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Glutamate Biosynthesis in an Organism Lacking a Krebs Tricarboxylic Acid Cycle. V. Isolation of α-Hydroxy-γ-ketoglutarate (HKG) in *Acetobacter suboxydans**

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ABSTRACT: An enzymatic condensation of glyoxylate with oxaloacetate has been studied in *Acetobacter suboxydans*, and differentiated at pH 6.0 from a nonenzymatic condensation occurring at 40° and pH 7.4.

The nonenzymatic reaction was employed for synthesis of oxalomalate and through it, by decarboxylation, for α-hydroxy-γ-ketoglutarate. From the latter γ-hydroxyglutamate was obtained, by reductive amination with glutamic dehydrogenase. α-Hydroxy-γ-ketoglutarate has been isolated from incubation mixtures of equimolecular amounts of glyoxylate and oxaloacetate with cell-free extracts under conditions where nonenzymatic condensation is virtually zero. The identity of this compound has been established by comparison with the product of the nonenzymatic transamination of γ-hydroxyglutamate and of the synthetic α-hydroxy-γ-ketoglutarate from oxalomalate.

In *Acetobacter suboxydans*, the Krebs tricarboxylic acid cycle appears to be largely nonfunctional (Cheldelin, 1961; Rao, 1958) and the nonessential amino acids can be formed through alternate pathways (Sekizawa *et al.*, 1962). The metabolic interest in α-hydroxy-γ-ketoglutarate arose from the fact that it was envisioned as an intermediate of glutamic acid biosynthesis (Sekizawa *et al.*, 1962; Maragoudakis *et al.*, 1964).

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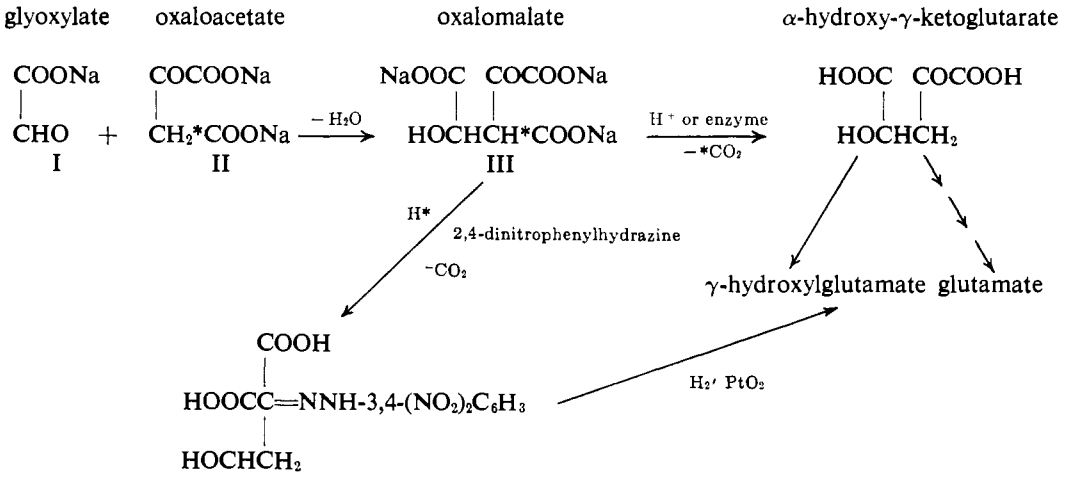
SCHEME I: Condensation of Glyoxylate with Oxaloacetate.



A mixture of oxaloacetate and glyoxylate can serve as precursors of glutamate in *A. suboxydans*. This observation in connection with the concurrent formation of γ-hydroxyglutamate and α-hydroxy-γ-ketoglutarate (Sekizawa *et al.*, 1965) in the reaction mixtures, and the expected condensation of glyoxylate with the acidic CH_2 group of oxaloacetate (Ruffo *et al.*, 1962b), led us to the speculation that glutamate may arise through an enzymatic sequence of reactions involving oxalomalate, α-hydroxy-γ-ketoglutarate, oxaloacrylate, and α-ketoglutarate (Scheme I). It is of significance that the condensation of oxaloacetate with glyoxylate to form oxalomalate can also take place easily nonenzymatically (Ruffo *et al.*, 1962b) under suitable conditions. This paper is concerned with the study of the nonenzymatic *vs.* the enzymatic condensation in *A. suboxydans*.

The isolation and characterization of α-hydroxy-γ-ketoglutarate, a product of the decarboxylation of oxalomalate, the first intermediate of the suggested pathway, is described. Its configuration will be considered in relation to the steric properties of the isolated γ-hydroxyglutamate (Sekizawa *et al.*, 1965). Paper and column chromatographic evidence, conversion enzymatically or nonenzymatically to γ-hydroxyglutamate, as well as infrared spectroscopy, all point to the structure given. The structure of α-hydroxy-γ-ketoglutarate was further established by its chemical degradation to malic acid. Its steric properties were elucidated by comparison of the infrared spectra of the formed malic acid with that of authentic L- and DL-malic acid. It was found to be D-2-hydroxy-4-ketoglutaric acid and this fact was considered to be further support of the occurrence of stereospecific enzymatic condensation of oxaloacetate with glyoxylate in *A. suboxydans*.

Materials and Methods

Oxalomalic acid was prepared synthetically by the method described by Ruffo and co-workers (Ruffo *et al.*, 1962b). The same method was employed for the preparation of radioactive oxalomalate using [1,2-^{14}C]glyoxylate as the condensing partner of oxaloacetate (Maragoudakis *et al.*, 1964). The rate of the condensation was followed by determining the disappearance of the aldehyde group of glyoxylate at various times (Figure 1) (Kramer *et al.*, 1959).

α-Hydroxy-γ-ketoglutarate (HKG)[1] was synthesized by decarboxylation of synthetic oxalomalate. The decarboxylation takes place by acidifying the solution of oxalomalate; further purification is effected through a column packed with Dowex 1-formate X8, 200–400 mesh (see Figure 2). The same procedure was followed for the isolation of HKG from incubation mixtures with cell-free extracts of *A. suboxydans*.

HKG was converted to γ-hydroxyglutamate (HGA) by reductive amination with glutamic dehydrogenase (Adams and Goldstone, 1963) or by reduction of the 2,4-dinitrophenylhydrazone of HKG with H_2 in the presence of PtO_2 (Goldstone and Adams, 1962). Keto acids were measured by the method of Friedemann and Haugen (1943).

The *A. suboxydans* cells were grown and cell-free extracts were obtained by sonic disintegration in a Raytheon ultrasonic vibrator as reported previously (King and Cheldelin, 1954). Protein was determined by the method of Lowry *et al.* (1951).

The steric properties of HKG isolated from *A. suboxydans* were studied by oxidative degradation to malate with H_2O_2 and comparison of the infrared spectra of the formed malate (as the calcium salt) to the stereoisomers of calcium bimalate.

Paper chromatography was performed by an ascending method on Whatman No. 1 paper. The organic acids were separated in a solvent system consisting of *n*-butyl alcohol–formic acid–water (4:0.7:1) and for the phenylhydrazones of keto acids, the systems *n*-butyl alcohol–ethanol–ammonia (70:20:10) or *n*-butyl alcohol saturated with 3% ammonia were employed. Amino acids were separated in *n*-butyl alcohol–acetic acid–water (12:3:5) or phenol–water (85:15). Oxalo-

[1] The abbreviations used are: HGA, γ-hydroxyglutamic acid; HKG, α-hydroxy-γ-ketoglutarate; CFE, cell-free extracts of *A. suboxydans;* OAA, oxaloacetate; DPN$^+$ and DPNH, oxidized and reduced diphosphopyridine nucleotides.

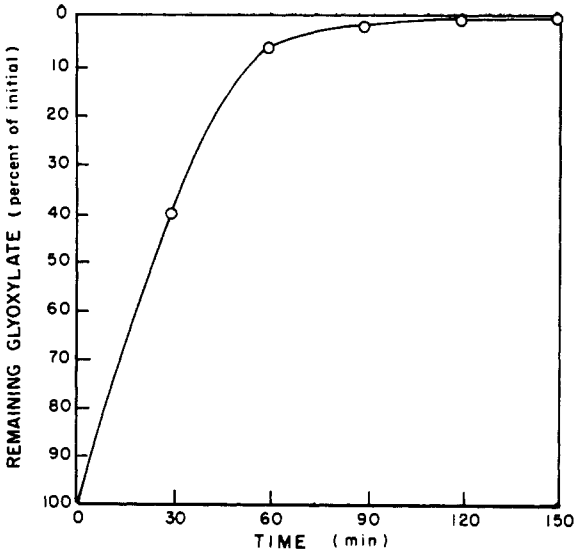


FIGURE 1: Nonenzymatic condensation of glyoxylate with OAA and preparation of oxalomalate. OAA (1 mmole) and 1 mmole of glyoxylate were incubated at 40° after adjusting the pH with 0.1 N NaOH to 7.4. The reaction proceeds under these conditions almost to completion (99.5% disappearance of glyoxylate).

acetic and glyoxylic acids, DPN^+, DPNH, and glutamic dehydrogenase were obtained from Sigma Chemical Co.

Results

Nonenzymatic Condensation of Glyoxylate with Oxaloacetate

Preparation of Oxalomalate. At pH 7.4 and 40°, condensation between sodium oxaloacetate and sodium glyoxylate occurs yielding a trisodium salt of a new tricarboxylic acid, which was characterized by Ruffo and co-workers (1962a) as α-hydroxy-β-oxalosuccinic acid (oxalomalic acid). Their procedure was followed in this work for the preparation of oxalomalic acid. Figure 1 shows the rate of the nonenzymatic condensation as followed by the disappearance of glyoxylate, measured according to the colorimetric method of Kramer *et al.* (1959). Trisodium oxalomalate thus formed appeared as a slightly yellow substance. It is very unstable as a free acid and is decarboxylated in acid medium, yielding HKG. The formation of HKG has been considered in this work as a criterion of the structure of oxalomalate, although Ruffo *et al.* (1962a) have established its structure by C, H, and N analysis, functional groups, infrared spectroscopy, etc. Any attempt to obtain free oxalomalic acid failed because even under mildly acidic conditions, it always released CO_2. The formation of the 2,4-dinitrophenylhydrazone also causes decarboxylation of oxalomalic acid and the phenylhydrazone of HKG is obtained (mp 164°).

Decarboxylation of Oxalomalate. Carbon dioxide evolved during decarboxylation of oxalomalate was flushed by CO_2-free air through 0.1 N NaOH and was measured by acidimetric titration. Addition of 1 M H_2SO_4 to pH 3.0 caused complete decarboxylation. Similar results were obtained by formation of the 2,4-dinitrophenylhydrazone. The ease of decarboxylation of oxalomalate may account for the fact that this compound has not been isolated as yet from an *A. suboxydans* medium or from incubation mixtures with CFE.

Preparation of HKG from Oxalomalate. Synthetic oxalomalate (500 mg) 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 subjected to column chromatography as described in Figure 2. 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. This appears to be a simple method for HKG preparation.

Nonenzymatic Conversion of HKG to HGA. A suitable amount (approximately 2 μmoles) of the 2,4-dinitrophenylhydrazone of HKG was treated for 3 hr with H_2 at room temperature and atmospheric pressure in the presence of 20 mg of PtO_2. The 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 have been considered as a further proof for the structure of HKG produced by the condensation of glyoxylate with OAA (Goldstone and Adams, 1962).

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 (Adams and Goldstone, 1963). The same method was employed for the enzymatic preparation of HGA from HKG. This method, however, eliminates the enzymatic condensation reaction step for the synthesis of HKG from glyoxylate and pyruvate (Kuratomi and Fukunaga, 1960). These methods of HKG and HGA preparations permit the keto or amino acid products isotopically labeled in any or all carbon atoms depending on the choice of specifically labeled glyoxylate and/or oxaloacetate.

Enzymatic vs. Nonenzymatic Condensation. HKG has been isolated 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 in *A. suboxydans.* As already mentioned, this condensation proceeds essentially to completion at pH 7.4 and 40°, but does not take place at pH 3.0 (Ruffo *et al.*, 1962b).

In the study of the enzymatic reaction sequence of Scheme I, it was of importance to establish whether or not the first reaction, namely the formation of oxalomalate, was exclusively enzymatic under the reaction conditions employed. Above pH 7.0 the two reactions cannot be differentiated because the nonenzymatic one is fast and complete. Below pH 7.0, however, the non-

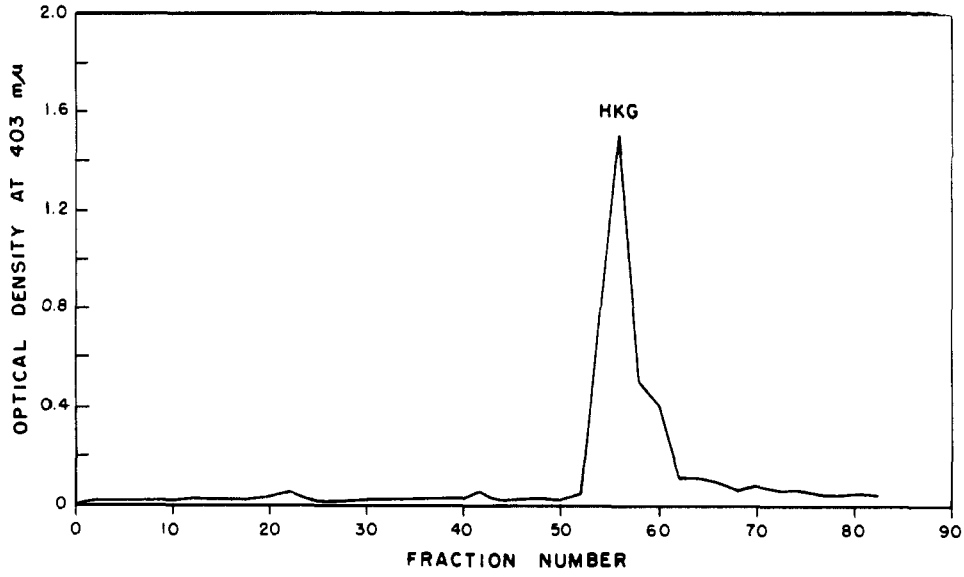


FIGURE 2: Isolation of α-hydroxy-γ-ketoglutarate (HKG). The solution of HKG was adjusted to pH 6.0 and adsorbed on a Dowex 1-formate column (66 × 1.5 cm), then subjected to gradient elution with 6 N formic acid in the reservoir and 200 ml of water in the mixing chamber. Fractions of 10 ml were collected and 0.1 ml of each fraction was assayed for keto acids (Friedemann and Haugen, 1943).

enzymatic reaction is virtually zero while the enzymatic one proceeds, as can be seen from the amount of keto acids formed as a result of OAA–glyoxylate condensation and as intermediates of the suggested glyoxylate–oxaloacetate pathway (Sekizawa *et al.*, 1962) (HKG and α-ketoglutarate). Table I summarizes the results of the znzymatic *vs.* nonenzymatic condensation (boiled eneyme) at pH 6.0. It can be seen that slightly acid pH completely stops the nonenzymatic condensation, while the enzymatic reaction proceeds to a significant extent. The stereospecificity of the isolated products, to be discussed later, is considered as unequivocal proof for the existence of stereospecific enzymatic reactions.

Isolation and Characterization of HKG from A. suboxydans. In the previous experiments, HKG, the decarboxylation product of oxalomalate, has been cited without structural identification, except in reference to the 2,4-dinitrophenylhydrazone and to its conversion to HGA. In the experiments described herein, the isolation and the characterization of HKG by chemical degradation have been undertaken. HKG used in these experiments was isolated from the following incubation mixture: OAA, 1 mmole; glyoxylate, 1 mmole; DPN^+, 5 mg; 0.02 M phosphate buffer, pH 6.0, 30 ml; CFE, 20 ml (protein 25 mg/ml); total volume 50 ml; incubated 30° for 6 hr. Proteins precipitated by boiling were centrifuged out and organic acids were extracted with ethyl acetate after acidifying the reaction mixture with 6 N HCl to pH 2.5. The organic acids extracted were separated, after removal of solvent, in a Dowex 1-formate column as described in Figure 2, but starting with 1.75 N formic acid in the mixing chamber. When gradient elution with 6 N formic acid (200 ml of 1.75 N formic acid in the mixing chamber and 6 N formic acid in the reservoir) was employed, and fractions of 10 ml were collected, the HKG was eluted between tubes no. 34–48 as a keto acid positive fraction. These fractions were pooled and evaporated under vacuum to remove formic acid and water. HKG thus obtained was a thick

TABLE I: Formation of Keto Acids from Enzymatic (as Compared to Nonenzymatic) Condensation of Glyoxylate with Oxaloacetate.[a]

Keto Acids[b] Formed	μmoles of Keto Acid Formed	
	Act. Enzyme	Boiled Enzyme
α-Ketoglutarate	6.3	0.0
α-Hydroxy-γ-ketoglutarate	4.4	0.0
Pyruvate	0.0	0.0

[a] The reaction mixture contained: glyoxylate, 100 μmoles; OAA, 100 μmoles; phosphate buffer, pH 6.0, 2 μmoles; cell-free extracts, 10 ml (protein 25 mg/ml). Total volume 30 ml, incubated at 30° for 6 hr. Boiled cell-free extract was obtained by autoclaving at 120° for 3 min. [b] 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 (Friedemann and Haugen, 1943).

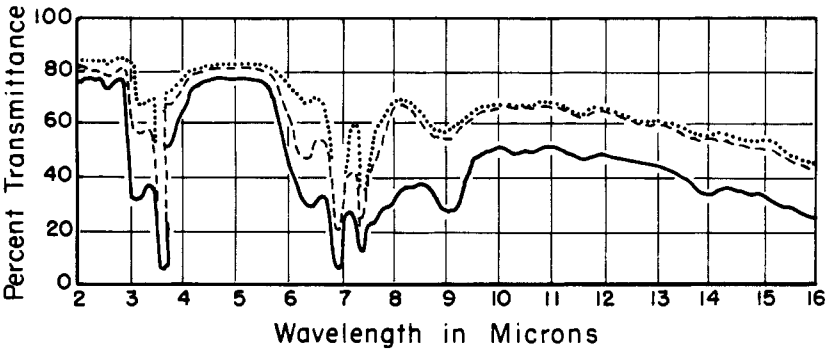


FIGURE 3: Infrared absorption spectra of the α-hydroxy-γ-ketoglutarate (HKG) formed enzymatically by *A. suboxydans*, compared to synthetic L- and DL-HKG. Samples of HKG as Ca salts were suspended in Ondina oil. ———, L-HKG; . . . . . , DL-HKG; ———, HKG formed by *A. suboxydans*.

syrup, which after drying in a vacuum desiccator appeared as a gumlike substance. This material was obtained in crystalline form by converting it to the calcium salt as follows: HKG was dissolved in a minimum amount of water and titrated to pH 7.0 with a solution of $Ca(OH)_2$. Acetone was then added to faint turbidity. Heavy white crystals obtained were washed with acetone–water and dried over P_2O_5 for several days. Ca-HKG (6.1 mg) was thus obtained.

This preparation gave a single spot in an *n*-butyl alcohol–formic acid–water system (R_F 0.19), showing that it was free from α-ketoglutarate (R_F 0.70). It also showed the same R_F value as HKG prepared from HGA by nonenzymatic transamination (Goldstone and Adams, 1962). The 2,4-dinitrophenylhydrazone of both *A. suboxydans* HKG and the reference sample of HKG showed the same R_F values [in *n*-butyl alcohol–ethyl alcohol–ammonia (R_F 0.01) and in *n*-butyl alcohol–acetic acid–water (R_F 0.53)]. The melting point of the isolated HKG 2,4-dinitrophenylhydrazone was 162° which is in good agreement with the melting point of HKG 2,4-dinitrophenylhydrazone of authentic HKG prepared from HGA (mp 165°) (Goldstone and Adams, 1962) or as previously described from oxalomalate (mp 164°). In addition, the infrared spectra of the isolated HKG are in good coincidence with both L- and D-HKG prepared from HGA (Figure 3).

Conversion of HKG to Malate. On treatment with H_2O_2, HKG is oxidatively decarboxylated to yield malate and 1 equiv of CO_2 (Goldstone and Adams, 1962). HKG (3 mg) with 1 ml of 36% H_2O_2 solution at room temperature for 1 hr yielded malate which was identified by paper chromatography (*n*-butyl alcohol–formic acid–water system, R_F 0.52). This is an additional unequivocal proof for the identity of HKG isolated from *A. suboxydans*.

Steric Properties of HKG. The configuration of the enzymatically formed HKG from *A. suboxydans* could not be established by comparing its infrared spectrum with those of the synthetic L- and DL-HKG prepared from L- and DL-HGA (Goldstone and Adams, 1962), because their spectra coincide closely; probably no racemic compound is formed. The steric properties of HKG formed enzymatically were therefore elucidated by chemical degradation of HKG to malic acid by H_2O_2 oxidation as previously described.

HKG has one asymmetric carbon atom, the γ or 4 carbon, shown in Scheme II. Oxidative removal of the 1-carboxyl group of HKG leads to the formation of D- or L-malate, depending on the configuration of HKG. The enzymatic HKG formed by *A. suboxydans* yields D-malate. The configuration of this product was elucidated as follows: 3 mg of HKG were oxidized as before with H_2O_2 and the malate formed was mixed with 4 mg of authentic L-malate. Calcium hydroxide solution was added to pH 4.0, followed by acetone, to faint turbidity. After standing overnight at 5°, crystals of calcium bimalate were obtained. After drying, the crystals were suspended in Ondina oil. The infrared spectrum obtained showed an excellent coincidence with that of authentic DL-calcium bimalate and different from that of L-calcium bimalate (Figure 4).

Since the calcium bimalate formed from the enzymatic HKG forms a racemic compound with L-calcium bimalate, it must be concluded that the oxidation product of HKG from *A. suboxydans* is D-malic acid. Since the asymmetric carbon atom is not involved in the oxidative degradation, it is established that HKG has also the D configuration and is therefore designated D_s-α-hydroxy-γ-ketoglutaric acid (Figure 4).

A similar approach was used by Goldstone and Adams (1962) to establish the configuration of HKG derived from HGA by transamination with OAA. The configuration of malate was established by the use of malate decarboxylase in their work.

A reductive amination of HKG to HGA leads to the formation of a second asymmetric carbon atom and consequently two possible isomers of HGA (Scheme II). The configuration of HKG was of interest not only because HKG is a metabolic product of *A. suboxydans*, but also because its configuration would confirm the configuration of HGA established previously (Sekizawa *et al.*, 1965) as being the L-*erythro* isomer. As shown in Scheme II, either the *erythro*-L-HGA or the *threo*-D-

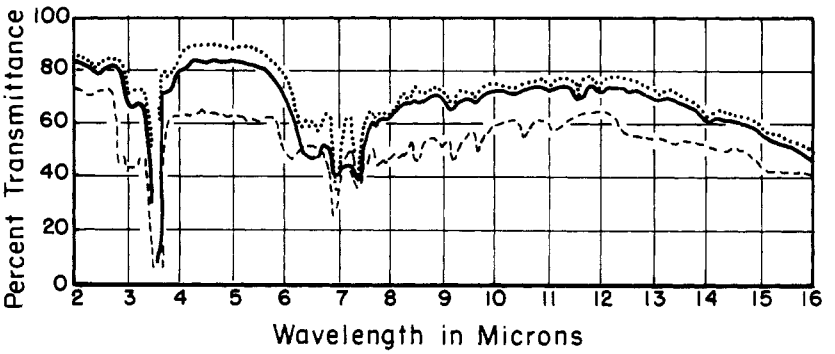
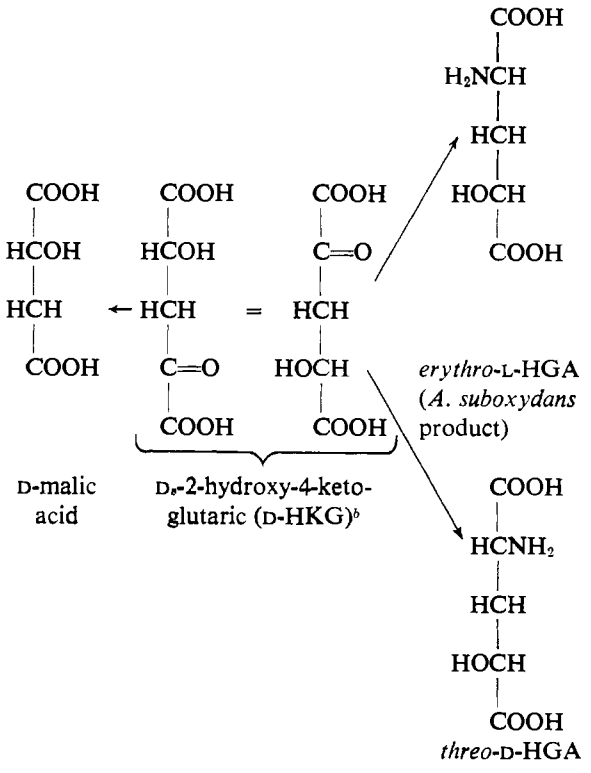


FIGURE 4: Steric identification of enzymatically formed HKG by conversion to malate and comparison of the infrared spectra with that of authentic L- and DL-malate. ———, oxidation product of enzymatic HKG and L-malate; (Ca salt) – – – – –, calcium L-malate; . . . . . , calcium DL-malate. Coincidence of the spectrum of DL-malate with those of a mixture of authentic L-malate and malate derived from enzymatic HKG reveals that the two forms must form a racemic compound; consequently, the product from *A. suboxydans* has the D configuration.

SCHEME II: Configuration of HKG in *A. suboxydans*.[a]



[a] The steric identification of enzymatically formed HKG by *A. suboxydans* as D-HKG is in agreement with the configuration of the *erythro*-L-HGA, derived from HKG and isolated in this organism. [b] The subscript s refers to serine (Commission on the Nomenclature of Biological Chemistry).

HGA can be formed from D-HKG by a nonselective (nonenzymatic) reductive amination or transamination. In *A. suboxydans*, however, only the *erythro*-L-HGA exists, which is an optical isomer derived from D-HKG.

Discussion

The observations described suggest that a nonenzymatic condensation reaction between OAA and glyoxylate can easily take place at pH 7.4 and 40° to form oxalomalate. This reaction is of similar nature to the formylation reaction (condensation of acidic CH groups with formaldehyde in its hydrated form to yield a C–C link (Fourneau *et al.*, 1930)).

In this study, this nonenzymatic condensation was differentiated at pH 6–6.5 from an enzymatic condensation reaction occurring in *A. suboxydans* which appears to be the first reaction of an enzymatic sequence leading to the aminodicarboxylic acids, glutamate and HGA (Sekizawa *et al.*, 1962). Oxalomalate has not yet been isolated from *A. suboxydans*, presumably because it is decarboxylated to HKG. HKG, the decarboxylation product of oxalomalate, has been i olated from incubation mixtures of OAA and glyoxylate with cell-free extracts.

HKG has been studied as a product of the transamination of HGA with OAA in the metabolism of hydroxyproline (Goldstone and Adams, 1962) and a product of the enzymatic condensation of glyoxylate with pyruvate (Kuratomi and Fukunaga, 1960). This condensation reaction has no been demonstrated in *A. suboxydans*, and the fact that no pyruvate can be detected under conditions where HKG is formed from glyoxylate and OAA (Table I) may be considered as evidence that the cleavage of HKG to glyoxylate and pyruvate does not occur in this organism.

It is of interest that *A. suboxydans*, although an obligate aerobe, has been reported as not having a demonstrable Krebs cycle (Cheldelin, 1961). On the other hand, Ruffo has studied oxalomalate as a potent inhibitor of aconitase (Ruffo *et al.*, 1962a,b). In addition, Payes and Laties (1963) 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 the apparent absence

of the Krebs cycle in this organism may in part be a result of a competitive inhibition of the key enzymes of the cycle by these compounds.

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