactive glutamate from pyruvate and acetate-1-^{14}C under conditions presumably favorable to the citramalate-mesaconate pathway. Their experiments are difficult to interpret because of the use of large amounts of undialyzed protein (28.5 mg/ml), the unexplained addition of large quantities of alanine (substrate level), the unusually long incubation time (6 hr), and the lack of published control experiments showing endogenous activities. After addition of known glutamate, separation of radioactivity by recrystalization, and ninhydrin degradation, they reported 56% of the label in the C1 position; however, they made no determination of radioactivity in the C5 position. We can neither repeat nor explain their results. On the contrary, recent results from our laboratory (Belly,

et al., Bacteriol. Proc., **70**:141, 1970) indicate that the citramalate-mesaconate pathway serves primarily as a route for isoleucine synthesis in a manner similar to that reported in *Escherichia coli* (1).

The absence of a complete functional tricarboxylic acid cycle in *A. suboxydans* has previously been substantiated by many types of experiments (18), and this absence is reconfirmed in this report by four types of evidence: (i) the failure of whole cells to oxidize acetate, (ii) the failure to detect succinate dehydrogenase activity in cell extracts, (iii) the lack of a random ^{14}C-label in glutamate isolated from cells grown in the presence of acetate-*1*-^{14}C, and (iv) the isolation of nonradioactive aspartate from cells grown in the presence of acetate-*1*-^{14}C.

The failure of resting cells to oxidize acetate is so well known that it has become part of the taxonomic characterization for the organism (4, 29); however, the cause of this "malfunction" has not been clear. Although our results are in conflict with the report of Williams and Rainbow (38), we have repeatedly been unable to demonstrate succinate dehydrogenase in cell extracts. We have also been unable to demonstrate succinate oxidation by whole cells, and this observation is confirmed by the work of others (12, 21, 26, 27, 35). Succinate has also been shown to accumulate in growth media (6, 27, 33) and in reaction mixtures incubated for a prolonged time in a rich milieu containing cell extracts (25). Therefore, we conclude that the lack of succinate dehydrogenase prevents full operation of a tricarboxylic acid cycle.

The isolation of nonradioactive aspartate from protein hydrolysates of cells grown in the presence of acetate-*1*-^{14}C indicates the lack of an anaplerotic glyoxylate cycle (24) that might have been used to bypass the succinate dehydrogenase "block." This observation from growing cells supports our continued inability to demonstrate isocitrate lyase and malate synthase in cell extracts. This finding also reinforces the suggestion of Claus et al. (7) that phosphoenolpyruvate carboxylation followed by amination of the newly formed oxalacetate is the predominant pathway for aspartate synthesis in *A. suboxydans*.

In 1961, Cheldelin (6) stated that, when isotopic glucose or $^{14}CO_2$ is administered, the label first appears among amino acids in aspartate and glutamate. These results can now be explained by the reactions presented in Fig. 2. Isotopically labeled glucose can be converted to phosphoenolpyruvate, then glutamate can be labeled via either oxalacetate or acetate, and aspartate will be labeled via oxalacetate amination. Carboxylation of phosphoenolpyruvate by $^{14}CO_2$ would yield labeled oxalacetate from which both aspartate and glutamate could be synthesized.

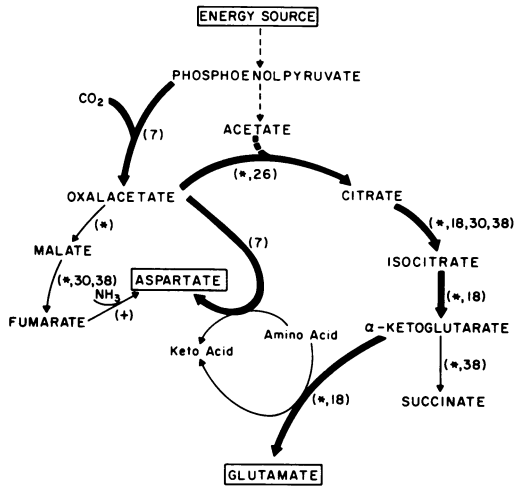

FIG. 2. *Glutamate and aspartate synthesis in Acetobacter suboxydans having a deficient tricarboxylic acid cycle and lacking a glyoxylate cycle. Numbers in parentheses refer to enzyme analyses reported in the cited literature. * refers to supportive data presented in this report; + refers to Cheldelin et al., Fed. Proc., 22:652, 1963.*

A. suboxydans has the capacity for limited oxidation of a large number of polyols and for excretion of the oxidation products into the growth medium (9). If one assumes that these oxidations provide the cell with energy for growth, then this obligate aerobe would have little need for an oxidative tricarboxylic cycle. The partial tricarboxylic acid cycle found in this bacterium (Fig. 2) then would appear to function primarily for glutamate, aspartate, and succinate biosynthesis.

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