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Isocitrate Dehydrogenase and Glutamate Synthesis in *Acetobacter suboxydans*[1]

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Acetobacter suboxydans is an obligate aerobe for which an operative tricarboxylic acid cycle has not been demonstrated. Glutamate synthesis has been reported to occur by mechanisms other than those utilizing isocitrate dehydrogenase, a tricarboxylic acid cycle enzyme not previously detected in this organism. We have recovered α-ketoglutarate and glutamate from a system containing citrate, nicotinamide adenine dinucleotide (NAD), a divalent cation, pyridoxal phosphate, an amino donor, and dialyzed, cell-free extract. Aconitase activity was readily detected in these extracts, but isocitrate dehydrogenase activity, measured by NAD reduction, was masked by a cyanide-resistant, particulate, reduced NAD oxidase. Isocitrate dehydrogenase activity could be demonstrated after centrifuging the extracts at 150,000 × *g* for 3 hr and treating the supernatant fluid with 2-heptyl-4-hydroxyquinoline *N*-oxide. It is concluded that *A. suboxydans* can utilize the conventional tricarboxylic acid cycle enzymes to convert citrate to α-ketoglutarate which can then undergo a transamination to glutamate.

Acetobacter suboxydans is usually regarded as unique among heterotrophic bacteria in that it is an obligate aerobe without a functional tricarboxylic acid cycle. Evidence for the lack of a tricarboxylic acid cycle is based on the inability of whole cells or cell-free extracts to oxidize intermediates of the tricarboxylic acid cycle (10, 17-19, 28, 35), on the lack of a complete complement of the enzymes of the cycle (37; M. R. R. Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955), and on the results of radioactive tracer experiments (22, 23). The pentose phosphate cycle has been reported to be the major pathway for carbohydrate dissimilation in this organism and is also thought to function as the main energy-generating system (5). The absence of a tricarboxylic acid cycle has led investigators to search for alternative mechanisms for the synthesis of certain amino acids which normally arise from carbon skeletons of the intermediates of the cycle. Aspartate has recently been shown to arise via carboxylation of phosphoenolpyruvate and subsequent transamination of the oxalacetate formed (7). The synthesis of glutamate has been reported to occur by two different mechanisms. One of these pathways is initiated by a condensation between glyoxylate and oxalacetate (33); the second, by a condensation between acetate and pyruvate (25). The latter pathway is essentially the reverse of the scheme for the fermentative dissimilation of glutamate in *Clostridium tetanomorphum* (1).

In this study, we report that citrate can serve as a precursor for glutamate synthesis in *A. suboxydans*, and that the mechanism involves the tricarboxylic acid cycle enzymes aconitase and isocitrate dehydrogenase in conjunction with an α-ketoglutarate-aspartate transaminase. This is the first report of the presence of isocitrate dehydrogenase in *A. suboxydans*.

MATERIALS AND METHODS

Organism and growth conditions. The organism used in this study was *A. suboxydans* ATCC 621 obtained from the American Type Culture Collection. It exhibited the typical characteristics of the strain, including inability to oxidize acetate and requirement of *p*-aminobenzoic acid, nicotinic acid, and pantothenic acid for growth (31). Stock cultures were prepared by incubating cells in 50 ml of the liquid medium described by Kitos et al. (23) and shaking in 500-ml flasks at 28 C for 24 hr. A 1-ml amount of this broth culture was mixed with 2 ml of glycerol and stored at −20 C.

For the preparation of large numbers of cells, 0.2 ml of the glycerol stock culture was inoculated into 50 ml of the above medium and grown with shaking at 28 C for 24 hr. The contents of the flask were used

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to inoculate a 14-liter New Brunswick Microferm fermentor containing 10 liters of growth medium at 28 C and aerated at a rate of 8,000 ml of air per min. Foaming was controlled by the automatic addition of GE-60 Antifoam (General Electric Co., Waterford, N.Y.). After 21 hr of growth (late exponential phase), the cells were harvested by centrifugation at 5 C and 14,000 × *g*, washed twice with cold 0.1 M potassium phosphate buffer (*p*H 7.5), and stored at −20 C.

Cultures of *A. aceti* ATCC 15973 were treated in the same manner except that growth was allowed to proceed for 31 hr in the fermentor to reach the late exponential phase.

Preparation of extracts. Cells were suspended in 0.1 M potassium phosphate buffer (*p*H 7.5) at a ratio of 1:1 (w/v). For sonic-treated extracts, 50-ml portions were treated with a 10-kc Raytheon sonic oscillator for 6 min at an output current of approximately 1 amp. Ice water was circulated through the cooling jacket during the period of sonic treatment. Extracts were also prepared by extrusion through a precooled French pressure cell at 10,000 psi. In this latter case, deoxyribonuclease had to be added to reduce viscosity and facilitate centrifugation. The suspensions resulting from either method of breakage were centrifuged twice at 34,000 × *g* for 30 min to remove cellular debris. For some experiments, the extracts were centrifuged again at 150,000 × *g* for 3 hr in a Spinco model L-50 ultracentrifuge. All extracts were dialyzed in the cold for 6 hr against 150 volumes of 0.01 M potassium phosphate buffer (*p*H 7.5). Dialyzed extracts were stored in small portions at −20 C. Protein concentrations were determined by the biuret method (13) with bovine serum albumin fraction V as a standard.

Enzymatic assays. All assays were performed spectrophotometrically at room temperature with a Beckman DU spectrophotometer.

Aconitase was measured by observing the increase in absorbancy at 240 nm in the presence of citrate (29). Reduced nicotinamide adenine dinucleotide (NADH) oxidase was measured by following the decrease in absorbancy at 340 nm in the presence of NADH. Isocitrate dehydrogenase was assayed by following the reduction of nicotinamide adenine dinucleotide (NAD) at 340 nm in the presence of isocitrate (24).

Chromatography. For identification of glutamate, the standard reaction mixture (Table 1) was extracted with hot ethyl alcohol as described by Flipse and Dietz (11) and was subjected to one- or two-dimensional ascending chromatography; the paper and solvent systems used are described in the Results. Amino acids were demonstrated with the ninhydrin spray reagent (34). α-Ketoglutarate was isolated as the 2,4-dinitrophenylhydrazone derivative from reaction mixtures not containing aspartate or pyridoxal phosphate according to the procedure of Friedemann and Haugen (12), and was chromatographed on paper and thin-layer cellulose (Eastman Chromagram Sheet 6064). α-Ketoglutarate was isolated as the free acid by overnight ether extraction of acidified reaction mixtures not containing aspartate or pyridoxal phosphate, and was chromatographed in two dimensions on paper and thin-layer silica gel (Eastman Chromagram Sheet 6060). The free acids were demonstrated with the aniline-xylose reagent (34).

Chromatograms containing radioactive reaction products were exposed to Kodak No-Screen X-ray film prior to being treated with spray reagents.

Chemicals. Citric acid, α-ketoglutaric acid, trisodium DL-isocitric acid, L-glutamic acid, L-aspartic acid, L-alanine, DL-α-amino-*n*-butyric acid, β-methyl-DL-aspartic acid, NAD, NADH, oxidized sodium nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form (NADPH), 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), and L-glutamic decarboxylase (type II, from *E. coli*) were obtained from Sigma Chemical Co., St. Louis, Mo. Pyridoxal phosphate, barium DL-fluorocitrate (converted to the sodium salt prior to use), and citric acid-*1(5)*-^{14}C (checked for purity by chromatography) were purcased from Calbiochem, Los Angeles, Calif.

RESULTS

Synthesis of glutamate from citrate. The conditions for glutamate synthesis from citrate in cell-free extracts are presented in Table 1. For maximal activity, the system was dependent on citrate, NAD, aspartate, pyridoxal phosphate, and Mn^{++} ions. Mg^{++} could replace Mn^{++}, but NADP

TABLE 1. *Synthesis of glutamate by cell-free extracts of A. suboxydans*

Expt	Components	Glutamate formed[a] (μmoles)
1	Complete system[b]	4.9
	minus citrate	0.1
	minus NAD	0.9
	minus ASP, PLP	0.8
	minus Mn^{++}	3.1
2	Reaction mixture[c]	
	plus α-KG	0.2
	plus α-KG, ASP, PLP	13.7

[a] As determined with L-glutamic acid decarboxylase (36).

[b] The complete system contained 50 μmoles of citrate, 4.2 μmoles of nicotinamide adenine dinucleotide (NAD), 25 μmoles of L-aspartate (ASP), 0.6 μmole of pyridoxal phosphate (PLP), 4 μmoles of $MnCl_2$ (Mn^{++}), 100 μmoles of potassium phosphate buffer (*p*H 7.3), dialyzed cell-free extract (22 mg of protein), and water in a total volume of 3.0 ml. Reaction mixtures were incubated at 30 C for 2.5 hr, deproteinized, and assayed for glutamic acid.

[c] Reaction mixture contained 100 μmoles of potassium phosphate buffer (*p*H 7.3), dialyzed cell-free extract (22 mg of protein), and 50 μmoles of α-ketoglutarate. Additions of aspartate, pyridoxal phosphate, and water as well as incubation, deproteinization, and assay procedures were identical to experiment 1.

could not substitute for NAD. Depending on the extract, as high as a 20% conversion of citrate to glutamate could be achieved under these conditions. Table 1 also demonstrates the ability of cell-free extracts to transaminate α-ketoglutarate to glutamate, as previously shown in this laboratory (7), and that this transamination was not the limiting reaction in the overall sequence. Aspartate could be replaced by alanine, β-methyl-aspartate, or α-aminobutyrate.

Eflect of fluorocitrate. If the mechanism of glutamate synthesis from citrate proceeds by way of the tricarboxylic acid cycle, then fluorocitrate, a specific competitive inhibitor of aconitase (27), should have a marked effect on the overall reaction. Figure 1 demonstrates fluorocitrate inhibition of glutamate synthesis. The extent of inhibition became greater as the concentration of fluorocitrate increased, approaching 80% at 5×10^{-3} M.

Aconitase. The presence of aconitase in *A. suboxydans* is demonstrated in Fig. 2. For comparison, cell-free extracts of *A. aceti*, a species with a complete functional tricarboxylic acid cycle (M. R. R. Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955), were prepared and treated in exactly the same manner as *A. suboxydans*. The specific activities, expressed as change in optical density at 240 nm per minute per milligram of protein were 0.03 for *A. suboxydans* and 9.45 for *A. aceti*; that is, *A. aceti* was 315 times more active

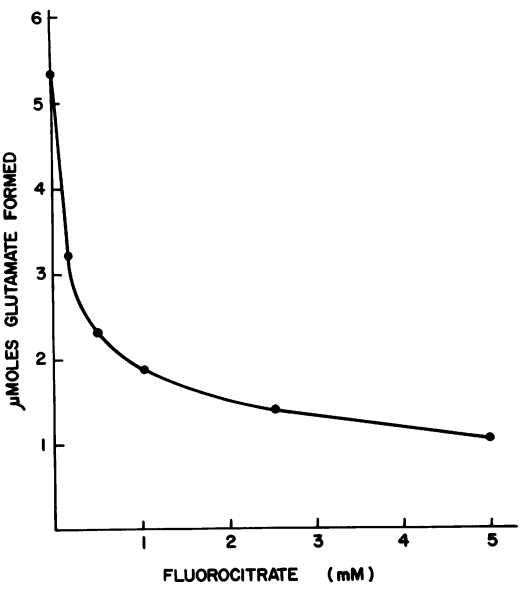

FIG. 1. *Effect of fluorocitrate on the conversion of citrate to glutamate in cell-free extracts of A. suboxydans. Conditions were the same as the complete system described in Table 1.*

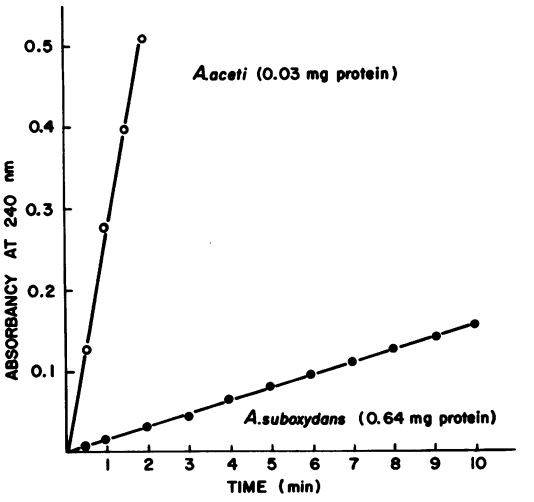

FIG. 2. *Aconitase activity in cell-free extracts of A. suboxydans and A. aceti. The reaction mixtures contained 50 µmoles of citrate, 150 µmoles of potassium phosphate buffer (pH 7.3), dialyzed cell-free extracts as indicated, and water in a total volume of 3.0 ml. The reactions were performed against a blank containing buffer and extract.*

than *A. suboxydans*. Similarly low activities for this enzyme in *A. suboxydans* have been reported by Rao (Ph.D. Thesis, Univ. of Illinois, Urbana, 1955) and by Williams and Rainbow (37). The addition of Fe^{++} to the dialyzed extract did not increase the activity as it does with the enzyme from mammalian sources (9).

Isocitrate dehydrogenase. Initial attempts to demonstrate isocitrate dehydrogenase by spectrophotometrically measuring NADH (or NADPH) production in the presence of isocitrate were unsuccessful. It was subsequently found that the NADH produced was being rapidly reoxidized, thereby avoiding detection by the assay system. Daniel and Redfearn (8) recently showed that *A. suboxydans* contains an "extremely active," particulate, cyanide-insensitive NADH oxidase, and that it could be inhibited by HQNO, piercidin A, and antimycin A. However, addition of HQNO to reaction mixtures containing sonically prepared extracts centrifuged at 34,000 $\times$ *g* or even at 100,000 $\times$ *g* did not allow detection of isocitrate dehydrogenase. Successful reduction of most of the NADH oxidase activity was achieved by using HQNO in extracts prepared with a French pressure cell and centrifuged at 150,000 $\times$ *g* for 3 hr (Fig. 3). Presumably, breaking the cells with the less drastic technique of the French pressure cell facilitated the removal of the particulate NADH oxidase when the extracts were centrifuged at high speeds. Under

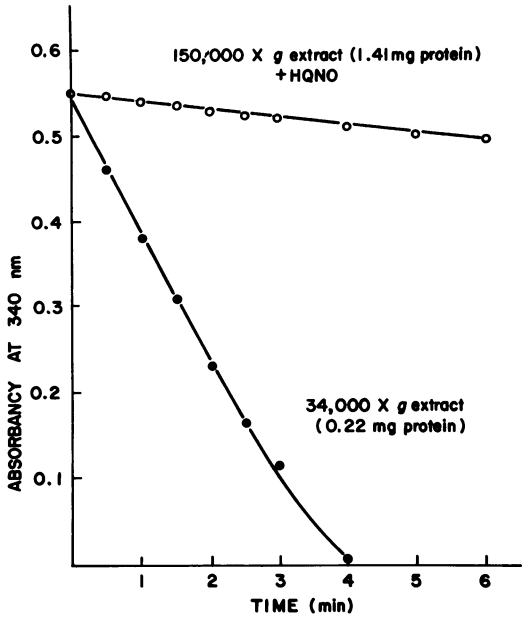

FIG. 3. *NADH oxidase activity in A. suboxydans extracts prepared with a French pressure cell. The reaction mixtures contained 0.3 μmole of NADH, 100 μmoles of potassium phosphate buffer (pH 7.3) plus additions, and water in a total volume of 3.0 ml. Additions were dialyzed extract centrifuged at 150,000 × g for 3 hr plus HQNO (prepared by dissolving 2 mg/ml in 0.01 N NaOH), or dialyzed extract centrifuged at 34,000 × g for 1 hr without the addition of HQNO. The reaction was performed against a blank containing buffer and NADH.*

these conditions, the presence of an NAD-dependent isocitrate dehydrogenase in *A. suboxydans* was demonstrated (Fig. 4).

Unlike the NAD-specific isocitrate dehydrogenase of yeast (24), *Neurospora* (32), or mammals (6), no requirement for adenine nucleotides could be established. No evidence could be found for an NADPH oxidase or an NADP-specific isocitrate dehydrogenase in the extracts of *A. suboxydans*.

In comparison, *A. aceti* had little NADH oxidase and contained an easily demonstrable NAD-linked isocitrate dehydrogenase (Fig. 4), the specific activity of which was 196 times greater than that of the enzyme found in *A. suboxydans*. An NADP-linked isocitrate dehydrogenase has also been reported for *A. aceti* (Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955).

When citrate was substituted for isocitrate, NAD reduction could be demonstrated by preincubating the extract with citrate for 2 hr prior to the addition of NAD. This preliminary incubation was presumably necessary to permit isocitrate to accumulate to a concentration at which its oxidation would not be masked by residual NADH oxidase.

Identification of glutamate and α-ketoglutarate. Glutamate and α-ketoglutarate were identified as products of the reaction sequence by chromatography and radioautography. From a complete reaction mixture, such as that described in Table 1, glutamate was isolated and identified by two-dimensional chromatography (Table 2). From similar reaction mixtures, but with the omission of aspartate and pyridoxal phosphate, α-ketoglutarate was isolated and identified either as the free acid or the 2,4-dinitrophenylhydrazone derivative (Table 2).

Experiments identical to those described above were carried out with radioactive citrate. The radioactive spot corresponding to glutamate on the two-dimensional chromatogram was eluted and co-chromatographed with authentic glutamate in pyridine-acetic acid-water, 50:35:15 (4). The resulting chromatogram was exposed to X-ray film for 2 weeks and then sprayed with ninhydrin. As shown in Fig. 5A, only one ninhydrin-positive spot appeared, and it coincided exactly with the radioactive area. Similarly, radioactive hydrazone formed in reaction mix-

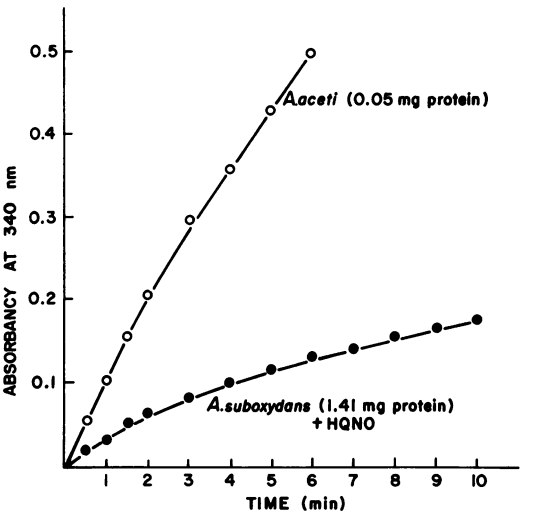

FIG. 4. *NAD-specific isocitrate dehydrogenase activity in cell-free extracts of A. suboxydans and A. aceti. The reaction mixtures contained 10 μmoles of DL-isocitrate, 1 μmole of NAD, 10 μmoles of $MgCl_2$, 0.8 μmole of HQNO where indicated, 100 μmoles of potassium phosphate buffer (pH 7.3), dialyzed cell-free extract of A. aceti or A. suboxydans (centrifuged at 150,000 × g for 3 hr) as shown, and water in a total volume of 3.0 ml. The reaction was performed against a blank containing all the components except isocitrate.*

TABLE 2. *Chromatographic identification of glutamate and α-ketoglutarate*

Product[a]	Solvent system[b]	R_F values	
		Known	Experimental
Glutamate	1 (2D) a	.36	.34
	b	.33	.35
α-Ketoglutarate (2,4-dinitrophenylhydrazone derivative)	2	.71	.72
	3	.45	.45
	4	.27	.27
	5	.39	.39
	6	.32	.32
	7	.35	.36
α-Ketoglutarate (free acid)	8 (2D) a	.39	.38
	b	.69	.71
	9 (2D) a	.29	.29
	b	.31	.32

[a] Isolated from reaction mixtures as described in Materials and Methods.

[b] Solvent system 1: two-dimensional (2D), Whatman no. 4 paper, (a) *n*-butyl alcohol-acetic acid-water, 12:3:5; (b) 80% phenol (34). Solvent system 2: Whatman no. 4 paper, *n*-butyl alcohol-acetic acid-water, 4:1:1 (3). Solvent system 3: Whatman no. 4 paper, ethyl alcohol-ammonia-water, 80:4:16 (3). Solvent system 4: Whatman no. 1 paper dipped in 0.1 N $NaHCO_3$ and dried, *n*-butyl alcohol-ethyl alcohol-0.1 N $NaHCO_3$, 10:3:10 (16). Solvent system 5: Whatman no. 4 paper dipped in 0.2 M potassium phosphate buffer (*p*H 6.2) and dried, *t*-amyl alcohol-ethyl alcohol-water, 5:1:4 (15). Solvent system 6: silica gel thin-layer sheets, *n*-butyl alcohol-ethyl alcohol-water, 7:1:2 (2). Solvent system 7: silica gel thin-layer sheets, benzene-tetrahydrofuran-acetic acid, 57:35:8 (2). Solvent system 8: cellulose thin-layer sheets, (a) ethyl alcohol-water-25% NH_4OH, 100:12:16; (b) *n*-propanol-formic acid-water, 96:12:12 (14). Solvent system 9: Whatman no. 4 paper, (a) ethyl alcohol-ammonia-water, 16:1:3; (b) *n*-butyl alcohol-acetic acid-water, 12:3:5 (34).

tures without aspartate or pyridoxal phosphate was isolated by chromatography in ethyl alcohol-ammonia-water, 80:4:16 (3), and was eluted and co-chromatographed with authentic α-ketoglutarate 2,4-dinitrophenylhydrazone in *n*-butyl alcohol-acetic acid-water, 4:1:1 (3). The resulting chromatogram was exposed to X-ray film. Figure 5B shows one hydrazone spot coinciding exactly with the radioactive area on the X-ray film.

DISCUSSION

The ability of *A. suboxydans* to utilize citrate for the synthesis of glutamate has not been previously demonstrated. Murooka et al. (28), working with dried cell suspensions, and King and Cheldelin (19), employing cell-free extracts, were able to show a limited degree of citrate oxidation; however, these workers did not explore the significance of this oxidation. We have also found that citrate can be oxidized by cell-free extracts (*unpublished data*). From the work presented here, it is clear that citrate can be enzymatically converted to glutamate by way of aconitase and isocitrate dehydrogenase working in concert with an α-ketoglutarate transaminase. The fact that isocitrate dehydrogenase could not be detected by previous workers (37; Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955) may have been due to the presence of the very active,

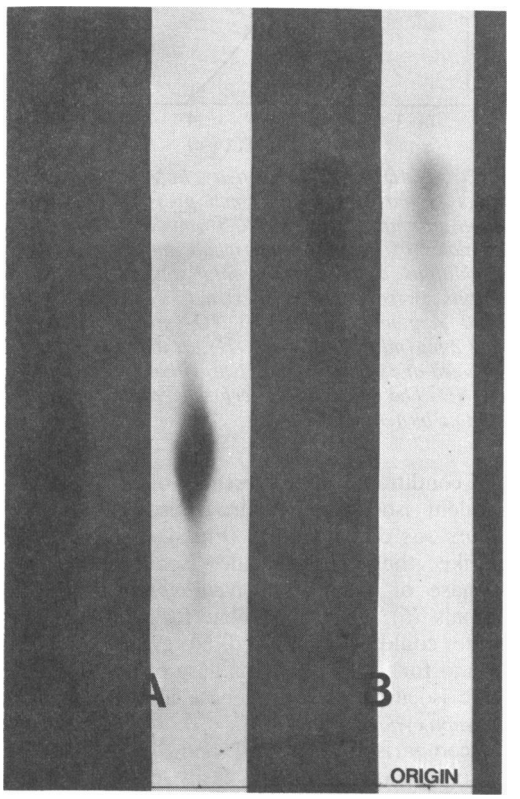

FIG. 5. *Comparison of chromatogram and radioautogram of products isolated from reaction mixtures containing ^{14}C-citrate. (A) Radioactive amino acid from complete reaction mixture (Table 1), isolated by chromatography, eluted, co-chromatographed with authentic glutamate, exposed to X-ray film, and sprayed with ninhydrin. (B) Radioactive hydrazone from reaction mixture not containing aspartate or pyridoxal phosphate, isolated by chromatography, eluted, co-chromatographed with authentic α-ketoglutarate hydrazone, and exposed to X-ray film.*

particulate, cyanide-insensitive, NADH oxidase which would destroy any reduced NAD formed during the synthesis of α-ketoglutarate, thus preventing the spectrophotometric assay of the enzyme. Although Ragland et al. (30) reported both the absence of isocitrate dehydrogenase and "significant levels" of cyanide-insensitive NADH oxidase, their cells were grown under different conditions and were harvested from cultures in the stationary phase (72 hr), whereas the cells used in this study were harvested from cultures in the exponential phase (21 hr).

The synthesis of acetate from pyruvate by pyruvate decarboxylase (20; Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955) and acetaldehyde dehydrogenase (21) is well documented in *A. suboxydans*. This organism is also able to form acetyl-coenzyme A from acetate via acetyl-coenzyme A synthetase (22). The condensing enzyme, thought to be absent in *A. suboxydans*, was recently discovered in cell-free extracts (26, 37) and shown to incorporate acetyl-coenzyme A into citrate (26). Thus, it appears that *A. suboxydans* possesses the enzymatic potential for the conversion of acetate to glutamate; yet, the oxidation of acetate, added to either intact cells or cell extracts, cannot be shown manometrically, either when added alone or in the presence of glucose or glycerol (5). In fact, the inability to oxidize acetate forms one of the bases for distinguishing *A. suboxydans* from other *Acetobacter* species (31). Acetate can, however, be incorporated in small amounts into cellular lipids, but only in the presence of an energy source (22).

There now exist three reported mechanisms for the synthesis of glutamate in *A. suboxydans*, the tricarboxylic acid pathway reported here and the two condensation reactions mentioned previously: glyoxylate plus oxalacetate (33), and acetate plus pyruvate (25). It must be mentioned, however, that evidence for the latter two pathways necessitated the use of both large amounts of protein (250 to 570 mg) and 6-hr incubation periods. In view of the ability of *A. suboxydans* to synthesize glutamate, it is difficult to explain why this organism grows poorly in the absence of added glutamate (31), but it may be a reflection of the limited amounts of active enzymes available to perform this function. This is suggested by the low specific activities of aconitase and isocitrate dehydrogenase found in *A. suboxydans* extracts.

The ability of whole cells to synthesize glutamate, as well as the presence of the remaining enzymes of the tricarboxylic acid cycle, is presently under study.

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