GLUTAMIC ACID BIOSYNTHESIS IN AN ORGANISM LACKING A KREBS TRICARBOXYLIC ACID CYCLE[1]

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Researches in this laboratory have been focused for several years upon the biosynthesis of glutamate in a vinegar producing bacterium, Acetobacter suboxydans. Glutamate is actively synthesized by this organism, yet the conventional Krebs cycle appears absent (Hauge, King and Cheldelin 1955; Kitos et al. 1958; Cheldelin 1961). The latter has been independently confirmed by Rao (1957) and by Fewster (1958). Individual precursors of glutamate have not been identified, although Röhr (1961) has suggested that the amino acid may arise through condensation of 2- and 3-carbon atom acid fragments in an unspecified Acetobacter species.

On the basis of our experimental evidence, it now appears that glutamate biosynthesis in this organism proceeds by at least two pathways. These may or may not be interrelated.

A. The condensation of oxaloacetate with glyoxylate.

B. The condensation of acetate with pyruvate.

In addition it has been demonstrated that glutamic acid is formed from several amino acids and several carbohydrate-nature intermediates.

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The present communication deals with glutamate formation from glyoxylate plus oxaloacetate.

MATERIALS AND METHODS

A. suboxydans ATCC 621 cells were grown and cell homogenates were prepared as previously described (King and Cheldelin 1954).

Paper chromatographic detection of amino acids has been carried out using 85 per cent phenol water in an atmosphere containing 3 per cent ammonia or n-butanol-acetic acid-water (12:3:5) on Whatman No. 1 paper. Acidic amino acids including methyl aspartic and γ-hydroxyglutamic acids were well separated in two-dimensional paper chromatography by these solvent systems.

RESULTS AND DISCUSSION

Oxaloacetate Plus Glyoxylate Condensation

The condensation of these acids resulted in glutamic acid formation. That these two compounds are preferred substrates for this condensation may be seen from Table I, since other condensing partners that might be suspected on a priori grounds, do not condense.

In a separate experiment, pyridoxal phosphate and alanine were omitted and formation of α-ketoglutarate was sought as the final product. This compound was separated by paper chromatography and characterized by the absorption spectrum of its 2,4-dinitrophenylhydrazone, with a maximum at 414 mμ. This reinforced our belief that the added alanine had merely served to furnish an amino group for transamination with α-ketoglutarate.

Formation of the C_5 skeleton was also indicated by the isolation of γ-hydroxyglutamate from the incubation mixture containing oxaloacetate, glyoxylate and L-alanine. The melting point was 180-184°, with decomposition (uncorrected); m.p. of authentic sample (epimeric mixture), 180-182°; mixed m.p., 180-184°.

Since formation of γ-hydroxyglutamate had probably been enhanced by the amino donor added, a separate condensation experiment was performed in the absence of added amino acid to isolate the keto acid precursor as a 2,4-dini-

Table I

Glutamic Acid Formation in Cell-Free Extracts of *A. suboxydans*

Oxaloacetate-Glyoxylate Pathway	Nitrogen Source	Amount µmoles	Conversion to Glutamic Acid per cent based on carbon source*
glyoxylate + succinate " + tartrate " + malate " + pyruvate	alanine + B_6-PO_4, 150 µg	100 + 100	0-5
glyoxylate + oxaloacetate	alanine + B_6-PO_4, 150 µg	100 + 100 + 100	19
α-ketoglutarate	---	100	13
α-ketoglutarate	$(NH_4)_2SO_4$	100 + 50	18
α-ketoglutarate	aspartate + B_6-PO_4, 150 µg	100 + 100	82
Citramalate Pathway**			
acetate + pyruvate	alanine	20 + 20	13
DL-citramalate	alanine	20 + 20	20
mesaconate	alanine	20 + 20	28
DL-β-methyl aspartate	alanine	20 + 20	10

Incubation period, 8 hrs. at 30° C; sonically digested cell-free extract (supernatant fraction after centrifuging at 4,000 x g for 30 min.); total vol. = 3 ml, with 0.05 M phosphate buffer solution pH 7.4. After incubation, the acidic amino acid fraction was separated on a column of Dowex 1 x 10 (acetate form) (Dekker *et al.* 1962).

*As determined with glutamic decarboxylase, either commercial preparations or after purification of the method of Najjar *et al.* (1954), from cells of *E. coli* 11216.

**Each flask contained the following cofactors: Vitamin B_{12}, 1 mµg; CoA, 0.1 µmole; ATP, 10 µmoles; $MgCl_2$, 5 µmoles, GSH, 3 µmoles; $FeSO_4 \cdot 7H_2O$, 0.1 µmoles; $(NH_4)_2SO_4$, 10 µmoles.

trophenylhydrazone. The dinitrophenylhydrazones were separated by paper chromatography, then an unknown band was eluted and reduced catalytically over palladized asbestos. The resulting amino acid was isolated and shown to be γ-hydroxyglutamic acid.

The precursor of γ-hydroxyglutamate was probably thus either γ-hydroxy α-ketoglutarate (HKG) (Kuratomi and Fukunaga 1960) or the corresponding unsaturated compound, oxaloacrylate, labeled D in Figure 1. The origin of these might in turn be expected from an appropriate six-carbon acid, listed in Figure 1 as oxalomalate, compound B. Formation of compound B, although speculative, is in line with the following experiments: when glyoxylate was incubated with uniformly labeled aspartate (used as a source of oxaloacetate), the resulting glutamic acid was highly radioactive; however, when aspartic acid-4-C^{14} was used, the glutamic acid bore no label, but respiratory CO_2 instead accounted for 90 per cent of the administered isotope. Ruffo _et al_. (1958) has suggested that a compound isomeric with compound B may be formed non-enzymatically from glyoxylate and oxaloacetate at pH 7.4. This has been confirmed by us; however, experiments with boiled _A. suboxydans_ cells at pH 6.0, measuring disappearance of reactants and formation of products, suggest that the non-enzymatic condensation is negligible under ordinary growth conditions.

The structural formulas of the various compounds involved are presented in Figure 1. While complete evidence for all of the individual transformations is not yet at hand, a possible reaction pathway is indicated; the compounds are arranged in a sequence that suggests this. It has been observed in several experiments that A and E and G separately give rise to glutamate in cell-free extracts of _A. suboxydans_. Finally, in addition to the observed conversion of hydroxyglutamate to glutamate, experiments with isotopes have shown (Table II) that when uniformly labeled aspartate is used as a source of oxaloacetate, then condensed with glyoxylate, radioactivity may be incorporated into either amino acid. The conversion of hydroxyglutamate to glutamate appears to be DPNH-dependent.

Figure 1

Compounds Related to Glutamate Formation in *A. suboxydans*

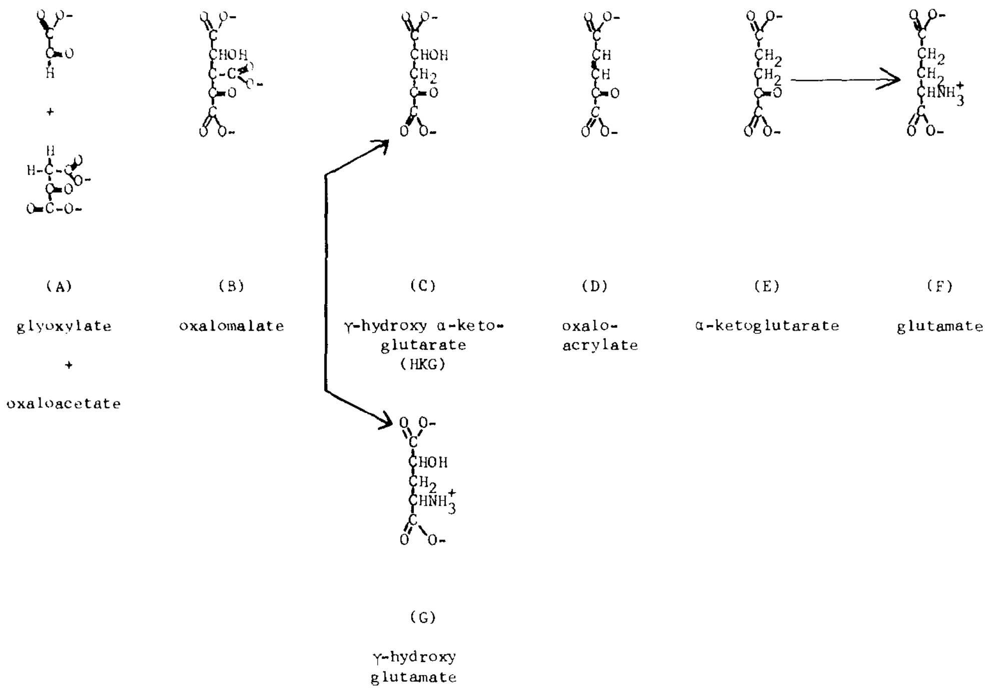

Table I also indicates that glutamate may be formed through condensation of pyruvate and acetate. Although no reaction scheme is offered here, it appears possible that a reversal may take place of the reaction sequence described by Munch-Petersen and Barker for the fermentation of glutamate in *Cl. tetanomorphum*. In addition to these compounds, malonate and oxalosuccinate have been shown by either tracer experiments or glutamic acid decarboxylase assay to yield glutamate in cell-free extracts. Finally, similar conversion has been observed from the amino acids arginine, ornithine, proline or histidine. The place of these compounds in the metabolism of *A. suboxydans* is being investigated further, as well as details of the principal conversion schemes outlined.

Table II

Formation of γ-Hydroxyglutamate and Glutamate by Dialyzed Cell-Free Extracts of A. suboxyd

System	Glutamate Synthesized* μmoles	Hydroxyglutamate Synthesized* μmoles	Isotope Incorporation per cent	
			Glutamate	Hydroxyglutamate
Complete	1.1	---	1.6	---
No DPNH	---	1.8	---	1.5

Each flask contained pyruvate, 5 μmoles; aspartate, 5 μmoles containing 5 μc U- aspartate; glyoxylate, 5 μmoles; ATP, 10 μmoles; $MgCl_2$, 10 μmoles; CoA, 0.5 μmole; glutathione, 0.5 μmole; DPNH, 5 μmoles where indicated; $B\text{-}PO_4$, 10 μg; cell-free extr dialyzed 96 hrs. vs. dist. H_2O; 1 ml; phosphate buffer pH 6.0, 100 μmoles. Total vo 5 ml. Incubated 24 hrs. at 30°C.

*Method of Moore and Stein (1954).

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