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Title Biosynthesis of Aminodicarboxylic Acids in Acetobacter suboxydans

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Acetobacter suboxydans, although an obligate aerobe, has no demonstrable Krebs cycle, the conventional route for the biosynthesis of vital cell components such as glutamate and aspartate. The ubiquity throughout nature of this metabolic scheme raises the question of the origin of these and other amino acids in this organism; for both of these amino acids may be synthesized by A. suboxydans. The observations described suggest the functional existence of two alternative reaction sequences which can lead to glutamate biosynthesis, without participation of the reactions of the Krebs cycle.

The first is the reversal of the unique reaction scheme which is involved in the fermentation of glutamate in Clostridium tetanomorphum, shown by Barker and his coworkers. It starts with the condensation of pyruvate with acetyl-coenzyme A to form citramalate, which is converted

to glutamate via mesaconate and β-methylaspartate. The formation of all these intermediate compounds has been demonstrated in Acetobacter suboxydans when labeled pyruvate or acetate were used as substrates, and all are converted to glutamate by cell-free extracts of this organism. Evidence was also obtained for some metabolic interconversions among the isolated intermediates.

A second reaction sequence by which glutamate can be formed in this organism starts with the condensation of glyoxylate with oxaloacetate; a tricarboxylic acid (oxalomalate) is thus formed, which by decarboxylation yields α-hydroxy-γ-ketoglutarate, a well characterized metabolic product of Acetobacter suboxydans. α-Hydroxy-γ-ketoglutarate is converted to γ-hydroxyglutamate either by transamination or reductive amination, or metabolized to α-ketoglutarate, presumably by a dehydration and reduction step. Glutamate and γ-hydroxy-glutamate, the terminal products of this reaction sequence, have been well characterized from the reaction mixtures and all the available evidence points to the existence of a direct enzymatic reaction sequence leading to their formation from the condensation of glyoxylate with oxaloacetate.

It is of significance that a similar non-enzymatic condensation of glyoxylate with oxaloacetate to form

oxalomalate can take place; the latter proceeds essentially to completion at pH 7.4 and 40°C within 2.5 hours. The non-enzymatic reaction, however, does not occur at pH 6.0 and 30°C, whereas the enzymatic reaction proceeds to a significant extent leading to stereospecific products. The non-enzymatic condensation has been advantageously employed for the synthesis of oxalomalate and from it, α-hydroxy-γ-ketoglutarate and γ-hydroxyglutamate.

Oxalomalate, although not isolated from Acetobacter suboxydans, probably participates in the biosynthesis of glutamate and γ-hydroxyglutamate, as suggested from the fact that synthetic oxalomalate is converted to these amino acids in this organism and also decarboxylated to α-hydroxy-γ-ketoglutarate.

The formation of oxalomalate and α-hydroxy-γ-ketoglutarate may be of significance not only in relation to glutamate and γ-hydroxyglutamate biosynthesis but also as at least partial explanation for the apparent absence of the Krebs cycle from this obligate aerobe, since both these acids have been reported recently to be potent inhibitors of the key enzymes of the Krebs cycle.

BIOSYNTHESIS OF AMINODICARBOXYLIC ACIDS IN *ACETOBACTER SUBOXYDANS*

by

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BIOSYNTHESIS OF AMINODICARBOXYLIC ACIDS IN ACETOBACTER SUBOXYDANS

INTRODUCTION

Despite the fact that A. suboxydans is an obligate aerobe, it has been reported by several investigators that no evidence for the Krebs tricarboxylic acid cycle activity or 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 (23, 37, 38, 40, 62). This fact probably accounts for the low oxidizing power that this organism possesses which is reflected in the name conferred upon this organism (42). Although this organism can oxidize a variety of carbohydrates and polyalcohols, it is unable to do so beyond the first or second oxidation step. Some of these oxidations are: alcohol to acetic acid, glucose to gluconic and ketogluconic acids, glycerol to dihydroxyacetone, sorbitol to sorbose, erythritol to erythrulose,

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The following abbreviations will be used:
ATP, adenosine triphosphate; ADP, adenosine diphosphate; CFE, cell-free extracts of A. suboxydans; CoA, Coenzyme A; DPN, diphosphopyridine nucleotide; HGA, γ-hydroxyglutamate; HKG α-hydroxy-γ-ketoglutarate; TCA (Krebs) tricarboxylic acid cycle; OAA, oxaloacetate; α-KG, α-ketoglutarate.

inositol to inosose, perseitol to perseulose, 2,3 butanediol to acetoin, etc., (15).

Some of the above well defined metabolic events characterize certain important industrial fermentations for which A. suboxydans has been employed for centuries (27, 71, 75).

In addition to the above direct oxidations, the phosphorylative scheme of carbohydrate dissimilation known as the pentose cycle (31) and to a small extent the Entner-Doudoroff pathway (22) account for the complete dissimilation of glucose and glycerol in A. suboxydans.

Except for the inability to supply immediate precursors of amino acids, the pentose cycle, which is the major dissimilation pathway of glucose in A. suboxydans, plays a role analogous to the tricarboxylic acid cycle in many other tissues, namely to satisfy the energy requirements.

The organism must, however, possess a mosaic of enzymes to account for the biosynthesis of numerous vital cell components (such as amino acids) which normally arise through the Krebs cycle, from relatively simple carbon skeletons of glucose and glycerol or their dissimilation products.

The fact that A. suboxydans has considerable and

varied synthetic capacities is illustrated by the simple composition of the chemically defined growth medium (70). A medium consisting of glycerol, salts, essential growth factors (pantothenic, nicotinic, and p-aminobenzoic acid) and the amino acids valine, isoleucine and alanine can support growth of A. suboxydans, although slowly.

In order to satisfy its nutritional requirements when growth in the minimal medium of Stokes and Larsen (70), A. suboxydans must be capable of converting the carbon skeleton of glycerol or its dissimilation products to those of the needed amino acids.

CO_2 fixation reactions have been reported (39) to partake in glutamic, aspartic and alanine biosynthesis in this organism, but of course, these cannot occur through the mediation of the citric acid cycle.

Several interesting possibilities may exist for the formation of glutamic and aspartic acids which are briefly noted in the review of the literature section. Among them the novel conversion of arabinose to α-ketoglutarate which was discovered by Doudoroff and coworkers (20), the carboxylation of γ-aminobutyrate (81) can yield glutamic acid without the participation of TCA cycle. Likewise, carboxylation of phosphoenol pyruvate to oxaloacetate could

lead to aspartic acid as in wheat germ (72). Through these or other possibilities, one would expect to explain the biosynthetic pathways leading to non-essential amino acids.

The objectives of this work have been to establish biosynthetic pathways existing in this organism which would account for the biosynthesis of amino dicarboxylic acids.

Work presented here indicates a functional existence of two pathways which may account for the formation of glutamic acid in A. suboxydans, without participation of the Krebs cycle. The first starts with the condensation of the regular metabolites, acetate and pyruvate, to yield citramalate which, by way of mesaconate and β-methylaspartate, is converted to glutamic acid. This pathway is named the citramalate-mesaconate pathway and is the reversal of Barker's scheme (55) for glutamic acid fermentation in Clostridium tetanomorphum. This, to the author's knowledge, is the first report which accounts for the participation of this pathway in glutamate biosynthesis; the pathway may be widespread in tissues in general.

A second pathway leading to glutamic acid through a series of intermediates starts with the condensation of glyoxylate and oxaloacetate, to form oxalomalate. The decarboxylation of oxalomate leads to α-hydroxy-γ-ketoglutarate.

The latter is the precursor of γ-hydroxy-glutamate which has been well characterized in A. suboxydans. α-Hydroxy-γ-ketoglutarate can also form α-ketoglutarate, probably through a dehydration and hydrogenation step. This pathway is called the glyoxylate-oxaloacetate pathway. It may be of significance that several intermediates of this pathway have been reported to be potent inhibitors of key enzymes of the Krebs cycle, which appear to be largely non-functional in this organism. In addition, glutamic acid can be formed in A. suboxydans from other carbohydrate- and amino acid-nature materials. Indirect evidence points to the formation of aspartic acid through malate synthetase and malate dehydrogenase. These reactions are probably of importance for the formation of oxaloacetate, one starting material of the glyoxylate-oxaloacetate pathway.

REVIEW OF THE LITERATURE

The important role of dicarboxylic amino acids in nitrogen metabolism, first of all indicated by the high content of these amino acids in the proteins and tissues in general, led to the investigation of the enzymatic mechanism of the biosynthesis of these amino acids.

Aspartic Acid

Aspartic acid biosynthesis was shown to arise from oxaloacetate. The latter compound is of widespread occurrence and is one of the most important known components of the known group of tricarboxylic and dicarboxylic acids. The ease with which this amino acid is related to the TCA cycle and/or can be synthesized from carboxylic acids has been shown in experiments with isolated rat diaphragm (51). In these experiments with all common amino acids, arising from protein, only alanine, aspartate and glutamate incorporated isotope when it was administered as citrate, succinate, isobutyrate, propionate or acetate.

In Staphylococcous aureus, although U-C^{14}-glucose leads to C^{14}-labeled aspartate, the glutamate and all intermediates of the cycle are unlabeled, indicated that the Krebs or glyoxylate cycles are incomplete. The most likely alternative route for the incorporation of carbon from glucose into aspartic acid appears to be via the formation of OAA from pyruvate, involving fixation of CO_2. Indeed the growth of this organism can be stopped by rapid passage of CO_2-free air through the medium and this can be reversed by the addition of aspartic acid. Such a mechanism has been shown to occur in a number of species of Lactobacilli and Streptococci (30). There have been reported five enzymes, or enzyme systems, which can form C_4

dicarboxylic acids from C_3 by CO_2 fixation (5, 30, 32, 66, 76, 82). Any of these reactions may form aspartate from C_3 carboxylic acids.

In microorganisms, there is proof available that aspartic acid can be formed through the participation of aspartase which catalyzes the combination of NH_3 with fumaric acid. Wilkinson and Williams (83) have purified aspartase and thus separated this enzyme from fumarase. In the case of plants, there are several reports indicating the possible participation of aspartase, but direct fixation from ammonia into aspartic acid seems unlikely in mammals since mammalian tissues are not known to have enzymes which form aspartate from NH_3 plus fumarate.

The enzymatic condensation of glyoxylate with acetyl-CoA to give malate was discovered by Wang and Ajl and the enzyme malate synthetase is widely distributed (43). This enzyme strongly favors malate synthesis and can therefore, in connection with malic dehydrogenase, yield OAA and thus aspartate.

Glutamic Acid

Glutamate usually is formed by reductive amination of α-ketoglutarate with ammonia (47).

In addition to this type of biosynthesis of glutamate, an apparently important role is also played in plants by the synthesis of it from the reaction of CO_2 and γ-

aminobutyric acid (81).

The novel conversion of arabinose to α-KG that was discovered by Doudoroff and coworkers (20) must also be mentioned as another alternative route leading to glutamic acid and not related to the Krebs cycle.

There are a number of observations in the literature which cannot be explained on the basis of the above mentioned pathways of glutamic and aspartic acid biosynthesis, suggesting the existence of alternative pathways (59, 73). Tomlinson (73), studying the distribution of label in isolated glutamic and aspartic acids from Clostridium kluyveri grown in a synthetic medium containing $C^{14}O_2$ or acetate-1-C^{14}, observed that the labeling pattern data are not consistent with the view that the two amino acids are formed through the conventional reactions of Krebs cycle. Similar studies by Nelson and Krotkov (59) with bean leaves showed similar inconsistencies. As a possible explanation, Tomlinson (73) assumed that the double bond produced in the aconitase reaction was formed between the central carbon atom of cis-aconitate and the carbon atom originating from the methyl group of acetate and not from the methylene carbon derived from OAA. This change in the position of the double bond formed in the aconitase reaction would explain the labeling patterns. Several other possible explanations are proposed, but without any experimental evidence. Similar explanations are also offered by Nelson and Krotkov (59). In addition, they also stated that "a $C_3 + C_2$

condensation to yield a precursor of glutamate would be consistent with the distribution of labeling data."

Metabolic Role of Amino Dicarboxylic Acids

One of the most important aspects of the role of dicarboxylic amino acids is that they are the source of amino groups for the reaction of enzymatic transamination with keto acids. Especially commonplace is the entry of glutamate into the transamination reactions. These reactions in connection with the oxidative deamination of glutamate to α-ketoglutarate (α-KG) by glutamic dehydrogenase illustrates the multiple functions of glutamic acid in the metabolism of the cell.

Krebs and Bellamy (45) and later others, showed that in a variety of tissues, under appropriate conditions, there is an accumulation of aspartate almost equal to the disappearance of glutamate. On the basis of these observations, they concluded that glutamate is oxidized to aspartic and CO_2 by catalytic amounts of OAA. These findings militate against the notion of the importance of glutamic dehydrogenase in the glutamic acid oxidation.

On the other hand, through the TCA cycle reactions and the urea cycle, a portion of glutamic acid can be oxidized through glutamic dehydrogenase to α-KG. This would then be expected to undergo further oxidization by the citric acid cycle to OAA and a portion of the OAA might then be converted to aspartate by transamination with some of the remaining glutamic acid. Glutamic acid thus furnishes both the nitrogen and the carbon skeleton for aspartic acid, and also supplies ATP through the oxidative steps (14).

Fermentations of Glutamate

In addition to the above mentioned metabolic processes in which glutamic acid is involved, a unique decomposition of glutamate by Clostridium tetanomorphum has been reported by Barker and coworkers (6, 7, 55, 79), which is entirely different from the TCA cycle.

Clostridium tetanomorphum ferments glutamate rapidly as follows:

L-glutamate ⇌ L-threo-β-methylaspartate ⇌ mesaconate

Acetate + pyruvate ⟵ citramalate

In addition to Barker, Wilson and Coeppe (84) have suggested that glutamate may be catabolized in rats by a pathway other than the direct conversion to succinate via α-KG.

Their conclusion is based on the fact that the methyl carbon of acetate was labeled when glutamate-2-C^{14} was injected into the cecum of rats and that this is compatible with the conversion via the mesaconate-citramalate pathway. This is presumably effected by the intestinal flora, and it has been invoked by way of explanation.

β-Methylaspartate. The conversion of glutamate to β-methylaspartate has been shown to require a vitamin B_{12}-containing coenzyme.

Benoiton (12) synthesized β-methylaspartate by the chemical method of Dakin by the condensation of diethyl benzamidomalonate with ethyl-α-bromopropionate. However, both Dakin and Benoiton observed the presence of only a single racemic form despite the two asymmetric carbons of the molecule. No evidence for a second racemate was obtained, either from melting point determinations of the recrystallized product from several solvents or from fractionations in a Dowex-1-acetate column.

The L-threo isomer was enzymatically prepared, isolated and characterized by Barker and coworkers (6). They were also able to observe the second diastereoisomeric racemate in the mother liquid of the crystallization of the synthetic product, by sensitive enzymatic and

electrophoretic criteria. Benoiton (12) separated the D,L isomers of β-methylaspartate by acylase II and thus provided independent evidence for the assignment of the Clostridium tetanomorphum product as L-β-methylaspartic acid.

In addition to the participation in the mesaconate-citramalate pathway of glutamate fermentation, the metabolic interest of β-methylaspartic acid was associated with the B_{12} requirement for the synthesis of deoxynucleic acids. This in turn suggested that β-methylaspartate and its carbamyl derivative may be intermediates in the thymine synthesis, analogous to the formation of uracil from aspartic acid (35). This possibility, however, when tested in various ways (1, 86), gave no evidence for such conversion. β-Methylaspartate has no growth activity for a thymine deficient strain of E. coli, but on the contrary, acts as a growth inhibitor of E. coli (1), apparently acting through competition with aspartate utilization for pyrimidine synthesis.

Experiments with β-methylaspartate-C^{14} showed that isoleucine-C^{14} can be formed in E. coli, and from the labeling patterns, it appears that β-methylaspartate is first converted to methyloxaloacetate by transamination

and then is decarboxylated to α-keto-butyric acid (1).

Mesaconate. The enzyme which catalyzes the reversible conversion of L-threo-β-methylaspartate to mesaconate has been purified by Barker et al (7) and a crystalline preparation of β-methylaspartase was reported by Bright and Ingram (13). Aspartate, which can also undergo a similar reaction to fumarate, is only 1/100 as active as β-methylaspartate. L-threo-β-methylaspartate is the normal substrate for β-methylaspartase although weak activity was seen with the L-erythro compound.

Citramalate. The hydration step of mesaconate to citramalate was not investigated in detail in Clostridium tetanomorphum (6). Katsuki and coworkers (36) partially purified the mesaconate hydrating enzyme from Pseudomonas fluorescens. In this purified preparation, Fe^{++} was required and the reaction proceeded stochiometrically and reached the equilibrium state when citramalate to mesaconate was present in a ratio of about 6:1.

Citramalate has been also isolated from peel of apple fruits and it was associated with the acetone given off by the apples (34). The hydration of citraconate to form citramalate has been reported recently in Pseudomonas fluorescens, when this organism was grown on citraconate

as the sole carbon source (63).

Evidence for the enzymatic interconversion of citramalate and acetate plus pyruvate has been obtained from studies in a variety of systems (17, 29, 55, 56, 80); but to our knowledge, the enzyme catalyzing the interconversion has not been characterized, nor has the mechanism of this reaction been established.

Gray and Konberg (29) demonstrated the reversible interconversion of acetate and pyruvate to citramalate in Pseudomonas ovalis chester, and showed that the equilibrium favors the cleavage rather than its formation. They described the enzyme catalyzing the interconversion as "pyruvate transacetase" by analogy with other enzymes catalyzing the transfer of acyl groups.

In contrast to the Pseudomonas ovalis chester, this reaction is not reversible in liver mitochondria as shown by Wang (80). In this system, although citramalyl-CoA was found to be an intermediate in the metabolic pathway of itaconate to pyruvate and acetyl-CoA, free citramalate was not metabolized. On the other hand, in Pseudomonas fluorescens, the same pathway, as in liver mitochondria, was found for itaconate metabolism (56), but in this system, the citramalyl-CoA, formed by CoA transfer from

succinyl-CoA, is cleaved to pyruvate and acetyl-CoA. This reaction was also found in Pseudomonas B_2 aba by Cooper and Kornberg (17).

Acetate plus pyruvate. The formation of citramalate from acetate-C^{14} and pyruvate has been also shown in Rhodospirillum rubrum by Benedict (11) and in chromatium extracts by Losada et al (49). In both reports, it is indicated that the formation of citramalate may represent an intermediate step in the synthesis of glutamate during the photometabolism of acetate. Later results of Fuller (26), however, based on the degradations of glutamate-C^{14} formed from acetate-C^{14}, are not consistent with a synthesis via citramalate and also the results exclude the operation of Krebs cycle.

Remarks

From the preceding short review of the literature, it appears that a complete pathway of glutamate fermentation exists in Clostridium tetanomorphum in which all the reactions involved except the cleavage of citramalate to acetate and pyruvate, have been shown to be reversible. The condensation of acetate and pyruvate, however, to form citramalate has been demonstrated in a variety of systems.

These have been considered as suggestive evidence that the mesaconate-citramalate scheme is of widespread occurrence in the variety of systems, where certain individual reactions have been demonstrated, and possibly in others where no extensive investigations have yet revealed these reactions.

It was intriguing to us to see the existence of a unique enzymatic reaction sequence in nature and yet to find it existing in only one system for fermentation (catabolic) purposes. Why does it not exist in other systems and moreover why has it not been employed by organisms as a synthetic route for glutamate and β-methylaspartate as well as citramalate and mesaconate biosynthesis?

This possibility was more than a matter of general curiosity in the present work since we were investigating the glutamate biosynthesis in A. suboxydans, an obligate aerobe, without a functioning Krebs cycle. Evidence to be presented herein indicates that glutamate can be formed in this organism from acetate and pyruvate through the citramalate-mesaconate pathway.

Glyoxylate-oxaloacetate Condensation

Early in 1958, Ruffo and coworkers showed that

aconitase was strongly inhibited when incubated in the presence of small amounts of glyoxylate and oxaloacetate (18, 64). This observation arose through the investigation of the long known toxic effect of glyoxylate to animals (4,10) and to the respiratory activity of various tissue homogenates, which Kleinzeller (41) attributed to a specific inhibition of decarboxylation of pyruvate. Several other investigators (58, 64) correlated the degree of inhibition with the concentration of glyoxylate.

Ruffo, Romano and Adinolfi (64), studying the effect of glyoxylate on the oxidation of components of the TCA cycle in rat liver homogenates, were the first to observe that the glyoxylate inhibition was strongly enhanced by oxaloacetate and that it is associated with the accumulation of citrate. They suggested that both the repression of oxygen uptake and the formation of citrate are related to the two different ways in which oxaloacetate may react in the liver cells with acetyl-coenzyme A to give citrate and with glyoxylate to give rise to an inhibitor of citrate oxidation. Chromatographic evidence showed that the inhibitory effect of glyoxylate is due to the formation of this new condensation product, namely oxalomalic acid, which is a potent inhibitor of both crude and purified

aconitase. Recently, they reported the preparation of oxalomalic acid synthetically and studied the mode of action of oxalomalate on aconitase (65).

The glyoxylate-OAA condensation is similar to the long known formylation, discovered by Tollens in 1884, in which acidic-CH-groups react with formaldehyde to yield a C-C link. This reaction has been employed for the preparation of amino alcohols and is catalyzed by alkali metals (24).

The condensation reaction taking place between the $-CH_2-$ group of OAA and the aldehyde group of glyoxylate to yield the C_6-tricarboxylic acid (α-hydroxy-β-oxalosuccinic) has not been considered, to our knowledge, in any system as an enzymatic reaction of biological significance, except for the inhibitory effect on aconitase. In A. suboxydans, however, glyoxylate and OAA can serve as a precursor of glutamate with the concurrent formation of γ-hydroxyglutamate. This observation in connection with the expected condensation of glyoxylate with OAA led us to the speculation that an enzymatic reaction sequence as the one outlined in Figure 11 may be the reactions leading to glutamate and γ-hydroxyglutamate (67). Evidence presented herein and elsewhere (52, 68, 69) indicates that the reaction sequence of Figure 11 may represent a new pathway for

the glutamic and γ-hydroxyglutamic acids as well as of the intermediates biosyntheses, which may be of widespread occurrence in tissues in general. γ-Hydroxyglutamate (HGA) formation might be considered suggestive of the participation of this pathway in other tissues, although HGA may also arise as a metabolic intermediate of the degradation of hydroxyproline in rats (28). HGA was found in Phlox decussata leaves, from which source Virtanen and and Hietala (78) first isolated this compound.

MATERIALS AND METHODS

Organism

Acetobacter suboxydans ATCC No. 621 was the organism used. The culture was maintained in a 0.5 percent agar slant containing 5 percent glycerol, one percent yeast extract and 0.5 percent KH_2PO_4 adjusted to pH 6.0 and transferred at weekly intervals.

Inoculum cultures were prepared by transferring a loopful of bacteria to a liquid medium of the foregoing composition (agar omitted) and incubating for 48 hours at 30°C on a shaker. Cells used in these experiments were grown in a 100 l. fermenter or in 10 l. carboys as

described by King and Cheldelin (37).

The cell homogenates were prepared by suspending 5 grams 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 minutes in a Raytheon ultrasonic vibrator at 1.25 ampere current output. To insure that the temperature did not exceed 3°C, the treatment was periodic, with 2 min. 1.25 amperes and 2 min. zero current output.

Cell-free extracts were obtained by centrifuging the above homogenate at 8,000 x g for 30 minutes.

Depletion of A. suboxydans Extracts of "Cofactor Activity"

Treatment of the CFE with charcoal yielded preparations deficient in cofactor activity (7, 53). Charcoal was prepared in the following manner: Nuchar 190 unground charcoal was suspended in 3N HCl and left standing overnight, then washed with distilled water to constant pH of about 5.0, washed once with 0.01M phosphate buffer pH 5.0 and again washed with water.

To 100 mg. of the above charcoal suspension were added 5 ml. of CFE containing 25-40 mg. of protein per ml. The mixture was stirred for 15 minutes in an ice bath and centrifuged at 25,000 x g for 15 minutes in a Servall

centrifuge. A clear charcoal treated extract was thus obtained.

Elution of Cofactor Activity from Charcoal

The charcoal, separated from the extracts by centrifugation, was suspended in 20 volumes of 50 percent aqueous acetone and stirred for 15 minutes in an ice bath in the dark. The suspension was centrifuged and the supernatant evaporated to dryness in vacuo. The residue was dissolved in a small amount of water, recentrifuged and the supernatant fraction was used as a source of the cofactor.

A culture of E. coli ATCC 11216, used for the preparation of glutamic decarboxylase was obtained from the American Type Culture Collection. Cells of E. coli were grown as described by Umbreit et al (74) and the glutamic decarboxylase was purified from these cells of E. coli by the method of Najar and Fisher (57).

Protein was measured by the method of Lowry (50), or turbidimetrically after the addition of trichloroacetic acid.

Paper Chromatography

Paper chromatographic detection of amino acids has been carried out using 85 percent phenol-water in an

atmosphere containing 3 percent ammonia or n-butanol-acetic acid-water (12:3:5) on Whatman No. 1 paper. Amino acids, including glutamic, β-methylaspartic, aspartic, HGA, were well separated in one or two dimensional paper chromatography by these solvent systems.

Organic acids were well separated in n-butanol-formic acid-water (4:0.7:1) system. For the 2,4 dinitrophenyl-hydrazones of the ketoacids, the solvent system n-butanol-ethanol-ammonia (70:20:10) or n-butanol saturated with 3 percent ammonia were employed.

Radioautograms 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 absorption on a Dowex-1-acetate column (67 x 1.0 cm.) followed by gradient elution with 0.5 N acetic acid (19). Fractions of 8 ml. were collected and assayed for ninhydrin reaction and radioactivity.

Organic acids were extracted from the reaction mixtures after precipitation of the proteins by boiling (at 90°C for 10 minutes) and acidifying the solution with 6N

HCl to pH 2.5 after centrifuging out the proteins. The organic acids were extracted with ether for two 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 (61), using chloroform-butanol mixture as eluant.

Amino acids were measured by a ninhydrin method (19). Glutamic acid was also determined manometrically by glutamic decarboxylase (commercial preparation) or purified from E. coli (57).

Keto acids were measured by the Friedemann-Haugen method (25) and other organic acids by acidimetric titration.

Citramalate was determined by the method of Egashira (21) spectrophotometrically, using cis-dichlorobis (ethylenediamine) chromium III chloride and mesaconate by measuring the absorbancy at 230 mμ (36).

Degradation of Glutamic Acid

Glutamic acid isolated from radioactive precursors was degraded after recrystallization with L-glutamic acid, to determine the localization of the label.

The Schmidt reaction was employed for measuring the

radioactivity on carbon No. 5 (2). One and .15 mmoles of hydrazoic acid (85) in 48 cc of chloroform was added in portions during 6 hours to glutamic acid (1 mmole dissolved in 10 ml. chloroform and 25 cc concentrated sulfuric acid), stirred at 43°- 46°C. Stirring was continued for four hours after the addition of hydrazoic acid. A suitable apparatus was employed for flushing out the CO_2 formed (from carbon No. 5 of glutamate) by CO_2-free dry air and trapping the CO_2 in a 0.5 N NaOH solution (CO_2-free).

For removing C_1 of glutamic acid, the Van Slyke reaction (77) was employed. An apparatus suitable for wet combustion was used. Glutamic acid equivalent to 0.5 mmoles of carbon and 100 mg of Van Slyke citrate buffer and 20 ml. of water were frozen solid. Three hundred mg. of ninhydrin was then added and the system was evacuated till the base started boiling. Then the reaction mixture was heated for 8 minutes in a boiling water bath and the CO_2 was trapped in 0.5 N CO_2-free base.

The total radioactivity was measured by persulfate combustion (16). γ-Hydroxyglutamate-C^{14} isolated was degraded after conversion to glutamic acid-C^{14} by reduction with red phosphorus and hydriodic acid (78). The $C^{14}O_2$

formed was counted as $BaC^{14}O_3$ in a thin window gas flow, low background counter (Nuclear-Chicago Corporation).

Other radioactive materials were counted in a Tri-carb liquid scintillation counter under counting efficiency 44 - 48 percent.

Chemicals

DL-citramalate (methylmalate) was synthesized from KCN and ethylacetoacetate by the method of Michael and Tissot (54).

$$CH_3COCH_2COOC_2H_5 + HCN \rightarrow CH_3\overset{CN}{C}(OH)-CH_2COOC_2H_5 \xrightarrow{H^+}$$

$$HOOC-CH_2\overset{CH_3}{\overset{|}{C}}(OH)COOH$$

The compound was recrystallized under vacuum several times from ethylacetate (mp 119°C).

DL-oxalomalate (α-hydroxy-β-oxalosuccinate) was prepared by a method described by Ruffo and coworkers (65). The same procedure was employed for the preparation of radioactive oxalomalate by using glyoxylate 1,2-C^{14} as the condensing partner of OAA. The rate of condensation was followed by determining the disappearance of the aldehyde group of glyoxylate at various times according to the colorimetric method of Kramer et al (44). By

decarboxylation of the synthetic oxalomalate, γ-Hydroxy-α-ketoglutarate (HKG) was synthesized. The decarboxylation was accomplished by acidifying the solution of oxalomalic acid and purification of the resulting HKG through Dowex-1-formate column (66 x 1.5) by gradient elution with 6N formic acid. The same chromatographic procedure was followed for the isolation of HKG from the growth medium of A. suboxydans or from incubation mixtures with CFE.

HKG was converted to γ-hydroxyglutamate (HGA) with glutamic dehydrogenase (3). The HGA was isolated as described previously by Dowex-1 chromatography. For identification purposes, HKG was also converted to HGA by reduction of the 2,4 dinitrophenylhydrazone with H_2 in the presence of PtO_2.

The other chemicals used herein were obtained commercially and used without further purification. These were obtained as follows: sodium glyoxylate, oxaloacetate, pyruvate, glutamate, alanine, β-methylaspartate, DPN, DPNH, ATP, coenzyme A, vitamin B_{12}, B_6-phosphate, glutathione, malic dehydrogenase, glutamic dehydrogenase, glutamic decarboxylase (from Sigma Chemical Company). Mesaconic acid was obtained from K and K Laboratories, Inc. Charcoal Nuchar C-190, unground, was a gift of West

Virginia Pulp and Paper Company.

Radioactive materials were obtained as follows: Acetyl-1-C^{14}-Coenzyme A, New England Nuclear Corp; acetate-1-C^{14} and acetate-2-C^{14} from California Corporation for Biochemical Research. Pyruvate-1-C^{14} and pyruvate-2-C^{14} was obtained from the California Corporation for Biochemical Research and from Merck and Co., Ltd., Canada. Glyoxylate-1,2-C^{14} was obtained from Nuclear Research Chemicals, and propionic acid-1-C^{14} from Nuclear Chicago Corp.

RESULTS AND DISCUSSION

The Citramalate-Mesaconate Pathway

The importance of the reaction sequence described by Barker and his coworkers as a fermentation scheme of glutamic acid in *Clostridium tetanomorphum* has been assessed in this study as a possible synthetic route for glutamic acid biosynthesis in *A. suboxydans*.

The occurrence of a number of the intermediates of this pathway in a variety of systems and the reversibility of the reactions involved has been suggestive in this respect along with the preliminary experimental evidence described herein.

Incorporation of Acetate-1-C^{14} and Pyruvate-2-C^{14} into Glutamate of the Cells of A. suboxydans Under Growing Conditions. It was observed that either acetate or pyruvate-C^{14} was incorporated into glutamate of the growing cells to a significant extent when present in the growth medium of A. suboxydans. Under the same conditions, α-ketoglutarate-C^{14} was largely incorporated into glutamate of the cells, whereas succinic-C^{14} incorporation was negligible.

Figure 1 shows the elution pattern of acidic amino acids obtained from acid hydrolysis of cells of A. suboxydans grown in glycerol-yeast extract medium containing pyruvate-2-C^{14}. Similar results were obtained when acetate-1-C^{14} was present, but incorporation of the label was obtained to a much smaller extent, presumably because of extensive dilution of the acetate.

Glutamate Formation from Intermediates of the Citramalate-Mesaconate Pathway.

The conversion of acetate and pyruvate as well as of citramalate, mesaconate and β-methylaspartate to glutamate has been considered as suggestive evidence for the possibility of participation of the citramalate-mesaconate pathway in glutamate biosynthesis in A. suboxydans.

Glutamate formation from all intermediates of the

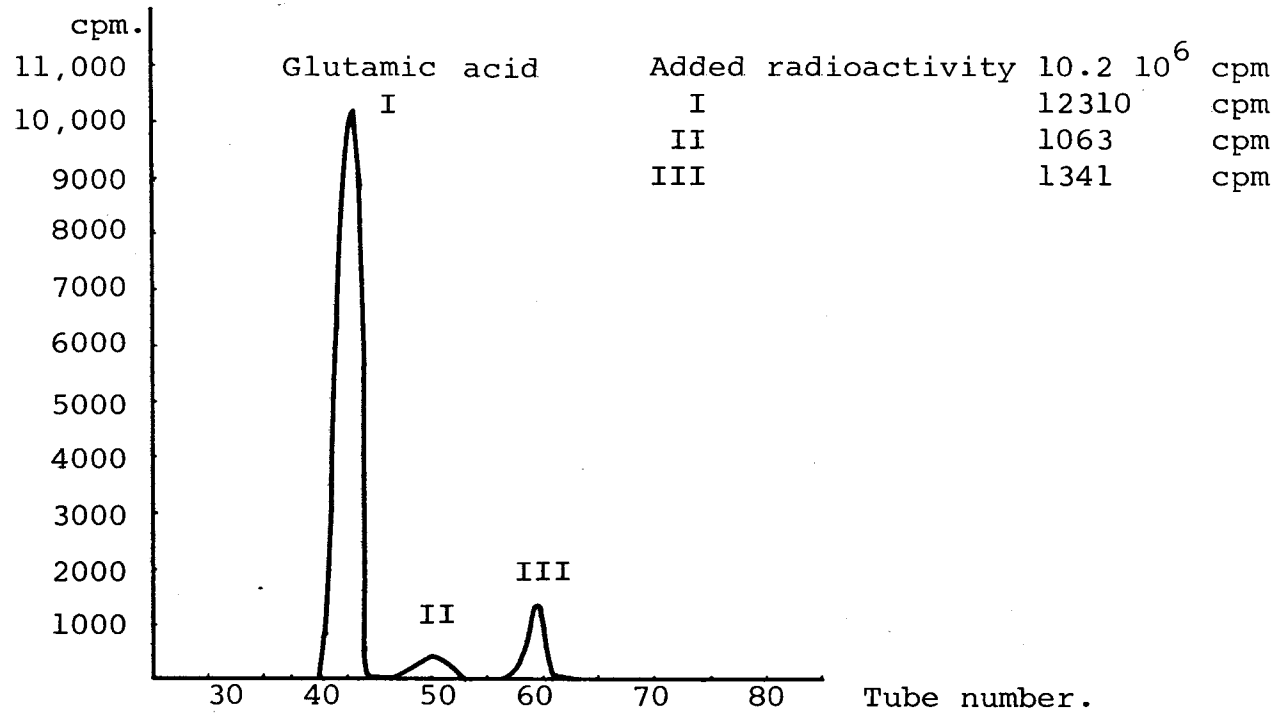

Figure 1. Acidic amino acids of a cell-hydrolysate of A. suboxydans grown in the presence of pyruvate-2-C^{14}.

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[1]Cells were grown in 12.5 ml, 5 percent glycerol, 0.5 percent yeast extract medium containing 10μC of pyruvate-2-C^{14}. After 48 hrs. of growth at 30°C, 8.4 mg of cells (dry weight) were obtained. Cells were washed with phosphate buffer, pH 6.5, several times and then hydrolysed for 18 hrs. in a sealed tube with 6N HCl in an autoclave. The hydrolysate was filtered and evaporated to dryness several times by adding small amount of water. Amino acids were separated on a Dowex-1-Acetate column and the fractions were counted.

pathway is shown in Table 1. The substrates in the presence of cofactors and under conditions detailed in the table are converted to glutamate, which was determined by glutamic decarboxylase and was detected by paper chromatography. The percent yield of glutamate is decreased at higher concentrations of each precursor. The reason is not

Table 1. Glutamate Formation from Intermediates of the Citramalate Pathway.

Substrate	Amount μmoles	Nitrogen Source: L-Alanine μmoles	Glutamate Formed* μmoles	Percent Conversion
Acetate + Pyruvate	20	20	2.65	12.6
	50	50	1.20	2.4
DL-Citramalate	20	20	4.00	20.0
	50	50	0.46	0.9
Mesaconate	20	20	5.65	28.2
	50	50	3.36	6.7
DL-β Methylaspartate	20	20	1.93	9.6
	50	50	0.79	1.6

* Substrates were incubated with one ml. of cell-free extracts (protein 25 mg/ml) in the presence of pyridoxal-PO_4, 0.5 mg; coA, 0.1 μmole; B_{12}, 1 mμg; ATP 10 μmoles; Glutathione, 3 μmoles; $MgCl_2$, 5 μmoles; $FeSO_4 \cdot 7H_2O$, 0.1 μmole; $(NH_4)_2SO_4$, 10 μmoles; L-alanine, 10 μmoles; phosphate buffer, pH 7.3, 100 μmoles; total volume 4 ml, incubated at 30°C for 6 hours.

Glutamate was determined by glutamic decarboxylase (74) manometrically (blanks with boiled enzyme were subtracted) and detected by paper chromatography.

yet known; the concentrations employed may, in fact, not be optimal.

Formation of citramalate and mesaconate from acetate plus pyruvate by CFE of A. suboxydans. The formation of citramalate and mesaconate from acetate plus pyruvate is necessary if glutamic acid is formed through these intermediates. The demonstration of this condensation has been difficult with non-radioactive substrates, presumably because they are not accumulated in significant amounts. Large amounts of CFE (50-100 ml) were incubated with acetate and pyruvate under the conditions detailed in Table 1, but without a nitrogen source. After precipitation of the proteins by boiling, the solution was adjusted to pH 2.5 with 6N HCl and extracted with ether in a liquid-liquid extractor for 48 hours. The organic acids thus obtained after drying the ether extract were chromatographed in a butanol-formic acid-water system for 24 hours. After drying the chromatograms, the positions of the different organic acids were detected by spraying with bromocresol blue (1 percent solution in acetone and adjusting the pH to 7.1 with base). The yellow spots of organic acids obtained corresponded to the R_f values of citramalic and mesaconic acids. The mesaconic acid spot was further identified by

the strong quenching of the ultra violet light.

Conversion of mesaconate to citramalate by A. suboxydans. Mesaconate incubated with CFE under conditions of Table 1, but in the absence of nitrogen source, yielded both citramalate and pyruvate along with the remaining mesaconate. A control experiment with boiled CFE gave no such conversion.

The hydration of mesaconate to citramalate was further demonstrated by incubation of mesaconate with CFE of A. suboxydans and measuring of both the remaining mesaconate and the formed citramalate at the end of the incubation period. The remaining mesaconate was determined by measuring the OD at 230 mμ, which is a very sensitive assay for mesaconate (36). Citramalate formed was measured by the method of Egashira (21). Table 2 summarizes the results. When the initial concentration of mesaconate was 20-50 μmoles, no citramalate could be detected, probably because it was further metabolized. However, when 150 μmoles of mesaconate were used, the amount of citramalate formed, accounts, at least partially, for the disappearance of mesaconate.

Incorporation of label into glutamate and β-methylaspartate from Acetate-1-C^{14} or pyruvate-1-C^{14}. Incubation

Table 2. Enzymatic Hydration of Mesaconate.*

Starting Amount of Mesaconate (umoles)	Final Amount of Mesaconate (μmoles)	Citramalate Formed (μmoles)
0	0	-
20	10	-
50	38	-
150	115	25

*Incubation of mesaconate with 2 ml of CFE (protein 24 mg/ml); Tris buffer, pH 8.2, 200 μmoles; $FeSO_4$, $7H_2O$, 6×10^{-2} μmoles; Total volume 6 ml; incubation period 3 hrs. at 30°C.

of CFE with acetate or pyruvate-C^{14} under conditions detailed in Table 3 gave rise to labeled glutamate and β-methylaspartate. These amino acids were separated on Dowex-1 column and the peaks were identified by paper chromatography and radiautography.

Pyruvate is extensively decarboxylated in this organism. When pyruvate-1-C^{14} was used under the conditions of Table 3, 40 percent of the radioactivity added was found as $C^{14}O_2$ trapped by base in the center well of the reaction flask. $C^{14}O_2$ was also formed from acetate-1-C^{14}, but only to a small extent (less than one percent).

Data shown in Table 3 suggest that glutamate and β-methylaspartate are metabolically related. Experiments

Table 3. Incorporation of Isotope into Glutamate and β-Methylaspartate in A. suboxydans from Acetate-1-C^{14} or Pyruvate-1-C^{14}.

Expt. No.	Substrate	Amount	Radio-activity	Glutamate Formed cpm	Glutamate Formed Percent Radioactivity Incorporated	β-Methylaspartate Formed cpm	β-Methylaspartate Formed Percent Radioactivity Incorporated
1	Acetate	20 μmoles	5 μc	6480	0.23	13000	0.46
	Pyruvate-1-C^{14}	5 μmoles					
	Acetate-1-C^{14}	5 μmoles	40 μc	30500	0.27	19350	0.17
	Pyruvate	20 μmoles					
2a	Acetate-1-C^{14}	10 μmoles	40 μc	46030	0.25	4770	0.025
	Pyruvate	20 μmoles					
2b	Acetate-1-C^{14}	10 μmoles	40 μc				
	Pyruvate	20 μmoles					
	β-Methyl-aspartate	20 μmoles		30060	0.16	12290	0.057

Experimental conditions and cofactors as in Table 1. pH of the reaction mixture was 8.0. Amino acids were separated on a Dowex-1-acetate column and identified by paper chromatography.

2a and 2b are identical except for the presence of 20 μmoles of β-methylaspartate in the reaction mixture of 2b; this causes an increase in the isotope incorporation into β-methylaspartic acid fraction (by a factor of about 2.5) by "trapping" the labeled compound formed in the reactions through dilution with unlabeled β-methylaspartate, added at the beginning of the incubation of pyruvate and acetate with the CFE. Glutamate is reduced in activity by about one-third presumably because of conversion to glutamate of the unlabeled exogeneous β-methylaspartate.

Amino dicarboxylic acids and organic acids formed from Acetate or pyruvate-C^{14}. In the experiments described herein, the complete elution patterns of acidic amino acids and organic acids formed from acetate and pyruvate condensation is investigated in detail.

Under the experimental conditions detailed in Figure 2, recovery of the added radioactivity, as acetate-1-C^{14} in the amino acid fractions, was as in Table 4-A. Radioactivity peaks of glutamate and β-methylaspartate had a perfect coincidence with the ninhydrin assay. Glutamate and β-methylaspartate were further identified by paper chromatography.

Similar elution patterns of amino acids were obtained

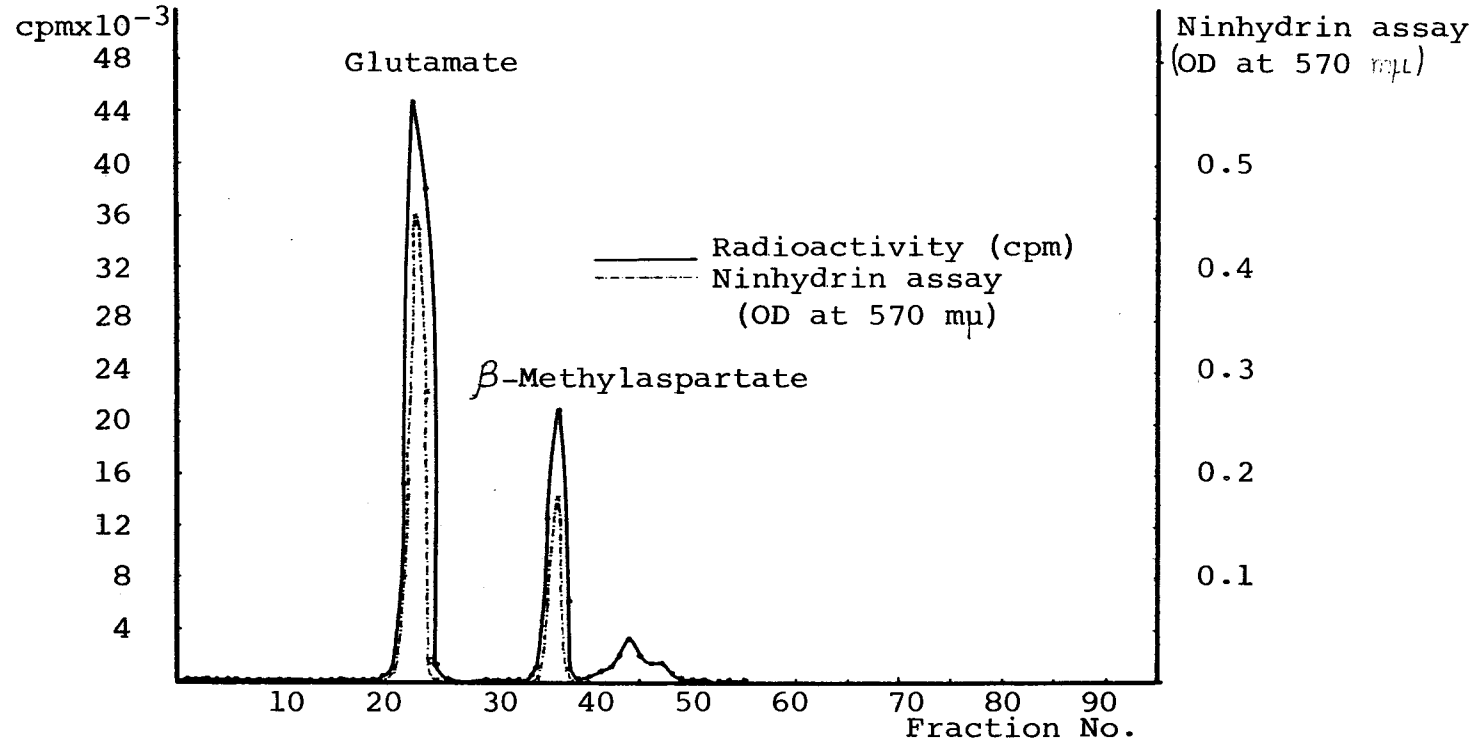

Figure 2. Amino Dicarboxylic Acids from the Condensation of Acetate-1-C^{14} with Pyruvate in A. suboxydans.

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[2]Reaction mixture: Acetate-1-C^{14}, 20 μmoles (43 μC); pyruvate, 20 μmoles; alanine, 10 μmoles; B_6-PO_4, 0.5 mg; CoA, 0.1 μmole; B_{12} 0.1 μg; ATP, 10 μmoles; glutathione, 3 μmoles; $MgCl_2$, 5 μmoles; $FeSO_4$ $7H_2O$, 0.1 μmole; $(NH_4)_2SO_4$, 10 μmoles; Tris buffer, pH 7.3, 100 μmoles; CFE 5 ml (protein 57 mg/ml). Incubation at 30°C for 6 hrs. Proteins were precipitated and centrifuged out. The solution was adsorbed on Dowex-1-Acetate column (66 x 1 cm) and the neutral compounds were washed away with 100 ml of distilled water. Acidic amino acids were then eluted exponentially with 0.5N acetic acid and fractions of 8 ml were collected. One ml of each fraction was taken for counting and 0.5 ml for ninhydrin assay.

Table 4. Recovery of Radioactivity in the Amino Acids and Organic Acid Fractions from the Condensation of Pyruvate with Acetate in A. suboxydans.

Amino Acids and Organic Acids Isolated	Substrates Incubated with CFE of A. suboxydans Other Substrates and Experimental Conditions as in Figure 2			
	A	B	C*	D*
	Acetate-1-C^{14} 20 µmoles (4.4×10^7 cpm) Pyruvate 20 µmoles	Acetate 20 µmoles Pyruvate-2-C^{14} 20 µmoles (2.08×10^7 cpm)	Acetate-1-C^{14} 20 µmoles (4.4×10^7 cpm) + Mesaconate 20 µmoles Citramalate 20 µmoles	Acetate 20 µmoles Pyruvate-2-C^{14} 20 µmoles (2.08×10^7 cpm) + Mesaconate 20 µmoles Citramalate 20 µmoles
Glutamate	1.5×10^5 cpm	1.05×10^5 cpm	not isolated	not isolated
β-methyl-aspartate	7.65×10^4 cpm	1.98×10^4 cpm		
Mesaconate	1.2×10^4 cpm	9.25×10^4 cpm	2.04×10^4 cpm	1.81×10^4 cpm
Citramalate	1.15×10^5 cpm	4.5×10^5 cpm	5.2×10^4 cpm	1.16×10^5 cpm
Pyruvate	---	6.0×10^4 cpm	2.3×10^4 cpm	2.16×10^4 cpm
Malate	---	---	4.1×10^3 cpm	3.02×10^4 cpm
Succinate	---	---	9.2×10^4 cpm	5.4×10^4 cpm

Under the above conditions in a control (with boiled enzyme) no radioactivity was incorporated into the amino acids.

*As A and B, but 20 µmoles each of mesaconate and citramalate are added at the beginning of the experiment and no nitrogen source.

from pyruvate-2-C^{14}. The amount of radioactivity incorporated in each fraction appears in Table 4-B.

Organic acids formed from acetate or pyruvate-C^{14} were extracted with ether after acidifying the solution (pH 3.0) with 6N HCl. Extraction was performed in a liquid-liquid extractor for 48 hours. The ether extract was evaporated under vacuum and the organic acid residue was redissolved in 0.5 ml. water and separated by means of a celite column (60 x 1.5 cm) as described by Phares (61). Figure 3 is the elution pattern of organic acids from acetate-1-C^{14} and pyruvate. It can be seen that acetate-1-C^{14} with pyruvate yields both organic acids expected from the condensation of pyruvate plus acetate. Citramalate and mesaconate peaks were identified by paper chromatography.

A similar elution pattern of organic acids would be expected from a condensation of pyruvate-C^{14} with acetate, if citramalate and mesaconate are formed in this organism through such a condensation reaction.

Organic acids formed from pyruvate-2-C^{14} and acetate separated as before on a celite column are shown in Figure 4. Except for the pyruvate-C^{14} peak, the elution pattern of organic acids is essentially the same as when

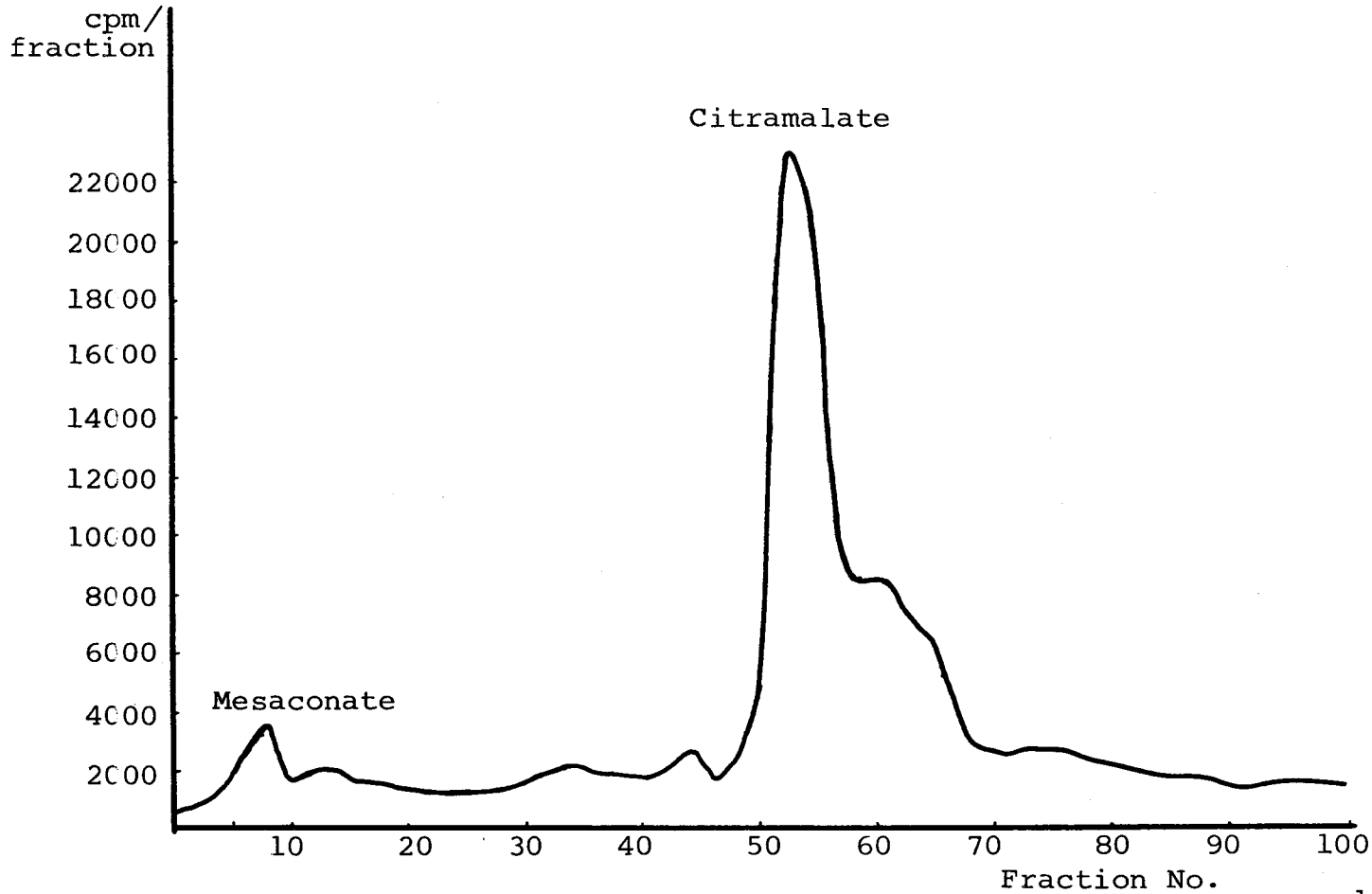

Figure 3. Organic acids from the Condensation of Pyruvate + Acetate-1-C^{14} in A. suboxydans.

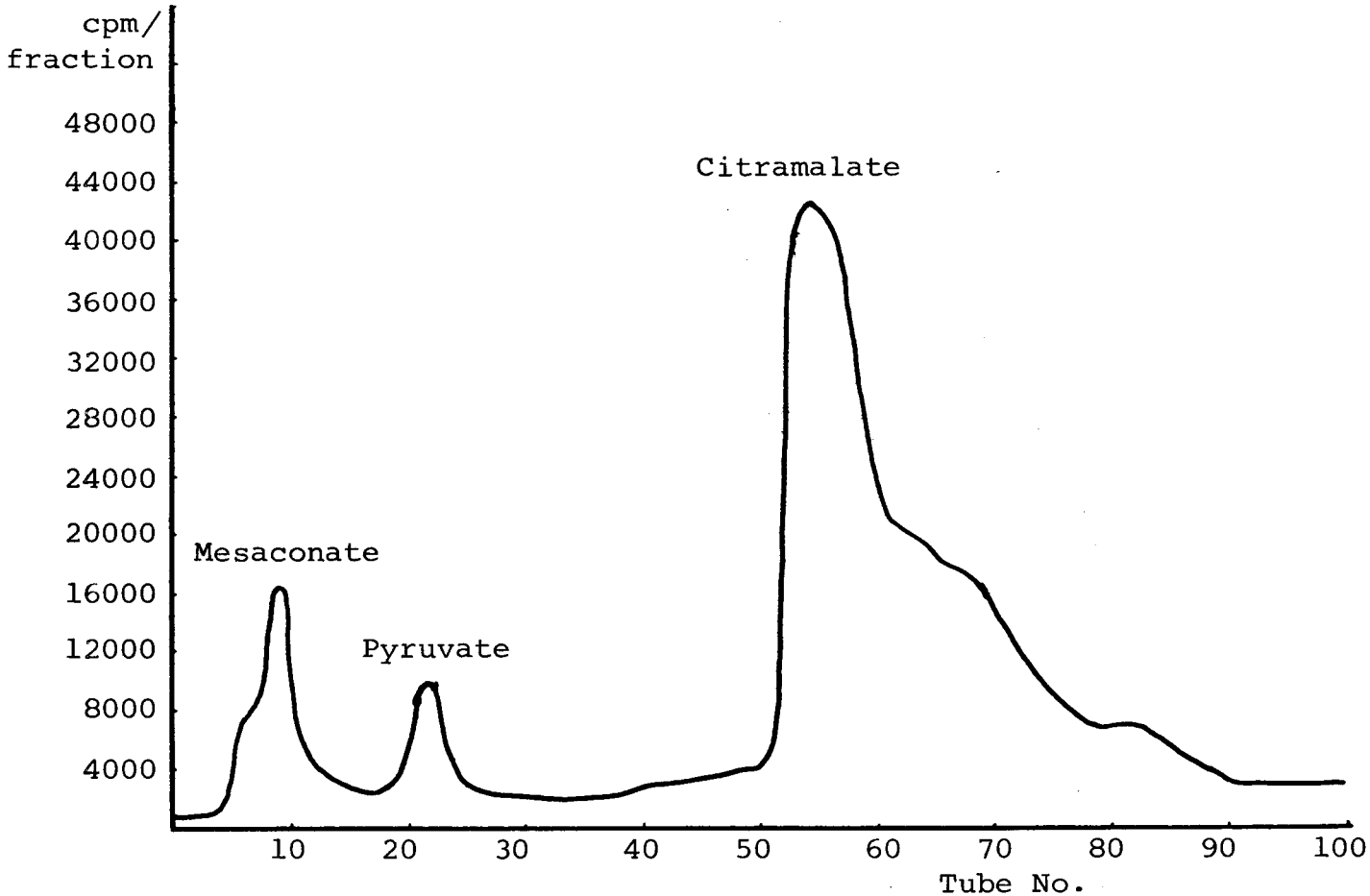

Figure 4. Organic acids from Condensation of Pyruvate-2-C^{14} + Acetate in A. suboxydans.

acetate-1-C^{14} was the radioactive precursor. The total radioactivity under each organic acid peak is obtained by pooling all fractions corresponding to each peak and assay for radioactivity a suitable fraction (Table 4-B).

The data suggest that under the conditions of these experiments the formation of citramalate is favored over mesaconate. This observation is in agreement with the results of Ketsuki (36) with the purified enzyme from P. fluorescens, which catalyses the reversible hydration of mesaconate.

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 radioactivity and recrystallization of citramalate to constant radioactivity as described later.

In an attempt to increase the incorporation of radioactivity into the citramalate and mesaconate fractions, "cold" citramalate and mesaconate were added at the

beginning of the experiment. It was expected thus that the radioactivity incorporated into mesaconate and citramalate fractions would be increased by "trapping" the formed labeled compounds with "cold" substrates. This would provide another indirect evidence for their formation and should supply enough radioactive materials for further experiments. However, when 20 μmoles each of mesaconate and citramalate were present in the reaction mixture of acetate-1-C^{14} and pyruvate (Figure 5 and Table 4-C), the radioactivity in the mesaconate fraction was increased by a factor of about two, but the radioactivity in the citramalate fraction was decreased to about one-half of the amount of radioactivity in the absence of mesaconate and citramalate. In addition, significant amounts of succinate, malate and pyruvate were formed from acetate-1-C^{14} and pyruvate (Figure 5). A similar effect was observed in the elution pattern of organic acids from acetate and pyruvate-2-C^{14} when 20 μmoles of each mesaconate and citramalate were present (Table 4-D).

The above findings cannot be explained on the basis of the known metabolic processes of A. suboxydans, and suggest that citramalate and mesaconate are formed in this organism only in small amounts, and that if their

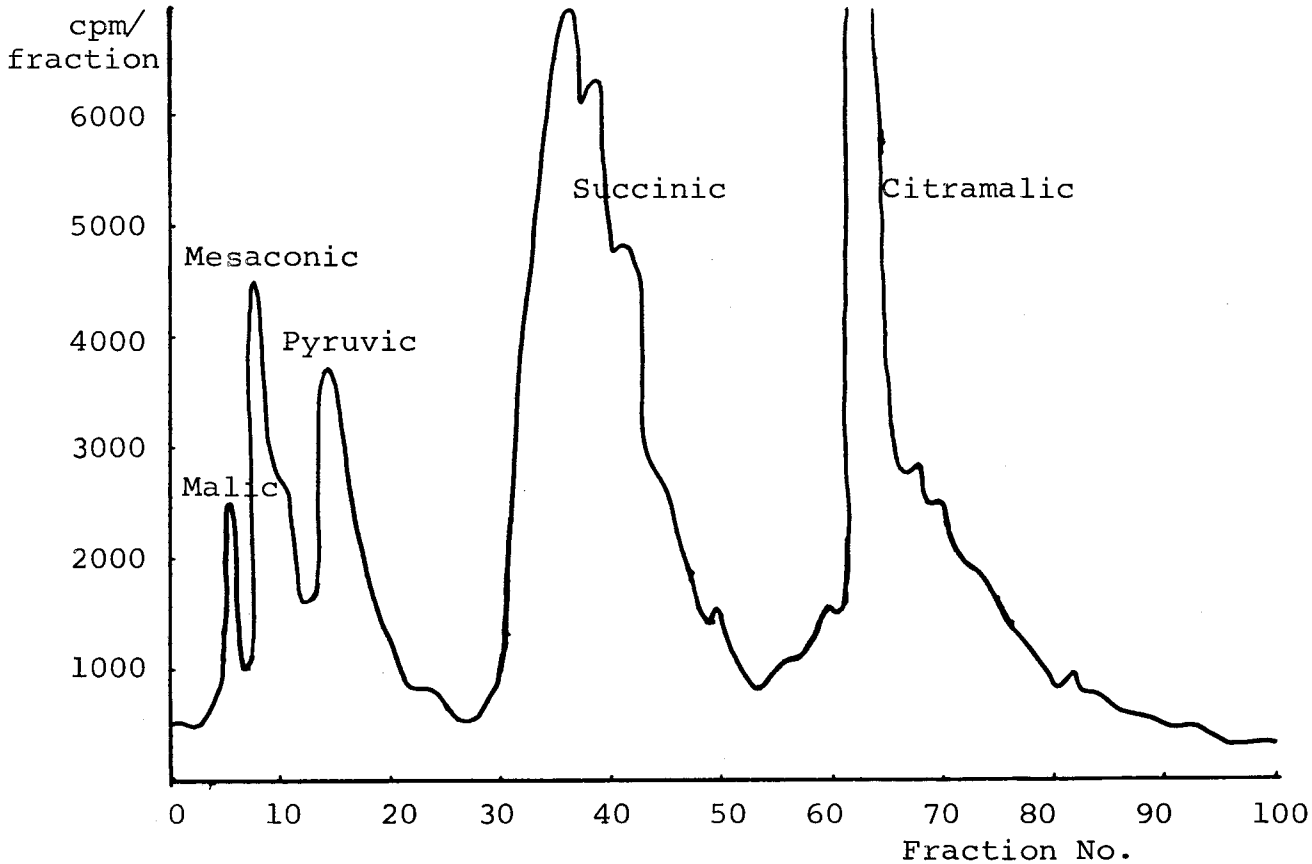

Figure 5. Organic acids from the Condensation of Acetate-C^{14} + Pyruvate when Mesaconate and Citramalate are present in the Reaction Mixture.

concentration increases, they are metabolized to other compounds or play an unknown role in the formation of other substrates.

The condensation of acetate with pyruvate takes place presumably with acetate in the form of acetyl-coenzyme A, and this is the reason for the addition of coenzyme A and ATP in the previous experiments. Therefore, it would be expected that acetyl coenzyme A should be a better precursor than acetate in the formation of glutamate and β -methylaspartate.

Acetyl-coenzyme A-1-C^{14} incubated with CFE under the conditions detailed in Figure 6, gave much higher incorporation of radioactivity into amino acid fractions than did acetate-C^{14} (Table 5). These results suggest that although acetyl-coenzyme A is formed by cells of A. suboxydans from acetate, CoA and ATP (39), the efficiency of this process is not high, 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 (15).

Organic acids remaining in the column after amino

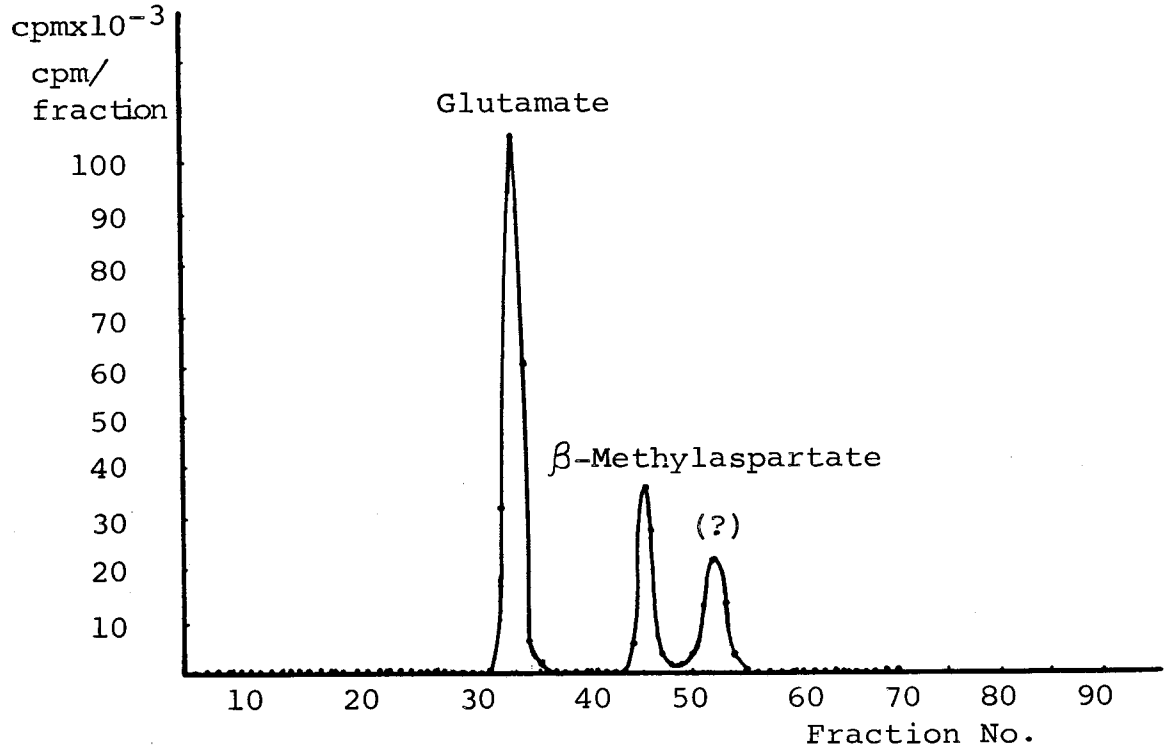

Figure 6. Amino acids from acetyl-coenzyme-A-1-C^{14} and Pyruvate in A. suboxydans.

[3]The following mixture of ingredients was incubated: Acetyl-coenzyme A-1-C^{14}, 20 μmoles, 6 μC; pyruvate, 20 μmoles; alanine, 10 μmoles; with 5 ml of CFE (protein 57 mg/ml) on the presence of cofactors as in Figure 2. Amino acids were separated as detailed in Figure 2, on Dowex-1-Acetate column.

acid separation (Figure 6) were eluted with 100 ml. of 5N acetic acid. The acetic acid was removed under vacuum and the organic acids formed from acetyl coenzyme A-1-C^{14} and pyruvate were separated as before on celite column. Figure 7 shows the results of this separation. Peaks of mesaconate and citramalate were obtained, but in addition, small amounts of pyruvate and a major peak of succinate appear to be formed.

Table 5 summarizes the amount of radioactivity incorporated in amino acid and organic acid fractions.

Table 5. Recovery of radioactivity in organic and amino acid fractions formed by condensation of acetyl-coenzyme A-1-C^{14} and pyruvate in A. suboxydans.

Acidic Amino Acids and Organic Acids Formed	Total Radioactivity cpm	Percent of the Added Radioactivity as Acetyl-CoA-1-C^{14}
Glutamate	2.70×10^5	4.6
β-methylaspartate	8.50×10^4	1.42
Mesaconate	7.60×10^3	0.12
Pyruvate	3.59×10^3	0.06
Succinate	4.25×10^4	0.71
Citramalate	3.11×10^4	0.52

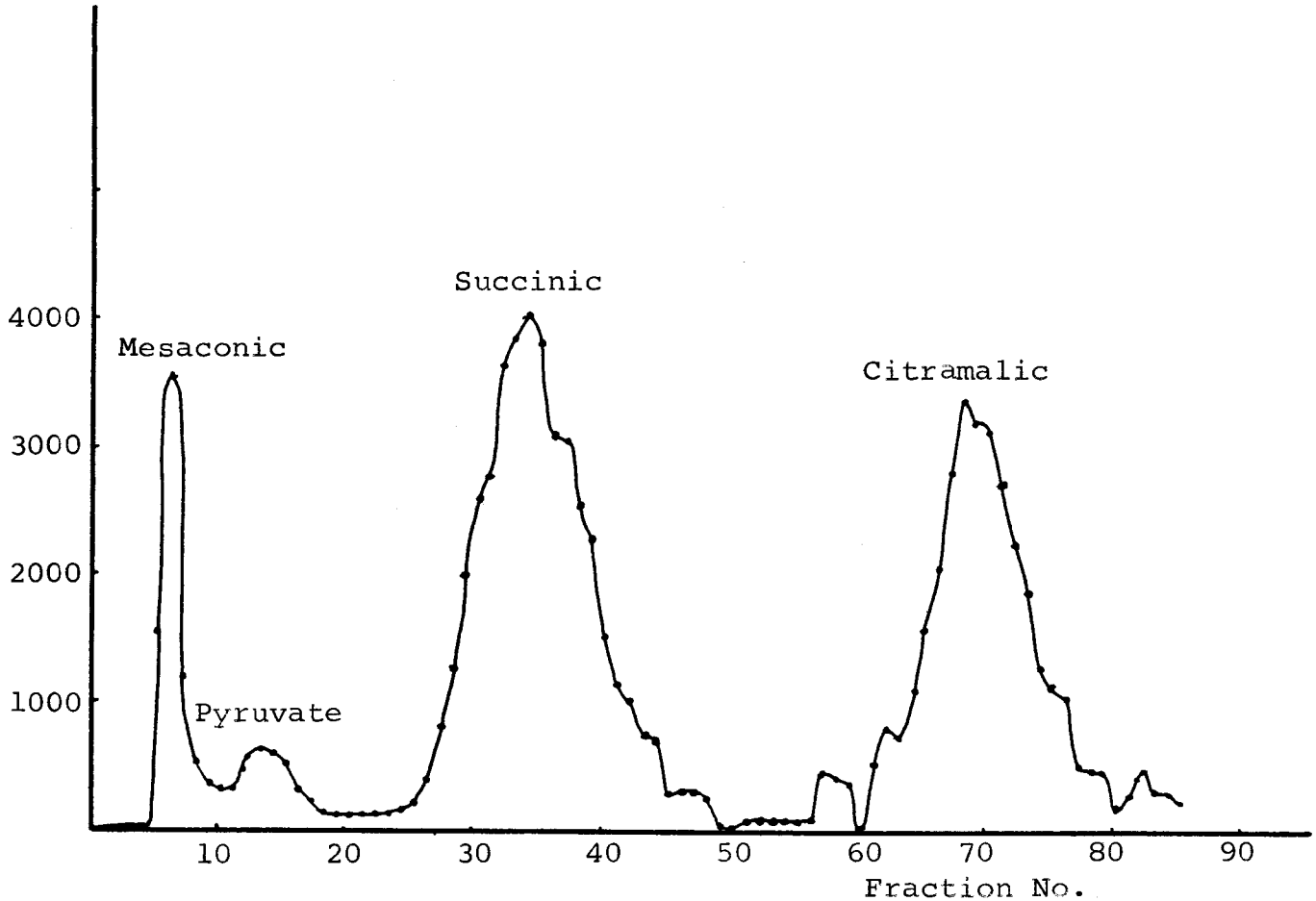

Figure 7. Organic acids from acetyl-1-C^{14} Coenzyme-A and pyruvate in A. suboxydans.

The formation of succinate, in several of the experiments already described and in others to be presented herein, cannot be explained on the base of the known metabolic processes of A. suboxydans, especially in view of the belief of the absence of the Krebs cycle. Indeed, the formation of succinate from acetate and pyruvate along with other observations to be discussed later suggest that further evidence is necessary before we can exclude the entire TCA cycle from this organism as active metabolic process leading to the TCA cycle related metabolites.

Characterization of the Isolated Products

Glutamic acid. The identity of the glutamate formed from radioactive substrates has been established as follows: 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 in one or two dimensional paper chromatography and radioautography. Finally by recrystallizing glutamate-C^{14} formed after mixing with authentic and thus showing that radioactivity remained in the glutamate after several recrystallizations.

β-Methylaspartate. The evidence for the formation of *β*-methylaspartate is the coincidence of the radioactivity peak of *β*-methylaspartate-C^{14} formed with the ninhydrin assay peak of authentic *β*-methylaspartate added to the reaction mixture prior to separation. Also, coincidence was observed with paper chromatography of the formed *β*-methylaspartate.

For some reason, the labeled *β*-methylaspartic acid obtained from several experiments could not be recrystallized to constant radioactivity upon mixing with synthetic DL-*β*-methylaspartic. Data shown in Table 6 indicate that although 50 percent of the radioactivity remained after four recrystallizations, the specific activity was gradually decreased.

Table 6. Recrystallization of *β*-methylaspartate-C^{14} formed from A. suboxydans with synthetic DL-*β*-methylaspartate.

1st recrystallization	1500	cpm/mmole
2nd "	1170	"
3rd "	950	"
4th "	750	"

Conditions of recrystallizations as described by Barker et al (6).

A possible explanation is that β-methylaspartate used as carrier of the radioactive substance was the synthetic DL, β-methylaspartic acid. It is known that this synthetic compound contains only one of the two possible racemates (12). Consequently, if A. suboxydans forms the stereoisomer which is not present in the synthetic racemate, and if the crystals are formed from the racemic forms, it would be then expected that the stereoisomer whose enantioisomer is not present in the solution (in this case, the radioactive β-methylaspartate) would remain in the solution preferentially.

Indirect evidence for β-methylaspartic acid participation is presented in Table 3 where by addition of 20 μmoles of β-methylaspartate, the radioactivity incorporated into β-methylaspartic acid fraction is increased 2.5 times, while the radioactivity of glutamate fraction is decreased. This is due, presumably, to the dilution of the β-methylaspartate-C^{14} formed with the non-labeled material added so that the conversion of a certain amount to glutamic acid leaves more radioactive β-methylaspartate unreacted; this yields a glutamate fraction with less radioactivity since it also arises from the non-labeled substrate employed.

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 citramalate-C^{14} fractions from several experiments were mixed with 500 mg. of authentic DL-citramalate, and then recrystallized from ethylacetate under vacuum several times. Changes of the specific activity with recrystallizations are summarized in Table 7. After seven recrystallizations, the specific activity remains constant.

A further uniquivocal proof for the identity of citramalate as well as its relative configuration, was obtained by isolating enough material (about 15 mg), from an

Table 7. Recrystallization of citramalate-C^{14} isolated from A. suboxydans to constant specific activity.

Recrystallization No.	Specific Activity
1	2640 cpm/mg
2	1665 "
3	580 "
4*	422 cpm/10 mg
5	595 "
6	452 "
7	420 "
8	410 "
9	440 "

*At this point, additional 100 mg. of DL-citramalate (non-radioactive) were added to continue the recrystallizations.

incubation experiment of mesaconate with CFE 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 (46), the $[a]_D$ of the citramalate isolated was +1215° (0.2% solution). The reported values of optical rotation of citramalate are $[a]_D$ = +1200-1410° (9).

The optical activity of the isolated product not only provides a good criterion for its identity, but also proves from the sign of the optical rotation that the product has the same absolute configuration as the product of Clostridium tetanomorphum and opposite to the citramalate isolated from apples (34). The optical rotation, however, does not help us in assigning the absolute configuration since the absolute configuration of citramalate isomers is not known.

Mesaconate. This acid has a double bond conjugated to a carbonyl group. As a result it has a high absorption coefficient at 230 mμ. This has been helpful for the identification and determination of this compound in this study. On paper chromatograms and radioautograms, it appears as a disc shaped spot with strong "quenching" of the ultraviolet light.

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 mesaconate-C^{14} fraction.

Uniquivocal proof of the identity of mesaconate formed was established by sublimation of the mesaconate-C^{14} to constant specific activity. The apparatus used is illustrated in Figure 8. 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 Hg pressure and temperature 110-140°C. Table 8 shows the change in specific activity with sublimations.

Table 8. Resublimation of mesaconate to constant specific activity.

Sublimation No.	Specific Activity
1	2920 cpm/mg
2	2350 cpm/mg
3	2200 cpm/mg
4	2200 cpm/mg

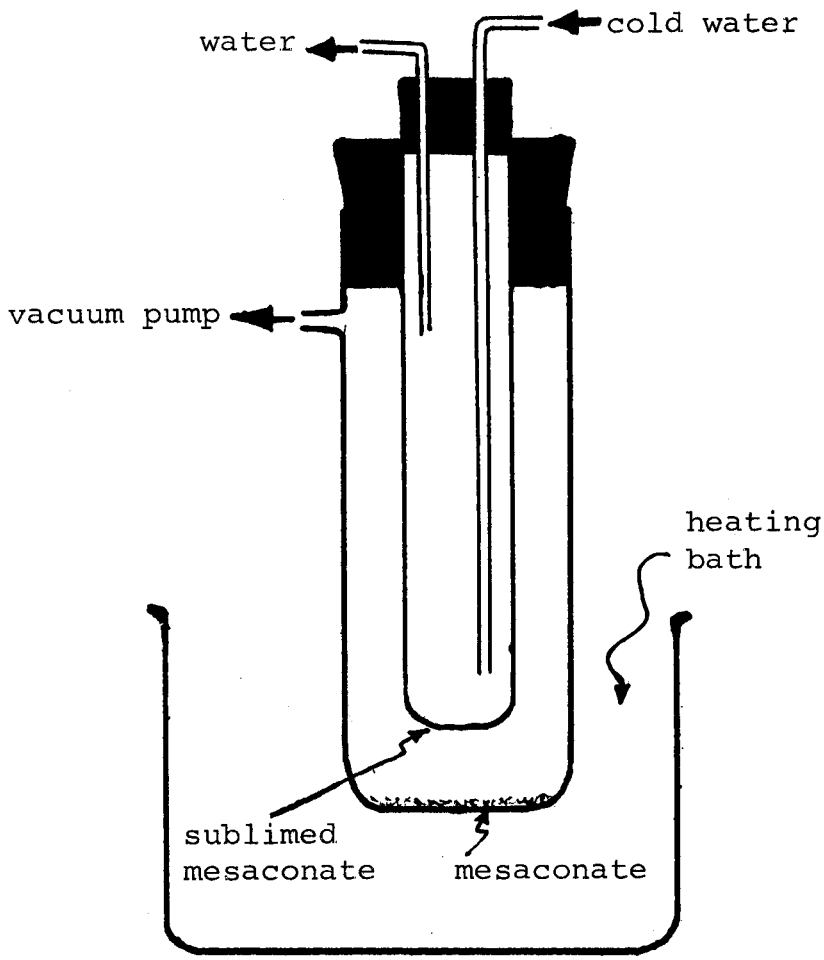

Figure 8. Apparatus used for sublimation of mesaconate.

Metabolic Interconversions

Although the main objective of this work has been to isolate and identify the intermediates of this pathway, certain interconversions among the intermediates have been well established.

It has already been mentioned that all intermediates including acetate plus pyruvate can be converted to glutamate which was quantitavely determined by glutamic decarboxylase and identified by paper chromatography. In most of these experiments, β-methylaspartate was also recognizable as a separate spot on the paper chromatograms.

In the absence of a nitrogen source, acetate plus pyruvate was converted by CFE to citramalate and mesaconate as identified by paper chromatography. Mesaconate was also converted to citramalate and pyruvate under similar conditions, implying that the condensation of acetate and pyruvate is reversible in contrast to liver mitochondria where it is not reversible (80).

The metabolic connection between glutamate and β-methylaspartate, suggested from the increase of radioactivity in the β-methylaspartate fraction and decrease of the radioactivity in glutamate fraction by addition of

cold β-methylaspartic, has been discussed earlier (pages 34-35). In addition to the above mentioned interconversions, evidence will be presented herein for the mesaconate metabolism in CFE of A. suboxydans and the effect of charcoal treatment of the CFE.

Mesaconate-C^{14} metabolism in A. suboxydans. Mesaconate-C^{14} fractions isolated from several experiments were mixed with 40 mg. of synthetic mesaconate and were purified as before by sublimation to constant specific activity. Thirty mg. of mesaconate-C^{14} (specific activity 2360 cpm/mg) were obtained. It was then incubated with 10 ml. of CFE (protein 57 mg/ml) in the presence of alanine, 100 μmoles; glutathione, 30 μmoles; $MgCl_2$, 50 μmoles; $FeSO_4.7H_2O$, 1 μmole; $(NH_4)_2SO_4$ 100 μmoles, Tris buffer, pH 8.0, 2 mmoles, at 30°C for 6 hours.

The organic acids formed were extracted and separated on a celite column as previously described. Figure 9 shows the results. In addition to the remaining mesaconate peak, pyruvate and citramalate are also formed as expected from the citramalate-mesaconate pathway. Two other acids are formed, one of which has been identified as succinic acid. The third peak, designated by a

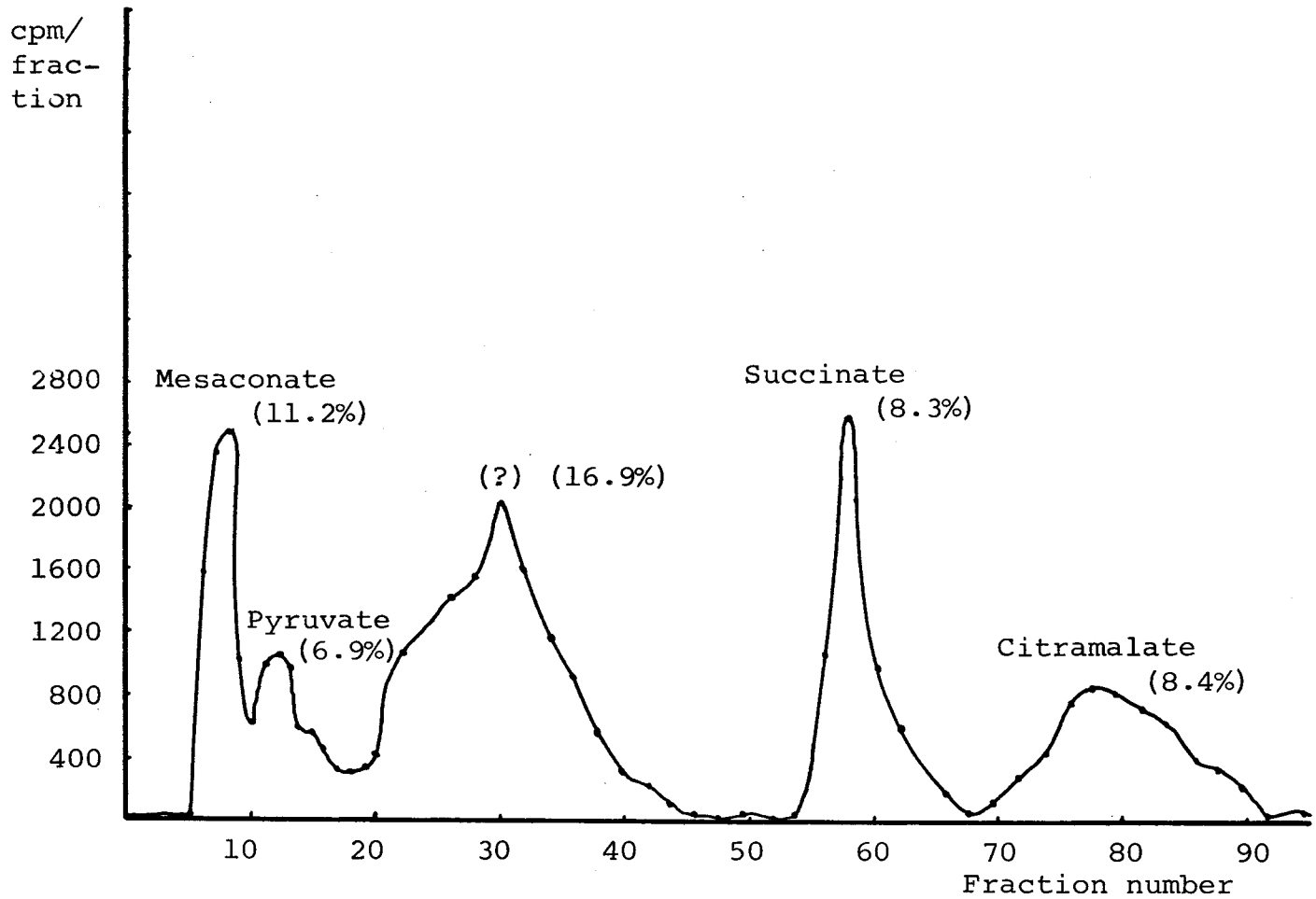

Figure 9. Organic acids from mesaconate-C^{14}.

question mark in Figure 9, although it has the R_f value of fumarate, it does not possess a high absorption coefficient at 230 mμ. The percent of the added radioactivity recovered in each organic acid fraction appears under each peak in Figure 9.

Citramalate has been identified on a paper chromatogram and was also crystallized. Its optical rotation was measured and found to be in agreement with the values reported in the literature. Isolated citramalate exhibited a molecular rotation $[a]_D = +1215^o$ under the conditions employed by Krebs and Eggleston (46).

From the same experiment, the acidic amino acids were separated on a Dowex-1-acetate column. Radioactivity was incorporated into both glutamate (1.2 percent of the radioactivity added as mesaconate-C^{14}) and in β-methylaspartate (1% of radioactivity incorporated as above). Both amino acids were also identified by paper chromatography.

Experiments with charcoal treated enzyme. Barker and coworkers (7), while studying the cofactor requirements of the reaction: glutamate$^-$ $\rightleftharpoons$ mesaconate$^=$ + NH_4^+ in Clostridium tetanomorphum, observed that treatment of the enzyme with charcoal removed a factor

essential for the decomposition of glutamate, without interfering with the formation of β-methylaspartate from mesaconate and ammonium ion. This observation enabled Barker and his coworkers to isolate β-methylaspartate and to characterize the cofactor removed by charcoal treatment which proved to be a B_{12} coenzyme.

In this study, charcoal treatment effects on the CFE of A. suboxydans have been considered as an indication that vitamin B_{12} is involved in the reaction sequence leading to glutamate formation.

Charcoal treatment of CFE with Nuchar 190 unground was performed as described under materials and methods. The "cofactor" was extracted from the charcoal used with 50 percent acetone at 0°C and the charcoal was removed by centrifugation. The "cofactor" then was obtained by removing the acetone under vacuum, in the dark and at 0°C.

In order to study the effect of charcoal treatment, the three experiments outlined in Table 9 were conducted. Experiments A and B are identical with only difference that B contains vitamin B_{12} and the "cofactor" extracted from the charcoal used for treatment of enzyme. Experiment C contained non-charcoal treated enzyme.

The amino acids glutamate and β-methylaspartate have

Table 9. Experiments with charcoal treated enzyme.

Substrates*	Incubation Mixture		
	A	B	C
Acetate-1-C^{14}	20 µmoles, 40 µC	20 µmoles, 40 µC	20 µmoles, 40 µC
Pyruvate	20 µmoles	20 µmoles	20 µmoles
Cell-free extract	5 ml. charcoal treated (protein 18 mg/ml)	5 ml.charcoal treated	5 ml.non-charcoal treated(native) (protein 20 mg/ml)
Other substrates*		+B_{12} 0.1 µg + "cofactor" extracted from charcoal	+ B_{12} 0.1 µg

Acidic Amino Acids Isolated	Radioactivity Recorded in Amino Acid Fraction (cpm)		
	A	B	C
Glutamate	3010 (1/9 of C)	10470 (3.5 times radioactivity in A)	26800
β-Methylaspartate	4020 (1/3 of C)	18780 (4.4 times radioactivity in A)	12720

*Substrates: alanine, 10 µmoles; B_6- PO_4, 0.5 mg; CoA, 0.1 µmole; ATP, 10 µmoles; glutathione, 3 µmoles; $MgCl_2$, 5 µmoles; $FeSO_4$ $7H_2O$, 0.1 µmole; $(NH_4)_2SO_4$, 10 µmoles; Tris buffer pH 8.0, 20 mmoles at 30°C for 6 hours.

been separated as before, and the data on radioactivity incorporation are shown in Table 9.

It is notable that the radioactivity in the glutamic acid fraction in Experiment A is about 1/9 of that of the control with the native enzyme, but the radioactivity in the β-methylaspartate fraction is about 1/3. Addition of B_{12} and "cofactor" extracted with charcoal increased the radioactivity incorporated into glutamate by a factor of 3.5 and about 4.5 in the β-methylaspartate fraction.

These experiments suggesting the removal of a "cofactor" by charcoal treatment of the enzyme, which is essential for the glutamate biosynthesis from acetate plus pyruvate, have been considered as indirect evidence for the participation of β-methylaspartate in the glutamate biosynthesis and that this process is possibly B_{12} dependent.

Degradation studies. It is of interest to note that the reactions of the Krebs cycle lead to the incorporation of the carboxyl group of acetate into the carboxyl group of glutamate farthest removed from the $-NH_2$ group (C_5). However, in a reaction sequence as the one described in Figure 10, it would be expected that a

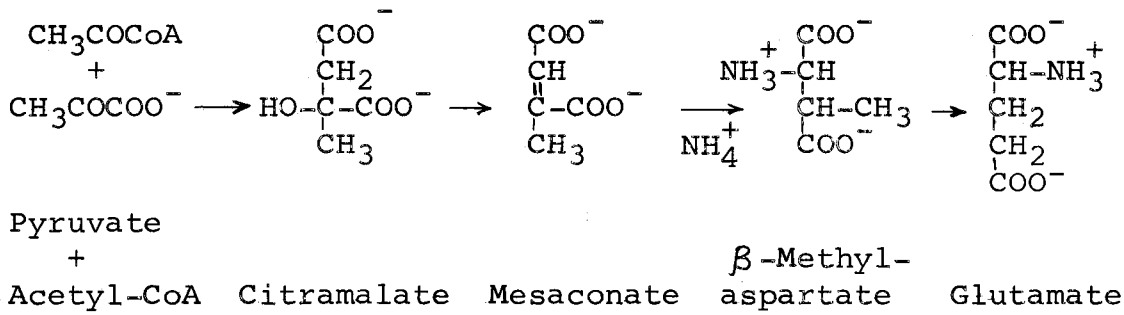

Figure 10. Citramalate-mesaconate pathway for Glutamate Biosynthesis in A. suboxydans.

different labeling pattern of glutamate should appear. Acetate-1-C^{14} should yield glutamate-1-C^{14} whereas pyruvate-1-C^{14} should give rise to glutamate-5-C^{14}. Therefore, it was of importance to establish the localization of the label in the glutamate-C^{14} isolated from the previously described experiments. Glutamate-C^{14} was recrystallized with authentic L-glutamate and degraded as described in the section on Materials and Methods. Glutamate-C^{14} isolated when acetate-1-C^{14} was the radioactive precursor yielded by ninhydrin degradation (C_1), 60-68 percent of the total radioactivity as $C^{14}O_2$ from carbon atom one of glutamate (adjacent to the amino group). The Schmidt reaction removed 62 percent of the radioactivity as $C^{14}O_2$ from glutamate-C^{14} isolated when

pyruvate-1-C^{14} was the labeled precursor.

Although the major portion of label was found at the carbon atoms expected from the participation of citramalate-mesaconate pathway in the glutamate biosynthesis, the fact that a considerable amount of randomization occurs indicates that other pathways, even including portions of the "Krebs cycle," must not be excluded from the glutamate biosynthesis in A. suboxydans. This is also suggested from the formation of succinate-C^{14} described in previous experiments from either acetate-C^{14} or pyruvate-C^{14} and also from the fact that α-ketoglutarate-3-C^{14} yields in addition to glutamate-C^{14} a considerable amount of aspartate-C^{14} when incubated with CFE of A. suboxydans.

The observations presented suggest that in A. suboxydans, acetate and pyruvate are condensing to form citramalate and mesaconate, and points strongly to the participation of these compounds along with β-methylaspartate in the glutamate biosynthesis (Figure 10), along with other pathways.

GLYOXYLATE-OXALOACETATE PATHWAY

Preliminary incubation experiments of CFE of A. suboxydans with α-ketoglutarate or oxalosuccinate showed

that glutamate can be formed from these substrates. However, under similar conditions, isocitrate does not serve as a precursor of glutamate in this organism as would be expected from ordinary Krebs cycle reactions (Table 10A). On the other hand, glutamate can be formed from a mixture of OAA and glyoxylate when incubated with CFE of A. suboxydans under the experimental conditions described in Table 10.

The concurrent formation of γ-hydroxyglutamate, and the isolation of the keto acids α-KG and HKG, in connection with the expected condensation of glyoxylate with the acidic-CH_2- group of OAA (65) to form oxalomalate led to the speculation (67) that an enzymatic sequence of reactions such as the one outlined in Figure 11 might be the reaction sequence leading to glutamate from the condensation of glyoxylate with OAA.

In the experiments described herein, the enzymatic condensation of glyoxylate with OAA will be considered and differentiated from a similar non-enzymatic reaction occurring at pH 7.4 at 40°C. The isolation of labeled HGA and glutamate, terminal products of the reaction sequence of Figure 11, will be discussed, as well as the isolation of HKG and the available evidence for the

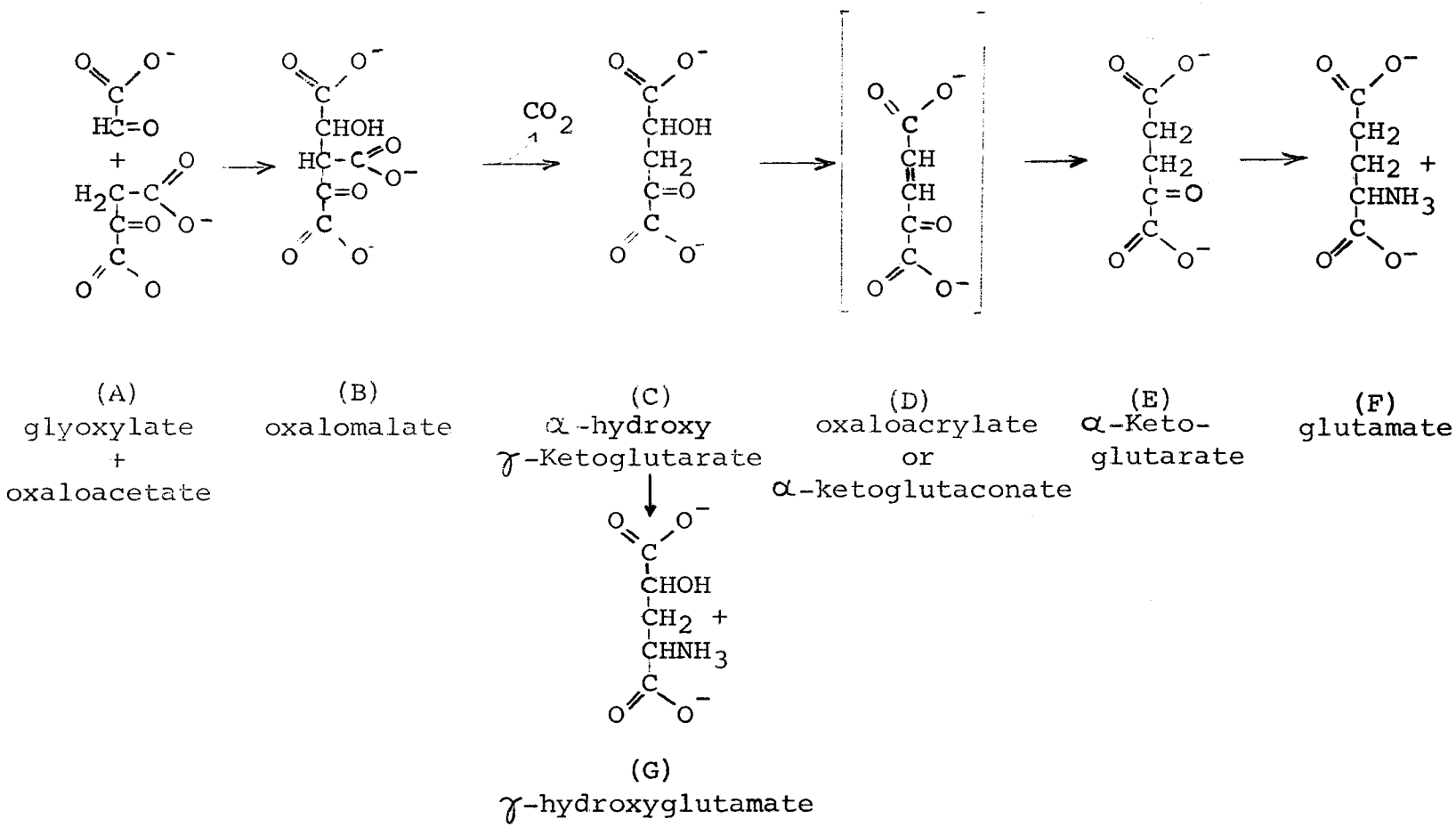

Figure 11. The Glyoxylate-Oxaloacetate pathway for Glutamic acid Biosynthesis in A. suboxydans.

Table 10. Glutamic acid Formation in Cell-Free Extracts of A. suboxydans.

	Substrates	Nitrogen Source	Amount (μmoles)	Conversion to Glutamic Acid* (% based on carbon source)
A	α-ketoglutarate + DPNH (3 mg)	$(NH_4)_2SO_4$ (50 μmoles)	100	17.6
	α-ketoglutarate + B_6-PO_4 (150 μg)	L-alanine (100 μmoles)	100	58.1
	α-ketoglutarate + B_6-PO_4 (150 μg)	L-aspartate (100 μmoles)	100	82.1
	oxalosuccinate + DPNH (3 mg)	$(NH_4)_2SO_4$ (50 μmoles)	100	21.2
	isocitrate + DPN (3 mg)	$(NH_4)_2SO_4$ (50 μmoles)	100	0.0
B	Glyoxylate + oxaloacetate	L-alanine (100 μmoles)	50+50	12.3
	Glyoxylate + oxaloacetate	-----	50+50	1.3
	Glyoxylate	L-alanine (100 μmoles)	50	0.4
	Oxaloacetate		50	1.0

Incubation of the substrates with one ml. of sonically digested cell-free extracts of A. suboxydans (protein 27 mg/ml) and 100 μmoles of phosphate buffer pH 7.4, at 30°C for 6 hours. Total volume 3 ml.

*Glutamate was measured manometrically with glutamic decarboxylase, either commercial preparations or enzyme purified from cells of E. coli 11216, according to the method of Najjar and Fisher (57).

participation of oxalomalate in this pathway. Further details on the isolation of glutamate, HGA, oxaloacrylate, α-KG are described elsewhere (52, 68, 69).

Formation of Glutamate

The elution pattern of acidic amino acids shown in Figure 12 was obtained under the conditions outlined, when the incubation mixture of equimolecular amounts of oxaloacetate, glyoxalate and a cell-free extract of A. suboxydans were incubated in the presence of an amino group donor and suitable cofactors. Usually the acidic amino acid fraction was separated from proteins and other neutral materials by a small Dowex-1-acetate column and then these were reseparated through the column described in Figure 12 (68). The glutamic acid fractions (tubes No. 30-35) were pooled and evaporated under vacuum. The product was identified by paper chromatography and assayed for radioactivity and amount of glutamic acid. In other experiments, sufficient amount of glutamic acid was isolated for melting point determinations, infrared spectra, etc., all of which agree with the properties of authentic L-glutamic acid (69).

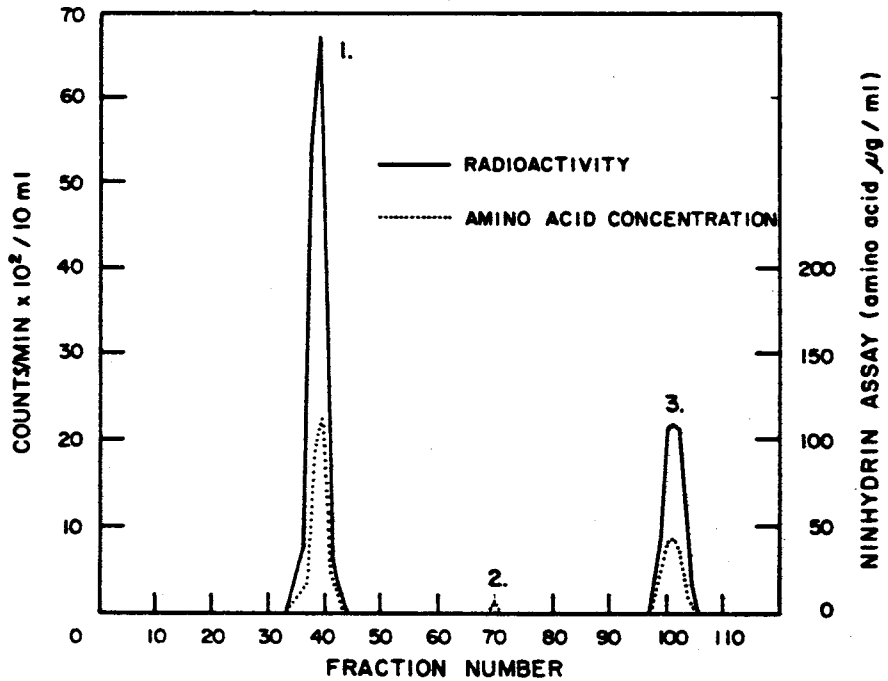

Figure 12. Acidic amino acids formed from glyoxylate-1-C^{14} and oxaloacetate by A. suboxydans.

[4]Elution pattern of acidic amino acids formed enzymatically by A. suboxydans. The reaction mixture contained: glyoxylate-1-C^{14}, 100 µmoles (1.75×10^6 cpm), oxaloacetate 100 µmoles, L-alanine 100 µmoles, NADH 1 mg, pyridoxal phosphate 1 mg, 2 ml M/50 phosphate buffer, pH 6.5, and 3 ml cell free extract (protein 25 mg/ml), total volume 10 ml; incubated at 30°C for six hours. Proteins were precipitated by boiling and centrifuged out. The supernatant was passed through a Dowex-1-acetate column (66 x 1.6 cm) and the acidic amino acids were eluted with 0.5N acetic acid. Peak 1 is glutamate total 1.7×10^6 cpm (1.5×10^4 cpm/µmole); peak 2 is aspartate and peak 3 is γ-hydroxyglutamate total 1.1×10^4 cpm (8.5×10^3 cpm/µmole).

Formation of γ-Hydroxyglutamate (HGA)

Another acidic amino acid is formed as shown in Figure 12 from the condensation of glyoxylate with oxaloacetate. The isolation and characterization of this amino acid have been described in detail elsewhere (68). It was established by infrared spectroscopy that the product of A. suboxydans is the L-erythro-isomer.

The concurrent formation of HGA with glutamate from the glyoxylate-oxaloacetate pathway (Figure 11) may be helpful in determining the participation of this pathway for the glutamate biosynthesis in other systems. The existence of HGA in a system should be considered only as suggestive evidence for the participation of the pathway, because HGA may be formed by other routes (28).

Degradation of the Isolated Glutamate and HGA

When glyoxylate-1-C^{14} is the condensing partner of OAA under the conditions described in Figure 12, it is expected from the reaction sequence outlined in Figure 11 that the label should be at position 5 of the glutamate and HGA (carbon atom farthest removed from amino group). This expectation was verified for both molecules, glutamate and HGA.

The experiment of Figure 12 was repeated several times in order to obtain sufficient amount of the amino acids from glyoxylate-1-C^{14} and OAA.

The radioactive glutamate was recrystallized several times after mixing with 500 mg. of L-glutamic acid. The Schmidt reaction (2) liberated carbon atom No. 5 which accounted for 70 percent of the total radioactivity. The ninhydrin degradation on the other hand liberated less than 3 percent of the total radioactivity of glutamic acid as $C^{14}O_2$ from carbon No. 1.

The same approach was used for HGA degradation. HGA was first converted to glutamic acid by reduction with hydriodic acid and red phosphorus (68). The resulting glutamic acid-C^{14} was mixed with authentic L-glutamic acid and recrystallized.

Table 11 summarizes the labeling patterns of both amino acids.

Enzymatic vs Non-enzymatic Condensation

The formation of both HGA and glutamate from OAA and glyoxylate proceeds presumably as outlined in Figure 11 through a reaction series; it is of significance that the first reaction, the condensation of OAA with glyoxylate,

Table 11. Degradations of Enzymatically Formed Glutamate-C^{14} and γ-Hydroxyglutamate-C^{14} from glyoxylate-1-C^{14} and OAA in A. suboxydans.

Carbon Atom and Method of Degradation	Radioactivity					
	Glutamate			γ-Hydroxyglutamate		
	cpm/mmole of carbon	cpm/mmole of glutamate	percent of total	cpm/mmole of carbon	cpm/mmole of γ-Hydroxyglutamate	percent of total
Total radioactivity (wet combustion)	97.2	486	100	28.6	143	100
C_1 (ninhydrin Degradation)	12.2	12.2	2.5	6.2	6.2	4.2
C_5 (Schmidt reaction)	356	356	73.5	115	115	80

can also take place non-enzymatically, and proceeds essentially to the completion at pH 7.4 and 40°C.

In a study of the enzymatic reaction sequence of Figure 11, it was of importance to establish whether or not the first reaction, namely the formation of oxalomalate, was enzymatic or not.

In the study of the enzymatic reactions, HKG is the first condensation product of glyoxylate plus OAA which has been isolated from both the growth medium (69) and

from incubation mixtures of CFE with an equimolecular mixture of glyoxylate and OAA as described later; all the available evidence points to the formation of HKG by decarboxylation of oxalomalate. The amount of HKG formed has been considered in this work as a measure of the extent of the condensation of glyoxylate with OAA.

Above pH 7.0 the two reactions cannot be differentiated because the non-enzymatic one is fast and complete. Below pH 7.0, however, the non-enzymatic reaction virtually ceases, while the enzymatic one continues to take place, as may be seen from the amount of keto acids expected to be formed as the result of OAA-glyoxylate condensation and as intermediates of glyoxylate-oxaloacetate pathway (Figure 11), namely HKG and α-ketoglutarate. Table 12 summarizes the results of the enzymatic vs non-enzymatic condensation (boiled enzyme) at pH 6.0. It can be seen that a slightly acid pH stops completely the condensation non-enzymatically, while the enzymatic reaction proceeds to a significant extent.

Although the physiological pH conditions within the cell are not known, it can be concluded from the above experimental evidence that enzymatic condensation can take place even under conditions where non-enzymatic reaction

Table 12. Formation of keto-acids from enzymatic, as compared to non-enzymatic condensation of glyoxylate with oxaloacetate.

Keto Acids Formed	Percent Yield at pH 6.0*	
	Active enzyme	Boiled enzyme
α-ketoglutarate	6.3	none
α-keto-γ-hydroxyglutarate (HKG)	4.4	none
Pyruvate	trace	trace

At pH 7.4 and 40°C the condensation proceeds essentially to completion within two and a half hours.

*Percent of the yield expected from condensation to completion. The reaction mixture contained: Glyoxylate 100 μmoles, OAA 100 μmoles, phosphate buffer (pH 6.0) 2 mmoles, cell-free extracts 10 ml. (protein 25 mg/ml). Total volume 30 ml. incubated at 30°C for 6 hours. Boiled cell-free extract was obtained by autoclaving at 120°C for 3 minutes.

Keto acids were separated from the concentrate of the above reaction mixture by paper chromatography. The zone of each keto acid was eluted by water or dilute ammonia solution. The eluates were made up to a certain volume and aliquots were taken for the colorimetric determination of the keto acids (25).

is nil.

The stereo-specificity of the isolated products, to be discussed later, is considered as unequivocal proof for the existence of stereospecific enzymatic reactions.

Whether or not the existence of a non-enzymatic

condensation has any evolutionary significance in the induction of the formation of such a condensing enzyme in A. suboxydans is not known. It is not known, either, how wide-spread this condensation reaction might be in tissues in general. It may well be, however, a good example of how nature has utilized favorable non-enzymatic reactions for its synthetic benefits.

Non-Enzymatic Condensation of Glyoxylate with Oxaloacetate. Preparation of Oxalomalate

The non-enzymatic reaction has been employed advantageously for the preparation of oxalomalate and from it HKG and HGA. Under controlled conditions of pH(7.4)and temperature (40°C), condensation between sodium OAA and sodium glyoxylate occurs yielding a trisodium salt of a tricarboxylic acid, which was characterized by Ruffo and coworkers (65) as α-hydroxy-β-oxalosuccinic acid (oxalomalic). Their procedure was followed in this work for the preparation of oxalomalic acid. Figure 13 shows the rate of the non-enzymatic condensation as followed by the disappearance of glyoxylate measured according to the colorimetric method of Kramer et al (44). Trisodium oxalomalate thus formed appeared as a slightly yellow

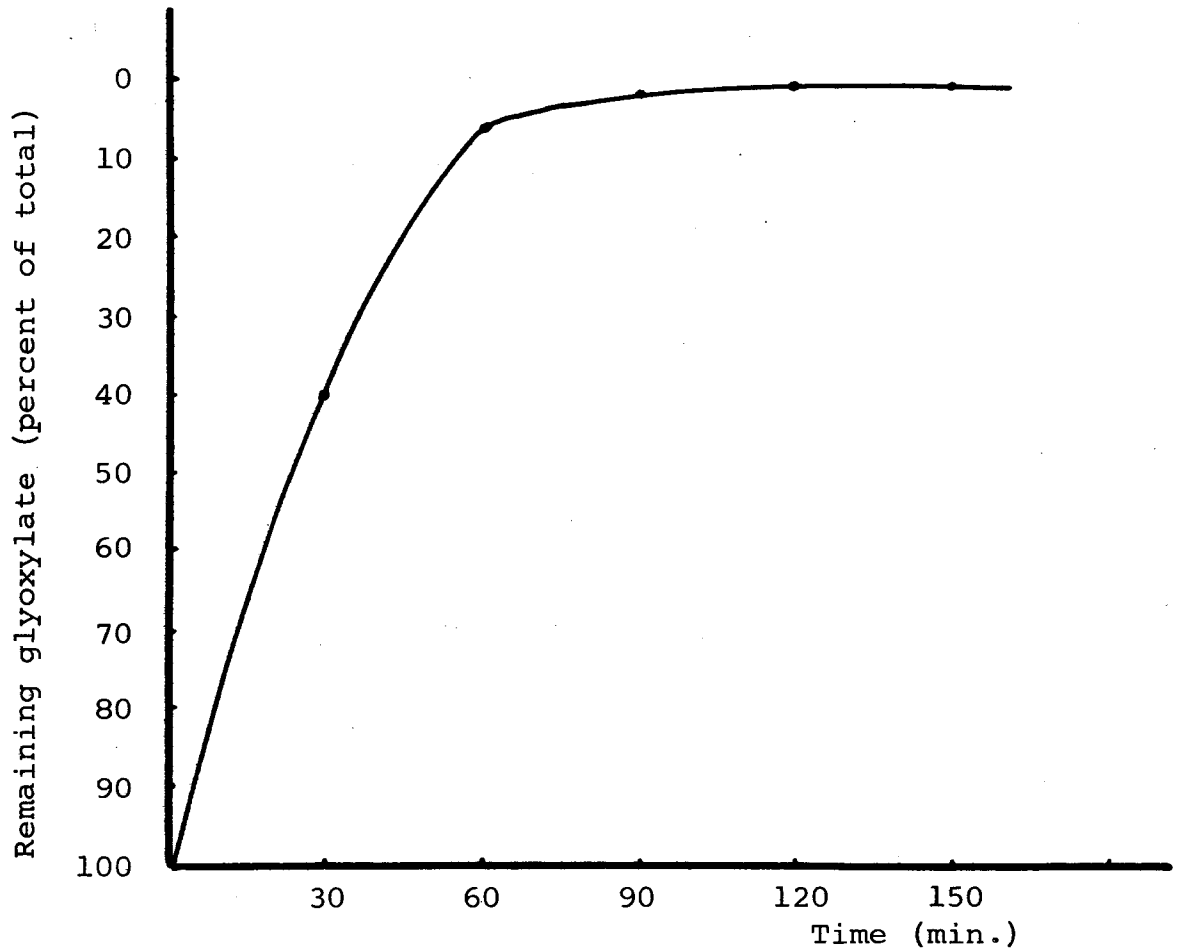

Figure 13. Non-Enzymatic condensation of Glyoxylate and Oxaloacetate.

substance which was used when possible immediately from the reaction mixture, or was freeze dried. It is very unstable as a free acid and is decarboxylated in acid medium yielding HKG. The formation of HKG from oxalomalate has been considered in this work as a criterion of the structure of oxalomalate, but Ruffo (65) has established its structure by C, H and N analysis, functional group, infrared spectroscopy, etc.

Any attempt to obtain the free oxalomalic acid failed because even under mild acidic conditions, it always released CO_2. The formation of a 2,4-dinitrophenylhydrazone also causes decarboxylation of oxalomalic acid and the phenylhydrazone of HKG is formed (mp 165°C).

Decarboxylation of Oxalomalate

Carbon dioxide evolved during decarboxylation of oxalomalate was flushed by CO_2- free air through 0.1N NaOH and was measured by acidimetric titration. Addition of 1M H_2SO_4 to pH 3.0 caused complete decarboxylation of oxalomalate. Similar results were obtained by formation of the 2,4-dinitrophenylhydrazone. CFE of A. suboxydans (5 ml, protein, 52 mg/ml) causes 55% decarboxylation of oxalomalate (10 mmoles) within 30 minutes. Under the same

conditions, however, boiled enzyme causes decarboxylation to a significant extent (20%). HKG, formed as a product of decarboxylation, was identified in the reaction mixtures.

The carbon dioxide evolved arises from the carboxyl group in the β-position of the keto group of the original oxaloacetate (C_4). This would be expected from the known tendency of OAA to lose CO_2 and form pyruvic acid, and it has been established by Ruffo (65) and in this work as described herein.

Ruffo (65) proved the mode of decarboxylation of oxalomalate by preparing the analogous compound, disodium α-hydroxymethyl-β-oxosuccinate from a similar condensation of formaldehyde with sodium oxaloacetate. When the phenylhydrazone of this compound was prepared, decarboxylation occurs and the phenylhydrazone formed was identified as the phenylhydrazone of γ-hydroxy-α-oxobutyric acid. We obtained more direct evidence concerning the loss of C_4 of OAA during the decarboxylation of oxalomalate. Table 13 shows the difference in the incorporation of label into glutamic acid and from aspartate-4-C^{14} plus glyoxylate as compared to that from aspartate-U-C^{14}. Seven and 6/10 percent of the added radioactivity in

Table 13. Incorporation of Aspartate-C^{14} into L-glutamic acid in A. suboxydans.

		Radioactivity	
		cpm	Percent
A	DL-Aspartate-4-C^{14} (added)	5.05×10^6	100
	L-Glutamate (formed)	1.51×10^4	0.3
	Carbon dioxide (recovered as $NaHC^{14}O_3$ in center well of reaction)	3.83×10^5	7.6
B	L-Aspartate-U-C^{14} (added)	5.35×10^6	100
	L-Glutamate (formed)	6.36×10^4	1.2
	Carbon dioxide (as above)	2.31×10^5	4.4
Control	L-Aspartate-U-C^{14}	5.35×10^6	100
	L-Glutamate	---	--
	Carbon dioxide	2.1×10^3	0.04

Aspartate-C^{14} 10 μmoles was incubated with 20 μmoles of glyoxylate in the presence of B_6-PO_4 2 mg, DPN 2 mg, Phosphate buffer pH 6.5 200 μmoles and 2 ml. of CFE (protein 51 mg/ml), at 30°C for 4 hours. The control contained instead of CFE 2 ml. boiled CFE. Amino acids were separated on Dowex-1-acetate column.

aspartate-u-C^{14} was recovered as $C^{14}O_2$, but only 0.3 percent was incorporated into the glutamic acid fraction from this substrate. With aspartate-U-C^{14}, however, 1.2 percent of the label was found in the glutamic fraction.

Further evidence was obtained by preparation of

oxalacetate-C^{14} from aspartate-4-C^{14} by non-enzymatic transamination as described by Goldstone and Adams (28) with pyridoxal phosphate. The OAA-4-C^{14} was used for the preparation of oxalomalate-C^{14} by condensation with glyoxylate as previously described. The presence of pyridoxal phosphate caused a considerable degree of decarboxylation of the formed oxalomalate-C^{14} under these conditions. The addition of CFE of A. suboxydans in the above preparation removed all radioactivity added (OAA-4-C^{14}) as $C^{14}O_2$.

Oxalacetate-4-C^{14} was also generated by transamination of aspartate-4-C^{14} with α-ketoglutarate in presence of glutamic-OAA transaminase. When CFE of A. suboxydans was present in this reaction mixture, it caused a decarboxylation of OAA-4-C^{14} to an extent of 72 percent. Addition, however, of glyoxylate under the same conditions caused an increase in the decarboxylation by 20% presumably due to the occurring condensation of OAA-4-C^{14} with glyoxylate and subsequent decarboxylation. Boiled enzyme under the same conditions yielded only 7 percent decarboxylation of OAA-4-C^{14}.

The above observations, along with the fact that oxalomalic acid yields HKG on decarboxylation, suggests

that the C_4 of OAA is lost during this process.

The ease of the decarboxylation of oxalomalate in acid medium and during the formation of the 2,4-dinitrophenylhydrazone, led Payers and Laties (60) to assume that the oxalomalate described by Ruffo (65) does not exist, but the product of the condensation is a mixture of bicarbonate and HKG. This assumption, however, does not seem to be true, because at pH 7.4, where the condensation is taking place, the concentration of oxalomalate is 0.2M and if decarboxylation had been taking place concurrently with the formation of oxalomalate, it would require either CO_2 evolution or the formation of a bicarbonate buffer (at pH 7.4) of 0.2M. Carbon dioxide, however, is not formed during the reaction, as shown by failure to trap any CO_2 formed. Moreover,a bicarbonate concentration of 0.2M at pH 7.4 is not possible.

The properties of oxalomalate, as an easily decarboxylated compound, may be an explanation for the fact that this compound has not been isolated as yet from A. suboxydans medium or incubation mixtures with CFE. The fact, however, that oxalomalate is decarboxylated by A. suboxydans to form HKG and that it is metabolized to form glutamate and HGA as expected from Figure 11, is fairly

convincing evidence that it is involved in the bioxynthesis of the above amino acids.

Preparation of HKG

As has already been mentioned, oxalomalic acid is decarboxylated to yield HKG. Five hundred mg. of the synthetic oxalomalate was dissolved in 2 ml. of water and the pH was adjusted to 4 by the addition of acetic acid. The solution was then dried and was subjected to column chromatography as described in Figure 14. HKG is eluted as a single peak under these conditions, and appears to be the only compound formed. Its identity has been established by paper chromatography of the 2.4-dinitrophenylhydrazone and by conversion by reduction to HGA.

Non-enzymatic Conversion of HKG to HGA

A suitable amount, approximately 2 μmoles of 2.4 dinitrophenylhydrazone, of the HKG was treated for three hours with H_2 at room temperature and atmospheric pressure in the presence of 20 mg. of PtO_2 (28). HGA formed was detected in the above reaction mixture by paper chromatography. Formation of HKG from oxalomalate and conversion of HKG to HGA as above has been considered as a further proof for the identity of the product of the non-enzymatic

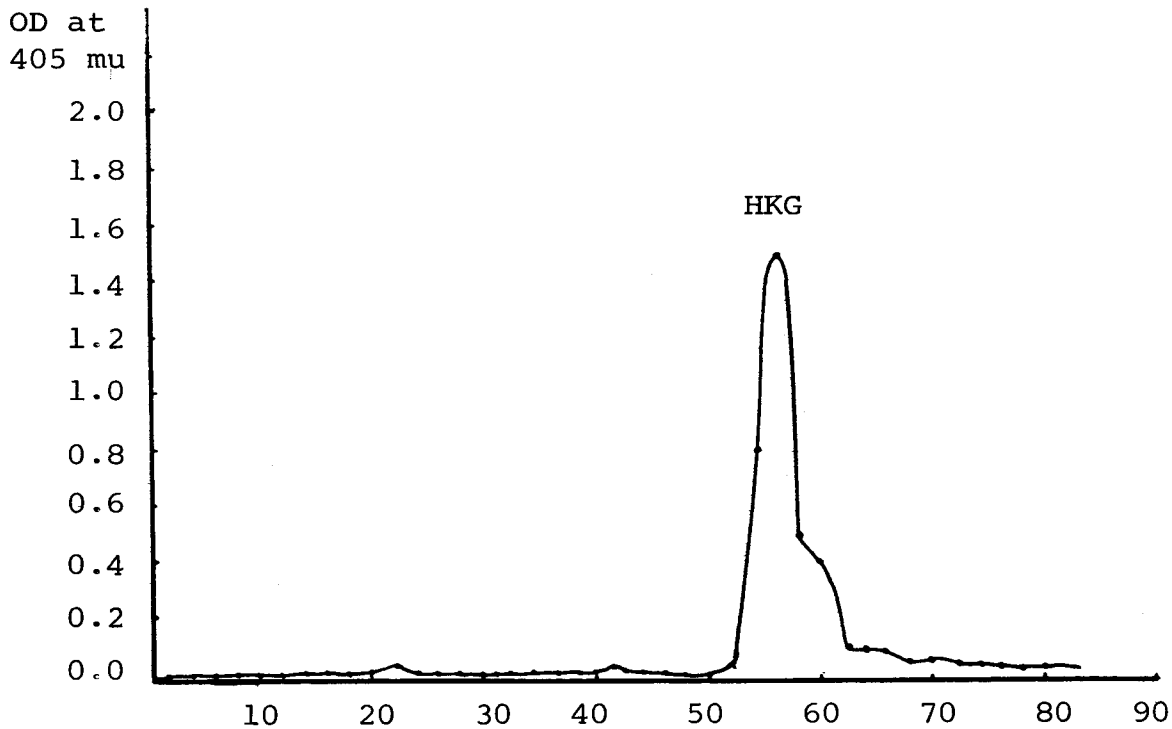

Figure 14. Separation of HKG on Dowex-1-formate column.

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[5]The solution of HKG was adjusted to pH 6.0 and absorbed on a Dowex-1-Formate column (66 x 1.5 cm), then subjected to gradient elution with 6N formic acid in the reservoir and 200 ml of water in the mixing chamber. Fractions of 10 ml were collected and 0.1 ml of each fraction was assayed for keto acids.

condensation of glyoxylate with OAA.

Enzymatic Conversion of HKG to HGA

The enzymatic reduction of HKG by glutamic dehydrogenase was first described by Goldstone and Adams. Both D and L-HKG are substrates for crystalline glutamic dehydrogenase (3).

Incubation of the synthesized HKG with crystalline glutamic dehydrogenase yielded HGA under the following conditions: HKG 0.14 mmoles, neutralized DPNH 0.15 mmoles, $(NH_4)_2SO_4$ 0.4 mmoles, phosphate buffer, pH 7.0, 1.0 mmole, crystalline glutamic dehydrogenase 10 mg., in a total volume 20 ml. incubation at $30^{\circ}C$ for 6 hours.

HGA formed was separated from other substances of the reaction mixture as described in Figure 15. This preparation of HGA is a simple preparative enzymatic synthesis. It is essentially similar to the method of Adams and Goldstone (3), but it eliminates the enzymatic condensation reaction step for the synthesis of HKG from glyoxylate and pyruvate.

These methods of HKG and HGA preparations permit the production of keto or amino acid products isotopically labeled in any or all carbon atoms depending on the

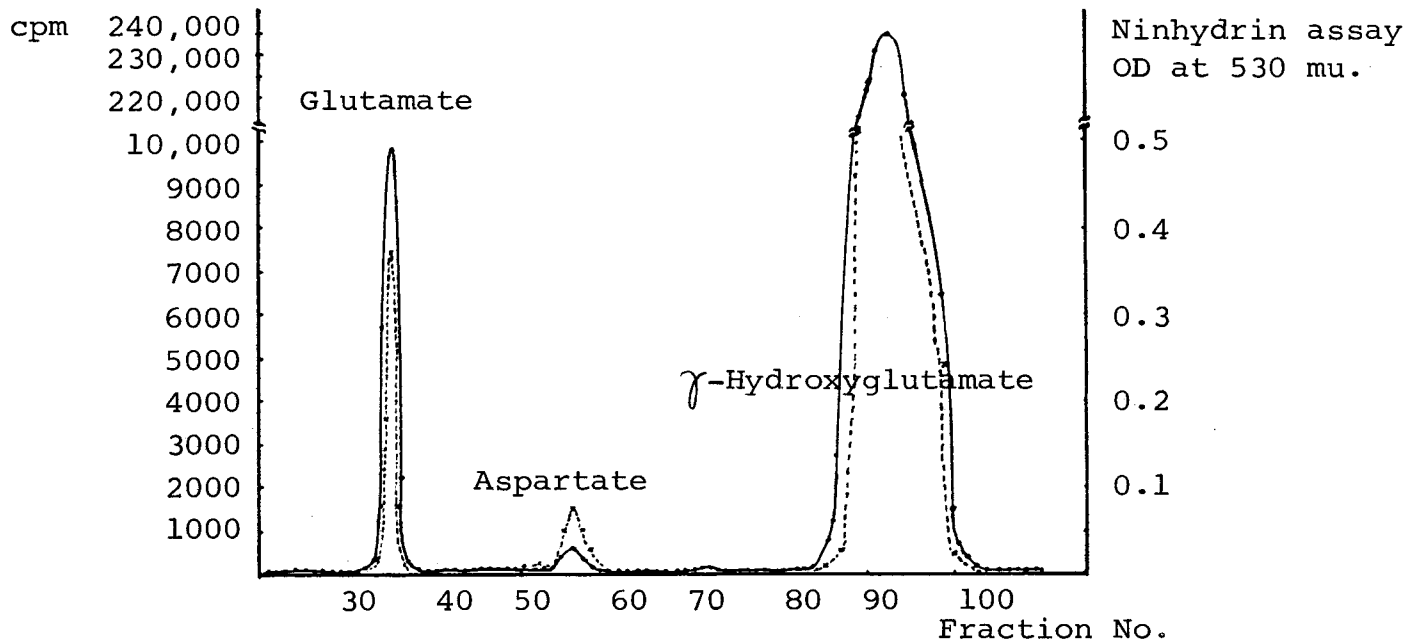

Figure 15. Elution pattern of the acidic amino acids formed from synthetically prepared oxalomalate-C^{14} by A. suboxydans.

[6]The reaction mixture contained: oxalomalate-C^{14} 400 µmoles (5.34×10^6 cpm), DPNH 140 mg (200 µmoles), $(NH_4)_2SO_4$ 1 mmole, L-alanine 1 mmole; Phosphate buffer, pH 6.5,1 mmole, CFE of A. suboxydans 20 ml (protein 55 mg/ml) in a total volume of 100 ml., incubated at 30°C for 6 hrs. Acidic amino acids were separated as outlined in Fig. 12. Radioactivity in the glutamic acid fraction (31,600 cpm) accounted for 0.6 percent of the radioactivity added. HGA-C^{14} fractions (1.57×10^6 cpm) accounted for 29.5 percent of the added radioactivity as oxalomalate-C^{14}. The same incubation mixture, but with boiled enzyme, gave no radioactivity in any of the acidic amino acids formed with active enzyme.

choice of specifically labeled glyoxylate or oxaloacetate.

Conversion of Oxalomalate-C^{14} to Amino Acids by A. suboxydans

Oxalomalate-C^{14} was prepared as already described from glyoxylate-1,2-C^{14} and OAA. A small excess of OAA was used to insure complete disappearance of glyoxylate; under these conditions after two and a half hours, no detectable amount of glyoxylate remained. Incubation of the oxalomalate-C^{14} solution with CFE of A. suboxydans under conditions detailed in Figure 15 yielded the two aminodicarboxylic acids, glutamic and HGA both labeled. Figure 15 is the elution pattern of radioactivity which coincides with the ninhydrin assay for both glutamic and HGA. A ninhydrin-positive peak, corresponding to aspartic acid, is not radioactive. Aspartate is probably formed from the small excess of OAA remaining from the preparation of oxalomalate-C^{14}.

Under the same conditions, boiled enzyme incubated with the oxalomalate-C^{14} gave no radioactivity in any of the amino dicarboxylic acid fractions.

The experiments described provide evidence for the participation of oxalomalate in the reaction sequence of

Figure 11. Although it has not been isolated as yet from the reaction mixtures of A. suboxydans, the fact that it is decarboxylated and converted to the aminodicarboxylic acids HGA and glutamate, under suitable conditions, strongly points to its participation in the reaction sequence of Figure 11.

Isolation of HKG-C^{14} from the Enzymatic Condensation of OAA and Glyoxylate 1,2 C^{14}

In the previous experiments, HKG has been cited as decarboxylation product of oxalomalate without structural identification. In the experiments described herein, the isolation of HKG from incubation mixtures of OAA and glyoxylate with CFE of A. suboxydans has been undertaken.

A reaction mixture consisting of: 1 mmole glyoxylate 1,2-C^{14} (17 μc), 1 mmole oxaloacetate, DPN 5 mg., M/50 phosphate buffer, pH 6.0, 30 ml., and 20 ml. of CFE (protein 47 mg/ml) in a total volume of 90 ml. were incubated at 30°C for 6 hours. At the end of this period, proteins were precipitated and any HKG-C^{14} formed was separated from other materials by Dowex-1-formate column. The HKG-C^{14} peak obtained from this separation amounted to 4.34 percent of the added radioactivity, while 90 percent

of the added glyoxylate-1,2 C^{14} was recovered. The identity of HKG was established by paper chromatography of its 2,4-dinitrophenylhydrazone in two solvent systems. Conversion of the hydrazone by reduction with H_2 in the presence of PtO_2 (28), or reductive amination with glutamic dehydrogenase, gave rise to a single amino acid corresponding to HGA on paper chromatography (R_f values agreed) (3).

The HKG-C^{14} formed from the above experiment is exclusively an enzymatic product, because under these conditions, pH 6.0 the non-enzymatic condensation is nil. (Table 12).

From similar experiments with non-radioactive substrates, a sufficient amount of HKG was isolated for melting point determination, infrared spectra and chemical degradation to malic acid (52). The properties of the enzymatic HKG were in agreement with the properties of HKG prepared by a non-enzymatic transamination with pyridoxal phosphate of HGA (28). Paper chromatographic properties of the 2,4-dinitrophenylhydrazone of HKG, melting point (162-164^{o}C) and infrared spectra of the isolated HKG are in good coincidence with both L and DL-HKG prepared from HGA.

Goldstone and Adams (28) established the absolute configuration of HKG formed in rats by oxidative decarboxylation to malic acid. On treatment with H_2O_2, HKG is oxidatively decarboxylated to yield malate and one equivalent of CO_2. The configuration of the formed malate was established by infrared spectroscopy (52) and from this the steric properties of the enzymatic HKG were deduced. D-malate is formed from the enzymatic HKG and since the asymmetric carbon is not involved in the oxidative degradation, it is concluded that HKG has also the D-configuration and is therefore designated D_s-α-hydroxy-γ-ketoglutaric acid.

Figure 16 summarizes the configuration of the enzymatically formed HKG in relation to the oxidation product D-malate and to the product of the reductive amination or transamination of HKG (HGA). It can be seen that the steric properties of HKG and HGA from A. suboxydans are in agreement and, as would be expected from a stereospecific enzymatic reaction sequence, pure isomers.

Concluding Remarks on the Glyoxylate-OAA Pathway

The observations described suggest that a non-enzymatic condensation reaction between OAA and

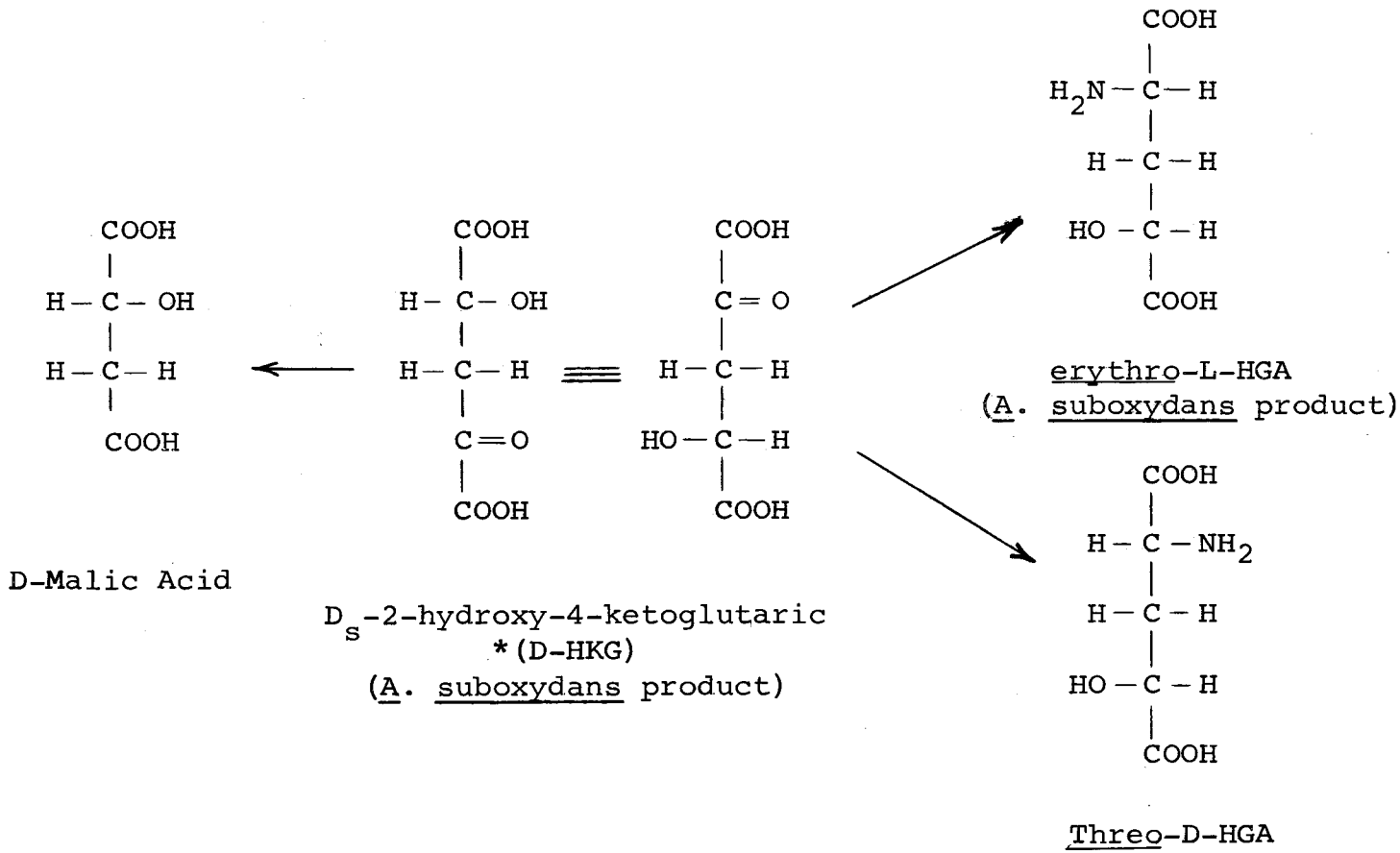

Figure 16. Configuration of HKG in A. suboxydans

*The subscript s refers to serine, the fundamental substance to which amino acids that bear structural resemblance to the carbohydrates can be formally related. The steric identification of enzymatically formed HKG by A. suboxydans as D-α-hydroxy-γ-ketoglutarate is in agreement with the configuration of the erthro-L-HGA, derived from HKG and isolated in this organism.

glyoxylate can take place easily at pH 7.4 and 40°C to form oxalomalate. This reaction is of similar nature to the formylation reaction discovered by Tollens in 1889 and used by Fourneau and coworkers for the preparation of amino alcohols (24). Formylation is the condensation of acidic-CH-groups with formaldehyde in its hydrate forms to yield a C-C link. The condensation of OAA with glyoxylate was investigated by Ruffo and coworkers (65).

In this study, this non-enzymatic condensation was differentiated at pH 6.0 from an enzymatic condensation reaction occurring in A. suboxydans which appears to be the first reaction of an enzymatic sequence leading to amino dicarboxylic acids, glutamate and HGA.

It must be noted that the fact that OAA and glyoxylate can be condensed non-enzymatically also, may have an evolutionary significance and it may serve as an example of the formation of enzymes to catalyze known non-enzymatic reactions.

Oxalomalate has not been isolated as yet from A. suboxydans, presumably because it is decarboxylated to HKG. The fact, however, that synthetically prepared oxalomalate is decarboxylated to HKG and it is metabolized in this organism to form glutamate and HGA under suitable

conditions, strongly points to its participation in the reaction sequence of Figure 11.

HKG, the decarboxylation product of oxalomalate, has been isolated from both the growth medium (69) and incubation mixtures of OAA and glyoxylate and cell-free extract. In considering the reaction sequence that leads to HGA and glutamic acid, it is notable that the decarboxylation step of oxalomalate must be irreversible and that the C_4 of OAA is lost during the decarboxylation. The use of OAA-4-C^{14} or aspartate-4-C^{14} as a source of OAA provides a direct proof that the carbon atom 4 is lost during the decarboxylation step, and degradation studies of the terminal amino acids, L-glutamate and HGA reveal that the localization of the label in the molecule is in accord with an enzymatic reaction sequence as in Figure 11. To our knowledge, this is the first report on the formation of HKG enzymatically from OAA and glyoxylate; this reaction may be widespread in tissues in general.

HKG has been studied as a product of the transamination of HGA with OAA in the metabolism of hydroxyproline (28) and a product of the enzymatic condensation of glyoxylate with pyruvate (28, 48). This condensation reaction has not been demonstrated in A. suboxydans, and

the fact that no pyruvate can be detected under conditions that HKG is formed from glyoxylate and OAA (Table 12) may be considered as evidence that the cleavage of HKG to glyoxylate and pyruvate (28) does not occur in this organism.

The determination of the steric properties of HKG was of fundamental importance, in view of the fact that some of the described reactions can also take place non-enzymatically under suitable conditions. It would be expected, however, that such non-enzymatic reactions should lead to racemic HKG and not to a pure isomer such as is formed in the reactions occurring in A. suboxydans. The stereospecificity of the reactions leading to L-erythro-HGA and L-glutamic acid is a further support of the occurrence of such enzymatic reactions. The steric properties of erythro-L-HGA are in agreement with the configuration of its precursor (D-HKG) in this organism.

It is of interest that A. suboxydans, although an obligate aerobe, has been reported as not having a demonstratable Krebs cycle. On the other hand, Ruffo has studied oxalomalate as a potent inhibitor of aconitase (65). In addition, Payes and Laties (60) reported that HKG also acts as a competitive inhibitor, in the respiration of

potato slices, not only for aconitase, but also for isocitric dehydrogenase and α-ketoglutaric dehydrogenase.

These observations suggest that since oxalomalate and HKG are formed and involved in the metabolism of A. suboxydans, the apparent absence of the Krebs cycle may well be a result of a competitive inhibition of the key enzymes of the cycle by these compounds.

FORMATION OF GLUTAMATE FROM OTHER SUBSTRATES

In addition to the previsously described two reaction schemes which can lead to glutamate formation in A. suboxydans, other compounds of carbohydrate or amino acid nature have been shown to be converted to glutamate in this organism. Table 14 summarizes the results of glutamate formation by incubation of several substrates with CFE of A. suboxydans, as determined manometrically with glutamic decarboxylase (57).

In addition, propionate-C^{14} plus glyoxylate, when incubated with CFE under the conditions of Table 9 yielded radioactive glutamate and aspartate.

Although none of the above reactions has been studied in any details, it appears that glutamate can be

Table 14. Conversion to glutamate of other substrates by cell-free extracts of A. suboxydans.

Substrates	Nitrogen Source	Amount (μmoles)	Conversion to Glutamic Acid* (percent based on carbon source)
Malate + glyoxylate + B_6-PO_4 (150 μg)	L-alanine (100 μmoles)	100+100	14.5
Malate + B_6- PO_4 (150 μg)	"	100	2.5
L-arabinose ± B_6- PO_4 (150 μg)	"	100	0.85
D-arabinose + B_6-PO_4 (150 μg)	"	100	1.8
5-ketogluconate + B_6-PO_4 (150 μg)	"	100	---
2-ketogluconate + B_6-PO_4 (150 μg)	"	100	3.6
L-histidine	---	100	13.1
L-proline	---	100	6.5
L-arginine	---	20	37.7
L-ornithine	---	20	2.7

*Reaction conditions and glutamate determination as detailed in Table 9.

formed from these substrates in this organism.

ASPARTIC ACID BIOSYNTHESIS IN A. SUBOXYDANS

The biosynthesis of aspartic acid in A. suboxydans is of interest not only in view of the apparent absence of the Krebs cycle in this organism, but also because it is related to oxaloacetate, one of the starting materials of the glyoxylate-OAA pathway.

OAA normally is formed through the Krebs cycle, while glyoxylate arises by the action of isocitrate lyase from isocitrate.

In A. suboxydans the most likely source of glyoxylate is from glucose by the action of transaldolase. OAA may arise from malate since malate is converted to aspartate in this organism (Table 15-A). If aspartate is arising from malate by way of OAA, then it would be expected that addition of glyoxylate, as in Table 15-B, should yield in addition to aspartate, labeled glutamate and HGA through the reactions of glyoxylate-OAA pathway described previously.

Malate may be formed by condensation of glyoxylate with acetyl-coenzyme A. Acetate-1-C^{14} plus glyoxylate yields all three amino acids expected from the

Table 15. Aminodicarboxylic Acids from Glyoxylate plus Acetate or Malate.

	Substrates*	Total Radioactivity in the Fraction of (cpm)		
		Glutamate	Aspartate	γ-Hydroxyglutamate
A	Malate-3-C^{14} 10 μmoles, 2 μC	-	85,000	-
B	Malate-3-C^{14}, 10 μmoles + 5 μc Glyoxylate , 20 μmoles	1020	44,541	880
C	Acetate-1-C^{14} 20 μmoles 20 μc Glyoxylate 10 μmoles	16347	13,632	4932

*Substrates were incubated with 2.5 ml CFE (protein - 45 mg/ml) in the presence of the following: B_6-PO_4, 0.5 mg; CoA, 0.1 μmole; DPN, 1 mg; ATP 10 μmoles; glutathione, 3 μmoles; $MgCl_2$, 5 μmoles; $FeSO_4 \cdot 7H_2O$, 0.1 μmoles; $(NA_4)_2SO_4$, 10 μmoles; L-alanine 10 μmoles; phosphate buffer pH 7.3 100 μmoles. Total volume 10 cc incubated at 30°C for 6 hours.

intermediate formation of malate and the participation of the glyoxylate-oxaloacetate pathway (Table 15-C).

SUMMARY

Although Acetobacter suboxydans is an obligate aerobe, the complete Krebs cycle has not as yet been demonstrated in this organism. Therefore, alternative routes were suspected to have a functional existence, in order to account for the biosynthesis of glutamate and of other vital cell components, which normally arise through the reactions of the Krebs cycle in other organisms.

1. The reverse of the reaction sequence described by Barker and his coworkers for the fermentation of glutamate in Clostridium tetanomorphum has been assessed in this work as a possible pathway of glutamate biosynthesis in A. suboxydans. Formation of glutamate from acetate plus pyruvate via citramalate, mesaconate and β-methylaspartate is suggested from the fact that all the foregoing compounds can be converted to glutamate by cell-free extracts of this organism and are formed, although in small amounts, as radioactive metabolites from either labeled acetate or pyruvate. Citramalate has been

isolated from the reaction mixtures by celite column chromatography and characterized by paper chromatography, radioautography, optical rotation determination and crystallization to constant specific activity. Mesaconate was also isolated and characterized by paper chromatography and resublimation to constant radioactivity. β-methylaspartate and glutamate have been isolated by Dowex-1-chromatography and their metabolic interrelation is suggested from the dilution effect of added "cold" β-methylaspartate, when these amino acids are formed from acetate-C^{14} plus pyruvate. Interconversions among the isolated products and other evidence, all point to the participation of citramalate, mesaconate and β-methylaspartate as intermediates of a reaction sequence leading to glutamate from acetyl-coenzyme A plus pyruvate.

2. Glutamate can also arise in A. suboxydans through another reaction sequence starting with the condensation of glyoxylate with oxaloacetate to form oxalomalate. The latter is decarboxylated to form α-hydroxy-γ-ketoglutarate, which is either converted to γ-hydroxyglutamate or metabolized to α-ketoglutarate through a dehydration and hydrogenation step. The first condensation reaction can also take place at pH 7.4 and 40°C

non-enzymatically and is complete within two and one-half hours under these conditions. At pH 6.0 and 30°C, however, the non-enzymatic reaction is insignificant and can be differentiated from the enzymatic condensation which proceeds to a significant extent yielding stereospecific products. Although oxalomalate has not been isolated from A. suboxydans, synthetic oxalomalate is decarboxylated to α-hydroxy-γ-ketoglutarate (HKG) and metabolized to glutamate and γ-hydroxyglutamate. α-Hydroxy-γ-ketoglutarate has been isolated and characterized by several methods. The terminal amino-acids of this reaction sequence, γ-hydroxyglutamate and glutamate, have been characterized in the reaction mixtures of A. suboxydans and the localization of the label and the specific activities, when glyoxylate-1-C^{14} is the radioactive precursor, suggest a direct reaction sequence leading to these amino acids from glyoxylate and oxaloacetate.

The participation of oxalomalate and α-hydroxy-γ-ketoglutarate in glutamate biosynthesis has been considered as possible partial explanation of the apparent absence of the Krebs cycle in A. suboxydans. Both these acids have been reported recently to be potent inhibitors of the key enzymes of the Krebs cycle.

Although this thesis covers the two reaction sequences outlined above, there may be still other means whereby glutamate may be made available to this organism. Thus, histidine, arginine, or proline may be converted to glutamate; also other carbohydrate-type precursors may exist. Evaluation of the two routes discussed herein in terms of their probable total contribution to the supply of cellular glutamate, is probably yet premature, since a total inventory of available routes to this amino acid does not yet exist.

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