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Glutamate Biosynthesis in *Acetobacter suboxydans*. VI. Formation from Acetate plus Pyruvate*

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ABSTRACT: *Acetobacter suboxydans* has been reported to lack a demonstrable Krebs tricarboxylic acid, the conventional route for the biosynthesis of vital cell components such as glutamate. In a study of possible precursors of glutamate in this organism, it was found that acetate plus pyruvate can serve as a source of glutamate.

The label in the C_1 carbon of glutamate, however, is not in accord with the formation of this amino acid *via* the Krebs cycle. As a possible route to glutamic acid formation from acetate plus pyruvate, the reverse of Barker's scheme for the fermentation of glutamate in *Clostridium tetanomorphum* has been assessed. The enzymatic formation of citramalate, mesaconate, and β-methylaspartate from acetate plus pyruvate in *A. suboxydans* point to the possibility of participation of this pathway in the biosynthesis of glutamate in this organism.

Despite the fact that *Acetobacter suboxydans* is an obligate aerobe, it has been reported by several investigators that no evidence for activity of either the Krebs tricarboxylic acid cycle or for the complete scheme of glycolysis can be obtained by standard manometric, chemical, and radioactive tracer experiments, to account for the complete terminal dissimilation of glucose (Fewster, 1958; Cheldelin, 1961; King and Cheldelin, 1952, 1954; Hauge *et al.*, 1955; Kitos *et al.*, 1957; Rao, 1957). The phosphorylative scheme of carbohydrate dissimilation known as the pentose cycle accounts for the complete dissimilation of glucose or glycerol in *A. suboxydans* (Kitos *et al.*, 1958; Cheldelin, 1961). Except for the inability to supply immediate precursors of amino acids, the pentose cycle, which is the major carbohydrate dissimilation pathway in *A. suboxydans*, plays a role analogous to the tricarboxylic acid cycle in many other tissues, namely to satisfy the energy requirements.

On the other hand the fact that *A. suboxydans* has considerable and varied biosynthetic capacities is illustrated by the simple composition of the chemically defined growth medium (Stokes and Larsen, 1945; Cheldelin, 1961). The organism must therefore possess an appropriate mosaic of enzymes, and alternate routes were suspected to have functional existence, to account for the biosynthesis of numerous vital cell components (such as amino acids) which would otherwise arise through the Krebs cycle from the carbon skeletons

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TABLE I: The R_F Values[a] of Glutamate and β-MA Isolated from *A. suboxydans*.

Solvents	Ratio (v/v)	Glutamate			β-Methylasparate		
		Enzymatic Product	Ref	Cochroma-tography	Enzymatic Product	Ref	Cochroma-tography
Pyridine–water	65:35	0.52	0.50	0.52	0.55	0.56	0.58
1-Butanol–pyridine–water	1:1:1	0.18	0.19	0.18	0.21	0.21	0.21
Pyridine–acetic acid–water	50:35:15	0.50	0.50	0.51	0.42	0.41	0.42
Ethanol–water	70:30	0.68	0.68	0.68	0.69	0.68	0.68
1-Butanol–acetic acid–water	12:3:5	0.32	0.32	0.32	0.35	0.36	0.35

[a] Paper chromatography (ascending) on Whatman No. 1 paper in chromatocab (Research Specialties Co.).

of glucose and glycerol of their dissimilation products. One such scheme has been described (Sekizawa *et al.*, 1966) utilizing a condensation between glyoxalate and oxalacetate. In this report the isolation of citramalate, mesaconate, and β-methylaspartate is described and their formation in relation to the biosynthesis of glutamic acid is discussed.

Materials and Methods

Organism. A. suboxydans ATCC No. 621 was the organism used. The culture was maintained on a 0.5% agar slant containing 5% glycerol, 0.5% yeast extract, and 0.25% KH_2PO_4, adjusted to pH 6.0 and transferred at weekly intervals.

Cells used in these experiments were grown in a 100-l. fermenter or in 10 carboys as described by King and Cheldelin (1952). The cell homogenates were prepared by suspending 5 g of lyophilized cells in 20 ml of 0.1 M phosphate buffer, pH 7.5, and 20 ml of distilled water and treating them for 30 min in a Raytheon ultrasonic vibrator at 1.25-amp current output. Cell-free extracts were obtained by centrifuging the above homogenate at 8000*g* for 30 min.

Protein was measured by the method of Lowry *et al.* (1951), or turbidimetrically after the addition of trichloracetic acid.

Paper Chromatography. Paper chromatographic detection of amino acids has been carried out using 85% phenol–water in an atmosphere containing 3% ammonia or 1-butanol–acetic acid–water (12:3:5) on Whatman No. 1 paper. Other solvent systems used are listed in Table I.

Organic acids were well separated in a 1-butanol–formic acid–water (4:0:7:1) system, and detected by spraying with brom cresol blue solution. Autoradiograms were obtained by exposure of X-ray films to the chromatograms for 1–5 weeks, depending on the amount of radioactivity on the chromatogram.

Column Chromatography. Acidic amino acids were separated by adsorption on a Dowex 1-acetate column (66 × 1.0 cm) followed by gradient elution with 0.5 N acetic acid (Dekker, 1962). Fractions of 8 ml were collected and assayed for ninhydrin reaction and radioactivity.

Nonvolatile organic acids were extracted from the reaction mixtures as follows. The proteins were precipitated by heating (at 90° for 10 min) and then removed by centrifugation. The solution was then acidified with 6 N HCl to pH 2.5 and the organic acids were extracted with ether for 2 days in a liquid–liquid extractor. The ether was then evaporated under vacuum and the organic acids were separated on a Celite column as described by Phares *et al.* (1952), using a chloroform–butanol mixture as eluent. Amino acids were measured by a ninhydrin method (Rosen, 1957).

Degradations of Glutamic Acid. L-Glutamic acid from [1-^{14}C]acetate was recrystallized to constant specific activity and then degraded to determine the position of the label. For removing C_1 of glutamic acid, the Van Slyke *et al.* (1943) reaction was employed, and an apparatus suitable for wet combustion was used. Glutamic acid equivalent to about 0.5 mmole of CO_2 was mixed with 100 mg of Van Slyke citrate buffer and 20 ml of water, then frozen solid. Ninhydrin (300 mg) was then added, and the system was evacuated till the base in the second flask started boiling. Then the reaction mixture was heated for 8 min in a boiling water bath and the CO_2 was trapped in 0.5 N CO_2-free NaOH. The total radioactivity was measured by persulfate combustion (Chen and Lauer, 1957). The [^{14}C]CO_2 formed was counted as [^{14}C]Ba$^{14}CO_3$ in a thin window, gas flow, low-background counter (Nuclear-Chicago Corp.). Other radioactive materials were counted in a Tri-Carb liquid scintillation counter under counting efficiency of 44–48%.

Chemicals. DL-Citramalate (methylmalate) (mp 119°) was synthesized from KCN and ethyl acetoacetate by the method of Michael and Tissot (1892) and recrystallized several times from ethyl acetate; the recrystalliza-

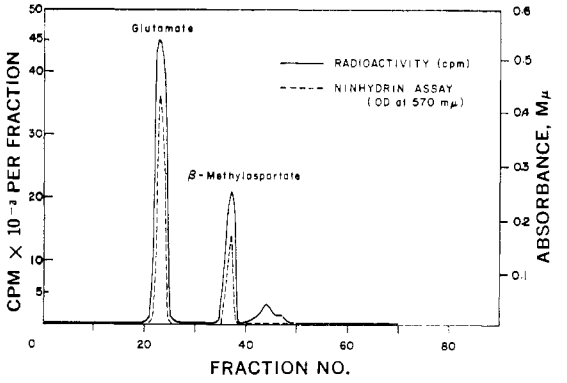

FIGURE 1: Aminodicarboxylic acids from the condensation of [^{14}C]acetate with pyruvate in *A. suboxydans*. Reaction mixture: [1-^{14}C]acetate, 20 μmoles (43 μc); pyruvate, 20 μmoles; alanine, 10 μmoles; vitamin B_6-PO_4, 0.5 mg; coenzyme A, 0.1 μmole; B_{12}, 0.1 μg; ATP, 10 μmoles; glutathione, 3 μmoles; $MgCl_2$, 5 μmoles; $FeSO_4 \cdot 7H_2O$, 0.1 μmole; $(NH_4)_2SO_4$, 10 μmoles; Tris buffer, pH 7.3, 100 μmoles; CFE, 5 ml (protein 57 mg/ml), total volume, 10 ml. Incubation at 30° for 6 hr. Proteins were precipitated and centrifuged out. Glutamate and β-methylaspartate (20 μmoles each) were added as carriers prior to the separation. The solution was adsorbed on Dowex 1-acetate column (66 × 1 cm) and the neutral compounds were washed away with 100 ml of distilled water. Acidic amino acids were then eluted exponentially with 0.5 N acetic acid and fractions of 8 ml were collected; 1 ml of each fraction was taken for counting of radioactivity and 0.5 ml for ninhydrin assay. Although the reaction mixture contained the constituents listed, it was not certain whether all of them were required and/or no attempt was made to test each cofactor added. Under the above conditions in controls with boiled enzyme no radioactivity was incorporated into the amino acid and organic acid fractions.

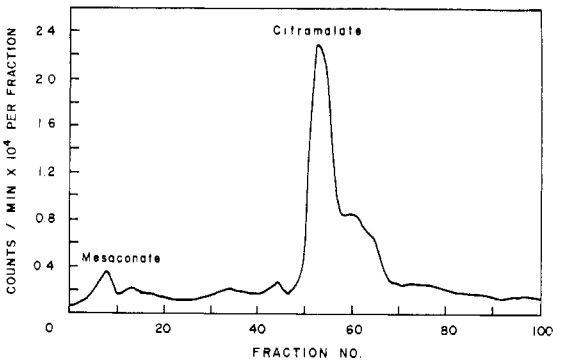

FIGURE 2: Organic acids from the condensation of [1-^{14}C]acetate plus pyruvate. Organic acids formed from an incubation mixture detailed in Figure 1 were extracted and separated on a Celite column as described in the text.

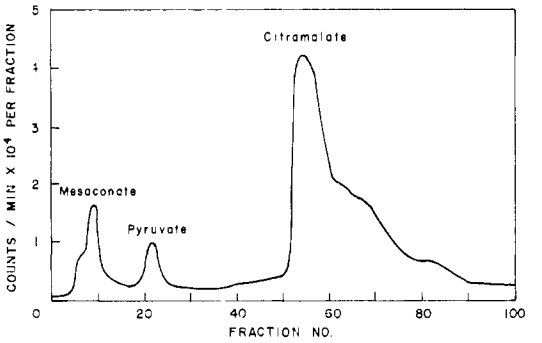

FIGURE 3: Organic acids from the condensation of [2-^{14}C]pyruvate plus acetate in *A. suboxydans*. Incubation mixture as in Figure 1. Instead of [1-^{14}C]acetate as radioactive precursor, [2-^{14}C]pyruvate was added with a total radioactivity of 2.08×10^7 cpm.

tion is best carried out by dissolving the substance in excess of ethyl acetate and then removing excess of solvent slowly *in vacuo*.

The other chemicals used herein were obtained commercially and used without further purification. These were obtained as follows: pyruvate, glutamate, alanine, β-methylaspartate, DPN$^+$,[1] DPNH, ATP, coenzyme A, vitamin B_{12}, B_6-phosphate, glutathione, and glutamic decarboxylase (from Sigma Chemical Co.). Mesaconic acid was obtained from K and K Laboratories, Inc. Charcoal Nuchar C-190, unground, was a gift of West Virginia Pulp and Paper Co.

Radioactive materials were obtained as follows: [1-^{14}C]acetyl coenzyme A, New England Nuclear Corp; [1-^{14}C]acetate and [2-^{14}C]acetate from California Corp for Biochemical Research. [1-^{14}C]Pyruvate and [2-^{14}C]-pyruvate were obtained from the California Corp. for Biochemical Research and from Merck and Co., Ltd., Canada.

Results

Aminodicarboxylic Acids Formed from [1-^{14}C]-Acetate plus Pyruvate. The elution pattern of acidic amino acids formed from an incubation mixture of [1-^{14}C]acetate plus pyruvate with CFE of *A. suboxydans* is shown in Figure 1. Under the experimental conditions detailed in the legends of Figure 1 the radioactivity peaks of glutamate and β-methylaspartate formed showed a coincidence with the peaks of ninhydrin assay of the carrier amino acids, which were added prior to the separation. The total amount of radioactivity incorporated into each fraction is shown in Table IIA. Glutamate and β-methylaspartate

[1] Abbreviations: β-MA, β-methylaspartic acid; CFE, cell-free extracts of *A. suboxydans;* DPN$^+$, DPNH, oxidized and reduced forms, respectively, of diphosphopyridine nucleotide; L-*erythro*- or D-*erythro*-β-MA and L-*threo*- or D-*threo*-β-MA, the respective antipodes of each racemate; ATP, adenosine triphosphate.

TABLE II: Radioactivity Recovered in the Acidic Amino Acid and Organic Acid Fractions from [1-^{14}C]Acetate or [2-^{14}C]Pyruvate.

Radioactive Substrate (cpm)	Total Radioactivity Recovered in Fraction (cpm)			
	Glutamate	β-Methyl-aspartate	Mesaconate	Citramalate
A [1-^{14}C]Acetate (4.4×10^7)	1.5×10^5	7.65×10^4	1.2×10^4	1.15×10^5
B [1-^{14}C]Acetyl coenzyme A (6.10×10^6)	2.7×10^5	8.5×10^4	7.6×10^3	3.11×10^4
C [2-^{14}C]Pyruvate (2.08×10^6)	1.05×10^5	1.98×10^4	2.25×10^4	4.5×10^5

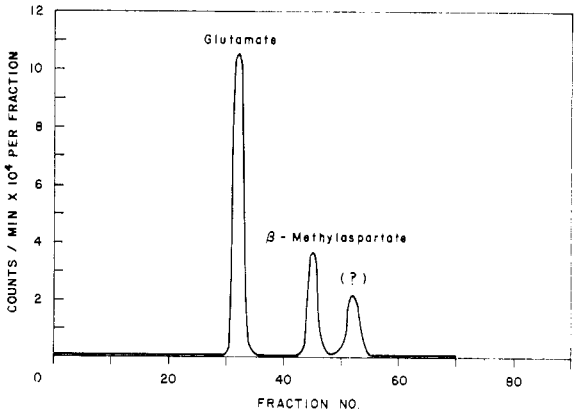

FIGURE 4: Acidic amino acids from [1-^{14}C]acetyl coenzyme A and pyruvate in *A. suboxydans*. The reaction mixture consisted of: [1-^{14}C]coenzyme A (sp act., 6 μc/mμmole); total amount added, acetate, 20 μmoles; pyruvate, 20 μmoles; alanine, 10 μmoles; CFE, 5 ml (protein 57 mg/ml). In the presence of the cofactors as in Figure 1, total volume, 10 ml, was incubated at 30° for 6 hr. Amino acids were separated as outlined in Figure 1.

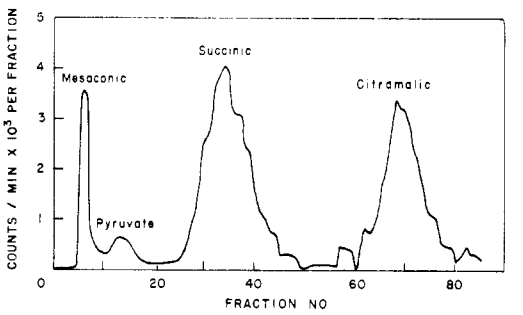

FIGURE 5: Organic acids from [1-^{14}C]acetyl coenzyme A and pyruvate in *A. suboxydans*.

fractions were identified further by paper chromatography and by recrystallization to constant specific activity as discussed later.

Isolation of Organic Acids from [1-^{14}C]Acetate Plus Pyruvate. From a concurrent experiment as outlined in Figure 1 the organic acids were extracted with ether and separated on a Celite column as described under *Materials and Methods*. Figure 2 is the elution pattern of the organic acids formed when [1-^{14}C]acetate is the radioactive precursor. The citramalate and mesaconate peaks were identified by paper chromatography. Total amount of radioactivity incorporated into each fraction is shown in Table IIA.

Isolation of Organic Acids from [2-^{14}C]Pyruvate Plus Acetate. A similar elution pattern of organic acids would be expected from a condensation of [^{14}C]-pyruvate with acetate, if citramalate and mesaconate were formed in this organism through such a condensation reaction. Organic acids formed from [2-^{14}C]-pyruvate and acetate separated as before on a Celite column are shown in Figure 3. Except for the pyruvate peak, the elution pattern of organic acids is essentially the same as when [1-^{14}C]acetate was the radioactive precursor. The total radioactivity incorporated into each organic or amino acid fraction was obtained by pooling all fractions corresponding to each peak and assaying for radioactivity suitable aliquots of the material obtained (Table IIC).

The identity of the peaks of citramalate and mesaconate from the above experiments was further established by mixing with authentic compounds, reseparation by paper chromatography, and elution of the compounds from the paper with ethanol. Under these conditions, most of the added radioactivity was recovered from the spots of the authentic compounds. The identities were further verified by resublimation of mesaconate to constant specific activity and recrystallization of citramalate and glutamate to constant specific activity as described later.

Evidence for the Participation of Acetyl CoA in Glutamate Formation. The condensation of acetate with pyruvate takes place presumably with acetate in the form of acetyl coenzyme A. Therefore, it might be expected that acetyl coenzyme A should be a better precursor than acetate for the formation of glutamate and β-methylaspartate if the formation of acetyl coenzyme A is the rate-limiting reaction.

[1-^{14}C]Acetyl coenzyme A incubated with CFE under the conditions detailed in Figure 4 gave much higher incorporation of radioactivity into amino acid

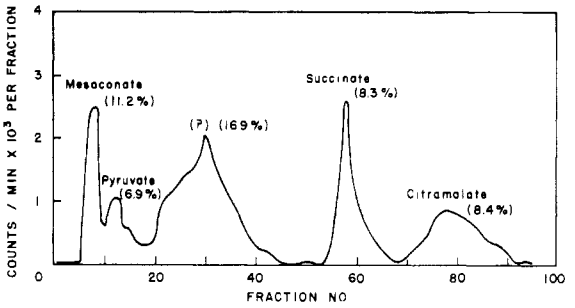

FIGURE 6: Organic acids formed from [^{14}C]mesaconate in *A. suboxydans*. The reaction mixture contained: [^{14}C]mesaconate, 30 mg (sp act. 2360 cpm/mg); alanine, 100 μmoles; glutathione, 30 μmoles; $MgCl_2$, 50 μmoles; $FeSO_4 \cdot 7H_2O$, 1 μmole; $(NH_4)_2SO_4$, 100 μmoles; Tris buffer, pH 8.0, 2 mmoles; CFE, 10 ml (protein 57 mg/ml). Incubation at 30° for 6 hr.

fractions than did [^{14}C]acetate (Table IIB). These results suggest that although acetyl coenzyme A is formed by cells of *A. suboxydans* from acetate, CoA, and ATP, the reaction rate is not high enough and may be the rate-limiting step in several reactions where acetyl coenzyme A is involved, including glutamate biosynthesis from acetate and pyruvate. This may also be related to the preference of this organism toward bound forms of pantothenic acid rather than toward the vitamin itself (Cheldelin, 1961).

Organic acids remaining on the column after amino acid separation (Figure 4) were eluted with 100 ml of 5 N acetic acid. The acetic acid was removed under vacuum and the organic acids formed from [1-^{14}C]-acetyl coenzyme A and pyruvate were separated as before on a Celite column. Figure 5 shows the results of this separation. Peaks of mesaconate and citramalate were obtained. Table IIB summarizes the amount of radioactivity incorporated in amino acid and organic acid fractions.

Characterization of the Isolated Products. GLUTAMIC ACID. The identity of the glutamate formed from radioactive substrates has been established as follows. The first indication was that the radioactivity peak of the formed glutamic acid coincided with the ninhydrin assay of authentic glutamic acid mixed with the reaction mixture prior to separation. It was further established by paper chromatography and radioautography. Finally, by recrystallizing [^{14}C]glutamate formed after mixing with authentic L-glutamate, the specific activity remained constant (Table III).

CITRAMALATE. The formation of this acid has been established by paper chromatography, radioautography, column chromatography as already described, and also by crystallization to constant radioactivity. The [^{14}C]citramalate fractions from several experiments were mixed with 500 mg of authentic DL-citramalate and then recrystallized from ethyl acetate. Changes of the specific activity with recrystallizations are summarized in Table IV. However, not until after seven recrystallizations did the specific activity remain constant.

A further indication of the identity of citramalate was obtained by isolating enough material from an incubation experiment of mesaconate with CFE, as described later, sufficient to measure optical rotation. In the presence of an excess of ammonium molybdate (29%) and under the conditions described by Krebs and Eggleston (1943), the $[\alpha]_D$ of the citramalate isolated was +1215° (0.2% solution). The reported values of optical rotation of citramalate are $[\alpha]_D$ +1200–1410° (see Barker, 1962).

MESACONATE. This acid, with a double bond conjugated to a carbonyl group, absorbs strongly at 230 mμ. Spots on paper chromatograms are readily observed under ultraviolet light, and these spots as well as those

TABLE III: Identification of [^{14}C]Glutamate and [β-^{14}C]-MA Formed by *A. suboxydans* by Recrystallization with Carrier.

No. of Recrystzn[a]	Sp Act. (cpm/mmole)	
	Glutamate	β-MA
1	2540	1500
2	2580	1170
3	2570	950
4	2520	750

[a] Conditions of recrystallizations as described by Barker *et al.* (1958). With glutamate the specific activity of the product of the fourth recrystallization is essentially the same as before recrystallizations.

TABLE IV: Identification of [^{14}C]Citramalate Isolated from *A. suboxydans* by Recrystallization with Carrier.

No. of Recrystzn	Sp Act.
	cpm/mg
1	2640
2	1665
3	580
4[a]	422
	cpm/10 mg
5	595
6	452
7	420
8	410
9	410

[a] At this point, an additional 100 mg of DL-citramalate carrier was added to continue the recrystallizations.

on the radioautographs derived therefrom were invariably compact and disk shaped.

On Celite column chromatography, the radioactive mesaconate formed coincides with the absorption at 230 mμ of the added authentic mesaconate. Reseparation by paper chromatography and elution of the mesaconate spot with alcohol recovers essentially all radioactivity in the [^{14}C]mesaconate fraction.

Additional proof of the identity of [^{14}C]mesaconate formed was established by sublimation of the [^{14}C]-mesaconate to constant specific activity. Radioactive mesaconate isolated from several experiments was mixed with 20 mg of authentic mesaconate dissolved in alcohol and dried. This preparation was then sublimed several times under 1.5 mm of pressure and temperature 110–140°. Table V shows the change in specific activity with sublimations.

TABLE V: Identification of [^{14}C]Mesaconate Isolated from *A. suboxydans* by Resublimation to Constant Specific Activity with Synthetic Carrier Mesaconate.

No. of Sublimation	Sp Act. (cpm/mg)
1	2920
2	2350
3	2200
4	2200

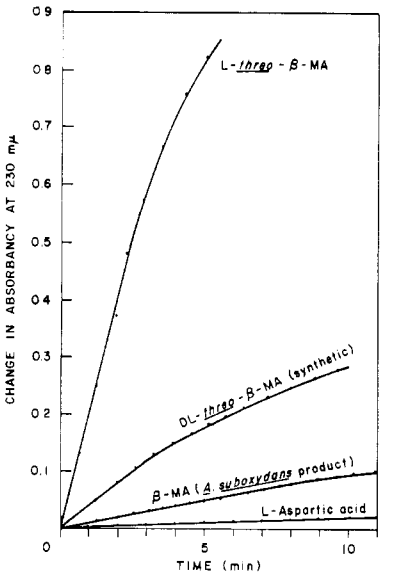

FIGURE 7: Comparison of the enzymatic reaction rates of L-*threo*-β-MA, DL-*threo*-β-MA (synthetic), aspartate, and *A. suboxydans* product by a purified preparation of β-methylaspartase. The reaction mixture for the β-methylaspartase assay contained ethanolamine chloride buffer, pH 9.7, 120 μmoles; amino acid, 1 μmole; KCl, 30 μmoles; $MgCl_2$ 3 μmoles; β-methylaspartase preparation (protein 0.1 μg/ml) and distilled water to a final volume of 3 ml. Readings were taken at 30-sec intervals after adding the enzyme.

Metabolism of Mesaconate in A. suboxydans. [^{14}C]-Mesaconate was prepared by mixing fractions of radioactive mesaconate obtained from several experiments with 40 mg of authentic mesaconate, followed by crystallization from alcohol, and then resublimation to constant radioactivity. [^{14}C]Mesaconate (30 mg) was thus obtained with specific activity 2360 cpm/mg. Incubation with CFE under the conditions detailed in Figure 6 yielded several organic acid peaks, when extracted as before with ether from the incubation mixture and separated on a Celite column. In addition to the remaining mesaconate peak it is shown that pyruvate and citramalate are also formed as expected from the citramalate–mesaconate pathway. Two other peaks are formed, one of which has been identified as succinic acid, but the other designated as (?) in Figure 6 has not been characterized. It has an R_F value on paper chromatograms the same as fumarate, but it does not have a high absorption coefficient at 230 mμ. The per cent of the added radioactivity recovered in each fraction is shown under each peak. Finally, as discussed above under "citramalate," the sample isolated from this experiment has been found to have optical rotation $[\alpha]_D$ +1215° under conditions prescribed by Krebs and Eggleston (1943).

Amino acids from the same experiment were separated on a Dowex 1-acetate column. Although only small amounts of radioactivity (about 1%) were incorporated into both glutamate and β-MA fractions because of the low specific activity of [^{14}C]mesaconate used and the small per cent conversion, both glutamate and β-MA formed were characterized by paper chromatography in several solvent systems (Table I).

β-Methylaspartate. The evidence for the formation of B-MA is the chromatographic coincidence of the radioactivity peak of [β-^{14}C]MA formed and the ninhydrin assay peak of authentic β-MA added to the reaction mixture prior to separation. Also, coincidence was observed on paper chromatography of the formed β-MA with the authentic compound in several solvent systems (Table I).

The labeled β-MA, however, obtained from the experiments described could not be recrystallized to constant specific activity upon mixing with synthetic DL-β-MA (Sigma Chemical Co.). Furthermore, DL-[β-^{3}H]MA, prepared by tritium gas exposure method of Wilzbach (1959) (New England Nuclear Corp., Boston), could not be converted to glutamate to any considerable extent. Data shown in Table III indicate that although 50% of the radioactivity remained after four recrystallizations, the specific activity gradually decreased. The synthetic DL-β-MA used contains almost exclusively the *threo* isomer (Benoiton *et al.*, 1954). Therefore it would be expected that only the *threo*

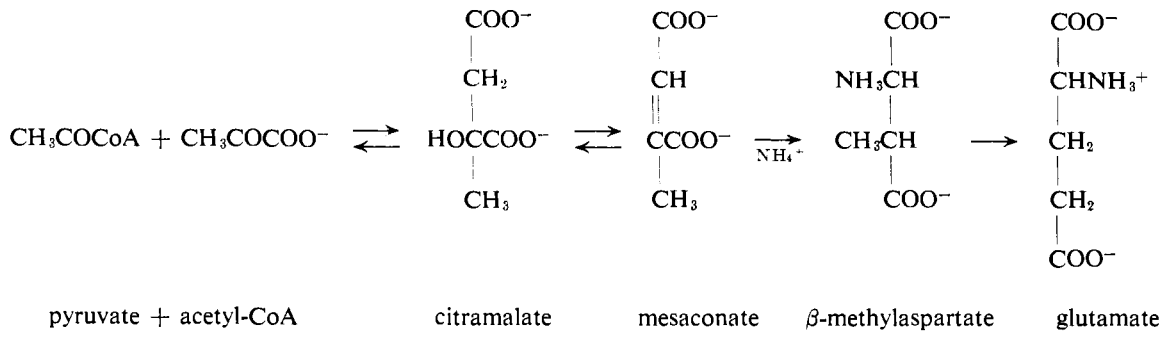

FIGURE 8: Proposed pathway for glutamic acid biosynthesis in *A. suboxydans*.

form would engage in recrystallization with the material added.

The identity of β-MA was further indicated by β-methylaspartase, a preparation from *Clostridium tetanomorphum*. Data shown in Figure 7 suggest that the β-MA isolated from *A. suboxydans* can serve as substrate for the purified β-methylaspartase preparation. The initial rate of conversion to mesaconate (change in absorbancy at 230 mμ), however, indicates that *A. suboxydans* product is not as good a substrate as is L-*threo*-β-MA. L-*erythro*-β-MA is known to be much less effectively metabolized to mesaconate by purified β-methylaspartase than is the L-*threo* isomer (Barker *et al.*, 1959). The product of *A. suboxydans* is probably L-*erythro*-β-MA.

This observation is also in agreement with our interpretation of the inability to crystallize the isolated [β-^{14}C]MA to constant specific activity upon mixing with synthetic DL-β-MA (Sigma product) which is known to contain essentially exclusively the *threo* racemate (Benoiton *et al.*, 1954).

Degradation of Glutamate. Localization of label in the glutamate molecule when a precursor such as [1-^{14}C]acetate is used would provide additional information for the reactions involved in its formation. Partial degradation of [^{14}C]glutamate formed from [1-^{14}C]-acetate with ninhydrin (Van Slyke *et al.*, 1943), after mixing with L-glutamate and crystallization to constant radioactivity (as in Table III), revealed that 56% of the total radioactivity was located on C_1. Glutamate formation through citramalate–mesaconate exclusively should yield 100% of the label on C_1 (adjacent to NH_2) but formation through the reactions of the Krebs tricarboxylic acid cycle should yield, after extensive recycling, 33% of the C_1 of glutamate. The observed value indicates that the Krebs tricarboxylic acid (or some similar cycling process) cannot yet be excluded completely as a participant in glutamate formation, and/or that other pathways may be operative along with the citramalate–mesaconate scheme.

Discussion

Several interesting possibilities may exist for the formation of glutamate without participation of the reactions of a functional Krebs tricarboxylic acid cycle in *A. suboxydans*. Such novel reactions have been demonstrated in other systems (Doudoroff *et al.*, 1956; Warburg *et al.*, 1957). In addition, a unique decomposition of glutamate by *Cl. tetanomorphum* has been reported by Barker and his co-workers (1958, 1959; Munch-Petersen and Barker, 1958; Wachsman, 1956), entirely different from the reactions of the Krebs tricarboxylic acid cycle. *Cl. tetanomorphum* ferments glutamate rapidly as follows

L-glutamate ⇄ L-*threo*-β-methylaspartate ⇄
mesaconate ⇄ citramalate ⟶ acetate + pyruvate

The intriguing possibility exists that this enzymatic reaction sequence, existing in nature in one system for fermentation purposes, may be employed by other organisms mainly as a biosynthetic route. In *A. suboxydans* it has been shown that either acetate or pyruvate can be incorporated into glutamate of the cells under growing conditions. Röhr (1961) has also suggested that glutamate may arise through an unspecified condensation of C-2 and C-3 carbon atom units.

The observations described in this paper suggest that acetyl coenzyme A and pyruvate condense in *A. suboxydans* to form citramalate and mesaconate, and point to the participation of these compounds along with β-MA in the biosynthesis of glutamate. On this basis, the proposed scheme in Figure 8 shows what may appear to be a logical order of their formation (essentially the reverse of Barker's scheme (1958) for glutamate consumption in *Cl. tetanomorphum*).

Although the intermediates involved are formed in relatively small yields, they have been characterized by several methods in the reaction mixtures with CFE. Experiments are continuing to attempt to evaluate the extent of these reactions in *A. suboxydans* as well as the existence of others which may give rise to important nitrogen sources within the cells of this organism.

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