2-Keto-4-hydroxybutyrate Aldolase. Identification as 2-Keto-4-hydroxyglutarate Aldolase, Catalytic Properties, and Role in the Mammalian Metabolism of L-Homoserine*

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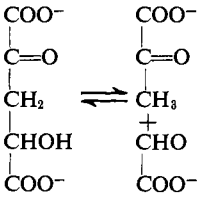
ABSTRACT: 2-Keto-4-hydroxyglutarate aldolase from bovine liver, previously known to catalyze the reaction, 2-keto-4-hydroxyglutarate ⇌ pyruvate + glyoxylate, has also been found to catalyze the reversible cleavage of 2-keto-4-hydroxybutyrate to yield equimolar amounts of pyruvate and formaldehyde. Several independent lines of evidence indicate that dealdolization of 2-keto-4-hydroxybutyrate and 2-keto-4-hydroxyglutarate is catalyzed by a single enzyme; these include a constant ratio of specific activity values throughout purification (1300-fold), similar responses in controlled inactivation studies with heat or by reduction of Schiff-base complexes with sodium borohydride, and parallel behavior in studies with competitive inhibitors where the K_i values for a given inhibitor are the same for either substrate. The equilibrium for the reaction, 2-keto-4-hydroxybutyrate ⇌ pyruvate + formaldehyde, favors cleavage of the ketohydroxy acid (K_{equil} = 4.1 × 10^{-3} M). The apparent Michaelis constants for 2-keto-4-hydroxyglutarate and 2-keto-4-hydroxybutyrate are 1.0×10^{-4} M and 3.1×10^{-3} M, respectively. Whereas the pH optimum for the aldolytic cleavage of 2-keto-4-hydroxyglutarate is 8.8, 2-keto-4-hydroxybutyrate is cleaved maximally at pH 8.1. Pig heart aspartate (L-aspartate: 2-oxoglutarate aminotransferase E.C. 2.6.1.1) and alanine (L-alanine:2-oxoglutarate aminotransferase E.C. 2.6.1.2) aminotransferases catalyze the interconversion of 2-keto-4-hydroxybutyrate and L-homoserine; either α-ketoglutarate, oxaloacetate, or pyruvate function as amino group acceptors. The formation of stoichiometric amounts of pyruvate and formaldehyde from L-homoserine has been demonstrated in a coupled enzyme system containing both aspartate aminotransferase and 2-keto-4-hydroxyglutarate aldolase. These findings establish a new sequence of reactions for the mammalian metabolism of L-homoserine involving an initial transamination (yielding 2-keto-4-hydroxybutyrate) followed by the subsequent cleavage of 2-keto-4-hydroxybutyrate to form pyruvate and formaldehyde.

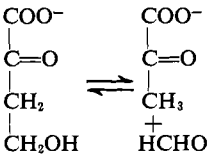
In 1960, Kuratomi and Fukunaga (1960) reported the presence of an enzyme in rat liver preparations that catalyzed the reversible cleavage of 2-keto-4-hydroxyglutarate yielding equimolar amounts of pyruvate and glyoxylate (eq 1).



(1)

Later studies by Adams and Goldstone (1960a,b, 1962), by Kuratomi and Fukunaga (1963), and by Maitra and Dekker (1961, 1962, 1963, 1964) demonstrated that this reaction represents a terminal step in the mammalian catabolism of L-hydroxyproline. In a recent paper (Kobes and Dekker, 1969), we outlined a procedure for the preparation of KHG[1]-aldolase (1300-fold purified) from extracts of bovine liver; some properties of the pure enzyme were also described (Kobes and Dekker, 1969).

In 1952, Hift and Mahler (1952) reported that bovine liver extracts contained an enzyme which catalyzed the condensation of pyruvate with formaldehyde yielding 2-keto-4-hydroxybutyrate, the α-keto analog of homoserine (eq 2).



(2)

In view of the marked similarity of eq 1 and 2, we decided to investigate the properties of these two aldolases. The studies presented here indicate that both KHG- and KHB-aldolase activities are common to the same protein. We conclude, therefore, that eq 1 and 2 are catalyzed by a single enzyme. The catalytic properties of the aldolase reaction with KHB as substrate are described.

We have also found that highly purified aspartate (L-aspartate:2-oxoglutarate aminotransferase, E.C. 2.6.1.1) and alanine (L-alanine:2-oxoglutarate aminotransferase, E.C. 2.6.1.2) aminotransferases catalyze the interconversion of L-homoserine with KHB; pyruvate, oxaloacetate, or α-ketoglutarate serve as amino group acceptors in this process. In a coupled enzyme system, containing both aspartate aminotransferase and KHG-aldolase, the net formation of pyruvate and formaldehyde from L-homoserine can be demonstrated. These findings establish a new pathway for the metabolism of L-homoserine and define a broader role for KHG-aldolase in mammalian metabolism.

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[1] The abbreviations used are: KHG, 2-keto-4-hydroxyglutarate; KHB, 2-keto-4-hydroxybutyrate; OAA, oxaloacetate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

Materials

DL-KHG, L-KHG, and D-KHG were prepared by the nonenzymatic transamination of *threo*-γ-hydroxy-DL-glutamate, *threo*-γ-hydroxy-L-glutamate, and *erythro*-γ-hydroxy-L-glutamate, respectively, according to the procedure of Maitra and Dekker (1963). 2-Keto-4-hydroxybutyrate was obtained in a similar manner starting with DL-homoserine, as described before (Lane and Dekker, 1969). These two compounds were determined to be homogeneous, both as the free keto acids and as their corresponding 2,4-dinitrophenylhydrazone derivatives (Kobes and Dekker, 1969; Lane and Dekker, 1969), by paper chromatography in several solvent systems. Alkaline hydrolysis of ethyl acetoacetate yielded acetoacetate (Hall, 1963) which was used immediately. 2-Keto-3-deoxygluconate was obtained by treatment of KDPG with alkaline phosphatase (MacGee and Doudoroff, 1954); KDPG was a gift sample from Dr. W. A. Wood, Michigan State University. 2-Keto-4-methyl-4-hydroxyglutaric acid was synthesized by the acid-catalyzed aldol condensation of pyruvic acid, as described by Shannon and Marcus (1962). The method of Keilin and Hartree (1938) was used to prepare calcium phosphate gel. Cellulose polyacetate (Sepraphore III) strips, 1 × 6.75 in., were obtained from Gelman Instrument Company and were used with a Gelman electrophoresis chamber.

Formaldehyde solutions were prepared by repeated acid hydrolysis of paraformaldehyde and distillation of the resulting formaldehyde. Stock solutions of formaldehyde were standardized gravimetrically by precipitation with dimedon (Weinberger, 1931). Chromotropic acid was purchased from Eastman Organic Chemicals and was recrystallized twice from ethanol–water (1:1, v/v); solutions of chromotropic acid were made fresh every month with 12.5 N H_2SO_4 (Frisell *et al.*, 1954) and were stored in the dark at room temperature.

The following materials were obtained from the sources indicated: NADH (disodium salt) from P-L Biochemicals, sodium borohydride from Metal Hydrides, Inc.; DL-[3,4-^{14}C]-glutamic acid (2.30 mCi/mmole), crystalline rabbit muscle lactate dehydrogenase, glutamate–aspartate and glutamate–alanine transaminases (both highly purified from pig heart) from Calbiochem, and [^{14}C]formaldehyde (15.00 mCi/mmole) from Nuclear Chicago. All other compounds were of the highest purity commercially available.

Methods

General Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard. Carbonyl compounds were quantitatively measured according to the procedure of Friedemann and Haugen (1943); KHB was also determined spectrophotometrically at 340 mμ by measuring the extent of NADH oxidation after complete reaction in the presence of excess lactate dehydrogenase (Lane and Dekker, 1969). 2,4-Dinitrophenylhydrazone derivatives were prepared and extracted in the usual manner (Block *et al.*, 1958) with ethyl acetate. 2-Keto-4-hydroxybutyrate dinitrophenylhydrazone was prepared as reported earlier (Lane and Dekker, 1969). Catalytic hydrogenations were performed at 50-pounds pressure of H_2 gas for 6 hr at 25° with PtO_2 as catalyst. Pyruvate concentrations were specifically measured in the presence of other keto acids by reaction with salicylaldehyde in alkaline solution using a modified Straub procedure (1936). Glyoxylic acid was routinely determined by the method of Dekker and Maitra (1962). Absorbancy measurements were made at 25° with a Gilford spectrophotometer equipped with a thermostated cuvet holder and a digital absorbance meter.

The concentration of HCHO in solution was measured colorimetrically by a modified chromotropic acid procedure (Frisell *et al.*, 1954). Aliquots (0.5 ml) of solutions containing HCHO were added to test tubes containing 5 ml of chromotropic acid reagent. The tube contents were mixed and heated in a boiling water bath for 6 min. After the samples were cooled to room temperature, the intensity of the characteristic violet color was measured in a Klett-Summerson colorimeter with a No. 54 filter. The color intensity remained constant for at least 1 hr after heating at 100°.

Enzyme Assays. KHB-aldolase activity was determined by measuring the amount of HCHO formed when the enzyme was incubated with KHB at 37°. The incubation mixture (1.0 ml) contained 100 μmoles of potassium glycylglycine buffer (pH 8.1); 5 μmoles of GSH; 10 μmoles of KHB; and appropriate amounts of the aldolase. After the mixtures were incubated for 30 min at 37°, the reaction was terminated by adding 0.5 ml of 12% metaphosphoric acid solution; any precipitated protein was removed by centrifugation. Aliquots (0.5 ml) of the supernatant fluid were withdrawn and assayed for HCHO content. The reaction could also be carried out in potassium phosphate buffer, but Tris-HCl buffer was found to interfere with the colorimetric determination of HCHO. High concentrations (20 mM) of KHB inhibited the reaction, presumably due to either substrate inhibition of aldolase activity or quenching of the color formed by reaction of HCHO with chromotropic acid. Within defined ranges, the initial rate of HCHO formation was linear with both time of incubation and protein concentration. No reaction occurred when either KHB or aldolase was omitted from the assay mixture nor when heat-denatured enzyme was added. One unit of enzyme activity is defined as the amount of protein that liberates 1.0 μmole of HCHO in 20 min at 37°. Specific activity is expressed as units per milligram of protein.

KHG-aldolase activity was routinely followed by colorimetric estimation of the amount of glyoxylate liberated when DL-KHG was incubated in the presence of appropriate amounts of the enzyme (Maitra and Dekker, 1964). For comparing the relative effectiveness of KHG with KHB as substrate, however, the assay was carried out in 0.1 M potassium glycylglycine buffer at pH 8.1 rather than in Tris-HCl buffer at pH 8.4. Specific activity with KHG as substrate is defined as the number of micromoles of glyoxylate formed in 20 min at 37° per milligram of protein.

Transamination of KHB, catalyzed by either glutamate–aspartate or glutamate–alanine transaminase, was followed by measuring the amount of radioactivity incorporated into 2-ketoglutarate when KHB was incubated in the presence of enzyme and ^{14}C-labeled glutamic acid. The assay mixture (1.3 ml) contained 200 μmoles of Tris-HCl buffer (pH 8.1); 5 μmoles of KHB; 10 μg of pyridoxal 5′-phosphate; 0.13 μmole of DL-[3,4-^{14}C] glutamate (1.61×10^6 cpm per μmole); 4 μmoles of carrier L-glutamate; and appropriate amounts of aminotransferase solution. After the mixtures were incubated for 40 min at 37°, the reaction was terminated by adding 0.2 ml of 50% trichloroacetic acid solution; the precipitated protein was removed by centrifugation. An aliquot (1.0 ml) of the supernatant fluid was added to 1 ml of 0.1% 2,4-dinitrophenylhydrazine solution in 2 N HCl and incubated for 30 min at 25°. This solution was then extracted with 2 ml of ethyl acetate. The ethyl acetate extract was washed with 0.3 ml of H_2O, and aliquots (0.5 ml) of the ethyl acetate were plated on planchets for the determination of radioactivity. The radio-

activity extractable into ethyl acetate was taken to be a measure of the extent of the enzyme-catalyzed reaction. A control sample containing no enzyme was treated in an identical manner. This assay procedure was linear with time for 1 hr when 3 international units of enzyme or less were used.

Electrophoresis. Polyacrylamide gel electrophoresis of KHG-aldolase preparations was carried out at room temperature in 0.19 M glycine–0.02 M Tris buffer (pH 9.3) for 1 hr at 5 mA per tube, according to the procedure of Davis (1964). Protein components were visualized by staining with a 0.5% solution of aniline blue-black in 7% acetic acid and destaining electrophoretically.

Electrophoresis was also carried out at 4° on Sepraphore III cellulose acetate strips in 0.05 M potassium phosphate buffer (pH 7.4) at 190 V for 2 hr. The strips were soaked in the same buffer at 4° overnight before use. Following electrophoresis, the cellulose acetate strips were stained with a 0.1% solution of Amido Black in 10% trichloroacetic acid and destained with 10% acetic acid solution.

Radioactivity Measurements. Levels of radioactivity were measured with a Nuclear Chicago thin-window gas flow counter having an efficiency for carbon-14 of approximately 32%. Radioactive compounds were detected on paper chromatograms with a Vanguard Autoscanner 880 as well as by exposure to Kodak No-Screen Medical X-Ray film.

TABLE I: Aldolase Activity with KHG and KHB as Substrates during Purification of the Enzyme from Bovine Liver.[a]

Fraction	Specific Activity (units/mg) KHG	KHB	KHG:KHB Activity Ratio
I Crude extract	0.028	0.0087	3.2
II Heated to 70°	0.255	0.079	3.2
III $(NH_4)_2SO_4$ precipitate (20–38% saturation)	4.47	1.35	3.3
IV DEAE-cellulose eluate	26.2	8.55	3.1
V Calcium phosphate–cellulose eluate	29.6	9.26	3.2
VI DEAE-cellulose eluate (eluted with 0.08 M pyruvate); then concentrated by $(NH_4)_2SO_4$ precipitation	36.7	10.6	3.4

[a] Enzyme assays were performed at 37° in 0.1 M potassium glycylglycine buffer (pH 8.1) as described in the section entitled Methods.

Results

Evidence for the Common Identity of KHG- and KHB-aldolases. PURIFICATION OF KHG- AND KHB-ALDOLASES. When we attempted to obtain KHB-aldolase in a highly purified state from bovine liver extracts, it soon became apparent that the purification of KHB- and KHG-aldolase activities paralleled one another. It became imperative, therefore, to ascertain whether the formaldehyde–pyruvate condensing enzyme described by Hift and Mahler (1952) was an enzyme distinct from KHG-aldolase or whether the aldolytic cleavage of KHG and of KHB was catalyzed by the same enzyme. The ratio of KHB- and KHG-aldolase activities was thus determined during the purification of KHG-aldolase by the method of Kobes and Dekker (1969). A 1300-fold purification is accomplished by the procedure outlined in Table I. Although the absolute specific activity values for this enzyme preparation with KHG as substrate happened to be lower than usual (Kobes and Dekker, 1969), it is clear that the KHG:KHB ratio of specific activities does not change significantly at any of the purification steps.

Further efforts to separate these two enzymatic activities by routine purification techniques were unsuccessful. The methods used included molecular sieve filtration with Bio-Gel P-200, DEAE-cellulose ion-exchange column chromatography, elution from a column of DEAE-Sephadex A-50 using a linear gradient ($0 \rightarrow 0.5$ M) of KCl, and adsorption to and elution from calcium phosphate gel. The ratio of KHG- to KHB-aldolase activities remained constant (3.2 to 3.7) throughout all of these procedures.

ACTIVITY OF TWO FORMS OF THE ALDOLASE WITH KHG AND KHB AS SUBSTRATES. Final preparations of KHG-aldolase obtained by the procedure of Kobes and Dekker (1969) were shown by polyacrylamide disc gel electrophoresis at pH 9.3 to contain two protein components, both of which are enzymatically active with KHG as substrate. However, under the conditions tested, these two protein bands were of very similar mobility (R_M values relative to tracking dye were 0.44 and 0.50), making it difficult to separate and isolate them from the gel. In the course of our studies, it was found that a modification of the purification procedure previously reported (Kobes and Dekker, 1969) provided a preparation of the aldolase which also showed two protein-stainable bands on polyacrylamide gel electrophoresis; the bands in this instance, however, were clearly resolved from one another.

The modified purification procedure included the following steps. All buffers contained 5 mM 2-mercaptoethanol and all procedures were carried out at 4°. The $(NH_4)_2SO_4$ precipitate (Fraction III, Table I) was dialyzed overnight against 0.025 M potassium phosphate buffer (pH 7.4). The dialyzed solution was then applied to a column (4.3 × 36 cm) of DEAE-cellulose which had been previously equilibrated with 0.05 M potassium phosphate buffer (pH 7.4). After the cellulose was extensively washed with 0.05 M buffer, the enzyme was eluted with 0.1 M potassium phosphate buffer. Crystalline $(NH_4)_2SO_4$ was added to the pooled fractions from the previous step to 40% saturation (234 g/l.), and the protein precipitate was dissolved in and dialyzed against 0.01 M potassium phosphate buffer (pH 7.4). This dialyzed solution was treated with calcium phosphate gel (gel/protein ratio = 1:1 on a dry weight basis) for 1 min, and the mixture was then centrifuged. KHG- and KHB-aldolase activities were found in the supernatant fluid (KHG:KHB activity ratio = 3.4). This three-step procedure provided an eight- to ninefold purification of the enzyme above that obtained at step III, Table I.

When this purified protein preparation was subjected to polyacrylamide gel electrophoresis at pH 9.3, as described in the Methods section, two protein-stainable bands were observed with R_M values of 0.43 and 0.59 relative to tracking dye. Each protein component could be isolated in homogeneous form by elution from the gel according to the following procedure.

Purified aldolase (250 μg) was electrophoresed on each of 6 cylinders of polyacrylamide gel at pH 9.3 and 4° (see Methods). Thereafter, two cylinders were stained for protein; using

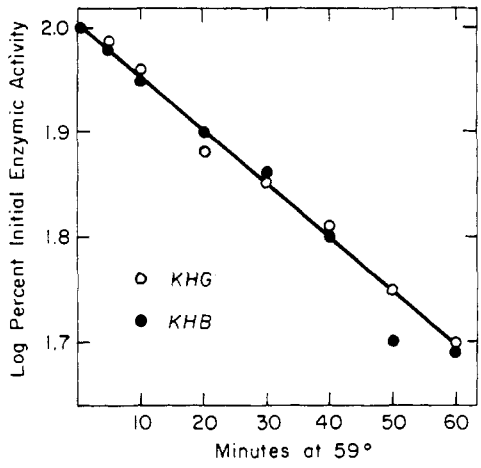


FIGURE 1: Controlled heat inactivation of KHG-aldolase and KHB-aldolase activities. The reaction mixture (0.5 ml) contained 0.11 mg of enzyme in 0.01 M potassium glycylglycine buffer (pH 8.1). After the mixtures were incubated at 59° for the time indicated, the tube contents were immediately chilled to 4° and aliquots were removed and assayed for KHG- and KHB-aldolase activities. The control tube was kept at 4° for the duration of the experiment.

these stained samples as references, the other four cylinders were cut horizontally into segments so as to completely isolate the one protein from the other. The four unstained pieces of gel containing the slower moving component were pooled in 2 ml of 0.01 M Tris-HCl buffer (pH 8.1) containing 5 mM 2-mercaptoethanol; the gel segments containing the faster moving protein were treated in an identical manner. After freezing and thawing once, the gel samples were macerated, and the mixture was centrifuged at 4°. The supernatant fluids were used to determine protein content and aldolase activity with KHG and KHB as substrates. A 90% recovery of protein could be obtained by this procedure, whereas the units of KHG- or KHB-aldolase activity recovered were approximately 70% of those applied to the gel. A portion of each supernatant fluid was subjected to electrophoresis a second time on fresh cylinders of polyacrylamide gel under identical conditions. In each instance, a single band of protein was observed with the same respective R_M value as determined prior to segmentation of the original gel cylinder.

The results of two such experiments performed with two different enzyme preparations are shown in Table II. In each case, KHG- and KHB-aldolase activities are associated with both protein-stainable bands; the levels of activity shown by these two forms of the aldolase are essentially the same with either substrate. Furthermore, the KHG:KHB activity ratio is not significantly different for either form of the enzyme. Similar results were obtained when the enzyme preparation was subjected to electrophoresis on Sepraphore III cellulose acetate strips, at pH 7.4, as described in the section entitled Methods.

HEAT INACTIVATION STUDIES. All studies described hereafter were performed with KHG-aldolase (1300-fold purified) prepared by the method of Kobes and Dekker (1969). Purified aldolase was subjected to controlled heat treatment at 59°. As shown in Figure 1, inactivation of aldolase activity occurs slowly at this temperature and proceeds at the same rate with either KHG or KHB as substrate; a constant KHG:KHB activity ratio (2.9 to 3.4) was found at each level of inactivation. Loss of enzymatic activity follows first-order kinetics over the time period studied; from these data the apparent first-order rate constant was calculated to be 0.011 min^{-1} at 59°.

INACTIVATION OF KHG- AND KHB-ALDOLASE ACTIVITIES IN THE PRESENCE OF BOROHYDRIDE AND SUBSTRATES OR SUBSTRATE ANALOGS. Previous studies by Kobes and Dekker (1966, 1967) showed that bovine liver KHG-aldolase is inactivated in the presence of $NaBH_4$ and any one of its three substrates. Moreover, several analogs of glyoxylate and of KHG also form Schiff-base complexes with the enzyme (Kobes, 1967; Kobes and Dekker, 1967). The effect of incubating the aldolase with $NaBH_4$ in the presence of various substrates or substrate analogs on KHG- and KHB-cleavage activities is shown in Table III; the loss of enzymatic activity is taken as a measure of the affinity with which the compound is bound *via* an azomethine linkage to the enzyme. In all cases, the degree of inactivation observed is virtually identical regardless of whether KHG or KHB is used as substrate.

INHIBITION STUDIES. The substrate analogs glycolaldehyde, 2-ketobutyrate, and 2-ketoglutarate all inhibit the aldolase in the presence of either KHG or KHB. The extent of inhibition by these compounds as a function of inhibitor concentration at two constant levels of KHG or of KHB was plotted according to the procedure of Dixon (1953). A representative experiment carried out with 2-ketobutyrate is shown in Figure 2; intersection of the two lines above the abscissa is indicative of competitive inhibition of aldolase activity by 2-ketobutyrate with either substrate.

A summary of the apparent dissociation constants obtained for the enzyme–inhibitor complexes is presented in Table IV. The constants listed were calculated from Dixon plots (1953) comparable to those presented in Figure 2. As shown in Table IV, the K_i values for a given inhibitor are virtually identical for both KHG and KHB. In all three cases studied, the substrate analog inhibited both KHG- and KHB-aldolase activities in a competitive manner; glycolaldehyde, an analog of glyoxylate, seems to be a more potent inhibitor of aldolase activity than either 2-ketobutyrate or 2-ketoglutarate which are analogs of pyruvate and of KHG, respectively.

Properties of the Aldolase Reaction with KHB as Substrate. IDENTIFICATION OF THE REACTION PRODUCTS. Formaldehyde was initially characterized as a product of the aldolase-catalyzed cleavage of KHB by its positive and specific reaction

TABLE II: Utilization of KHG and KHB as Substrates by Two Forms of the Purified Aldolase.[a]

Preparation	R_M Values[b]	Specific Activity (units/mg) KHG	Specific Activity (units/mg) KHB	KHG:KHB Activity Ratio
A	0.43	10.0	2.5	4.0
	0.59	11.4	2.2	5.1
B	0.41	13.6	3.3	4.1
	0.59	12.7	3.3	3.9

[a] Separation of the two forms of the enzyme was achieved by electrophoresis on 7.5% polyacrylamide gel in Tris-glycine buffer (pH 9.3) for 1 hr at 4°, as described under Methods. [b] R_M values were determined by staining for protein with a 0.5% solution of aniline blue-black in 7% acetic acid and are defined as the mobility of a protein band relative to the mobility of bromophenol blue as tracking dye.

TABLE III: Aldolase Activity with KHG and KHB as Substrates after Inactivation by Borohydride Reduction of Various Schiff-Base Complexes.[a]

Substrate or Substrate Analog	Inactivation after Reduction with $NaBH_4$ (%) KHG	KHB
None	0	0
Pyruvate	100	100
Glyoxylate	100	100
Formaldehyde	81	86
2-Keto-4-hydroxyglutarate	93	93
2-Keto-4-hydroxybutyrate	96	95
Glycolaldehyde	57	60
2-Ketobutyrate	57	56
2-Ketoglutarate	97	96
Acetoacetate	21	25
2-Keto-3-deoxygluconate	62	62
2-Keto-4-hydroxy-4-methylglutarate	99	98

[a] The reaction mixtures (0.30 ml) contained 50 μmoles of potassium phosphate buffer (pH 6.3), 9 μmoles of each substrate or substrate analog, and 0.20 mg of purified aldolase. Each reaction mixture was treated at 4° with four 0.01-ml portions of 1 M $NaBH_4$ solution added alternately with four 0.005-ml portions of 2 M acetic acid solution over a period of 30 min. The proteins were then precipitated with 168 mg of powdered $(NH_4)_2SO_4$, centrifuged, and washed two times with 1 ml of 80% saturated $(NH_4)_2SO_4$ solution. The washed precipitates were dissolved in 0.3 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol, and the resulting solutions were assayed for KHG- and KHB-aldolase activities.

with chromotropic acid. No color was detected in incubation mixtures lacking either KHB or active enzyme. Formaldehyde was further characterized by preparing its 2,4-dinitrophenylhydrazone derivative. A reaction mixture containing 200 μmoles of potassium glycylglycine buffer (pH 8.1), 6.2 μmoles of KHB, 5 μmoles of GSH, and 0.29 mg of KHG-aldolase in a final volume of 1.5 ml was incubated for 2 hr at 37°. The reaction was terminated by adding 15 ml of 0.1%

TABLE IV: Dissociation Constants of Aldolase–Inhibitor Complexes with KHG and KHB as Substrates.[a]

Inhibitor	Nature of Inhibition	K_i Value (M) KHG	KHB
Glycolaldehyde	Competitive	2.4×10^{-3}	2.7×10^{-3}
2-Ketobutyrate	Competitive	5.2×10^{-2}	5.3×10^{-2}
2-Ketoglutarate	Competitive	1.8×10^{-2}	1.8×10^{-2}

[a] K_i values were measured in 0.1 M potassium glycylglycine buffer (pH 8.1) at 37°. Appropriate corrections were made for glycolaldehyde interference in both colorimetric enzyme assays.

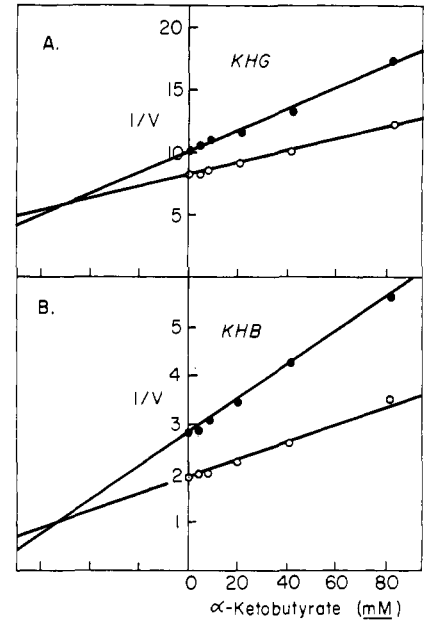


FIGURE 2: Competitive inhibition of (A) KHG-aldolase and (B) KHB-aldolase activities by α-ketobutyrate. Enzyme assays were performed as described in the Methods section: [A], (●) 2.5 mM KHG, (○) 5.0 mM, KHG; [B], (●) 2.8 mM KHB, (○) 5.6 mM KHB. Ikitial velocities are expressed as the number of micromoles of glyoxylate or of formaldehyde liberated in 20 min at 37°.

2,4-dinitrophenylhydrazine solution in 2 N HCl. The resulting mixture was stirred for 60 min at 25°. The precipitated protein was removed by centrifugation, and the supernatant fluid was extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness *in vacuo* at 42°, and the residue was dissolved in a minimal amount of absolute ethanol. The 2,4-dinitrophenylhydrazone of formaldehyde was identified by paper chromatographic techniques in the following solvent systems: (A) ethanol–water (7:3, v/v), R_F 0.83; (B) methanol–*n*-butyl alcohol–benzene–water (2:1:1:1, v/v), R_F 0.98; Whatman No. 1 filter paper was used with ascending solvent flow.

Pyruvate was also shown to be a product of KHB cleavage by paper chromatographic identification of its 2,4-dinitrophenylhydrazone; solvent A, R_F 0.63; solvent B, R_F 0.73. The dinitrophenylhydrazone derivatives of formaldehyde and pyruvate were resolved from KHB-dinitrophenylhydrazone in both solvent systems.

A portion of the ethyl acetate extract was also subjected to high-pressure catalytic hydrogenation. Again using paper chromatographic techniques, alanine was identified as a product in the following solvent systems: ethanol–water (7:3, v/v), R_F 0.52; pyridine–acetic acid–water (10:7:3, v/v), R_F 0.46; *tert*-butyl alcohol–water–methyl ethyl ketone–diethylamine (10:5:10:1, v/v), R_F 0.37; Whatman No. 1 filter paper was used with ascending solvent flow.

Pyruvate was further characterized by coupling the cleavage of KHB with glutamate–alanine transaminase. The reaction mixture (2.0 ml) contained 200 μmoles of potassium glycylglycine buffer (pH 8.1), 6.8 μmoles of KHB, and 0.31 mg of aldolase. The reaction was terminated, after incubating for 2 hr at 37°, by the addition of 3 ml of acetone; the protein was removed by centrifugation, and the supernatant fluid was concentrated *in vacuo* at 30° to remove the acetone. The incubation mixture was then supplemented with L-glutamate (8 μmoles), EDTA (5 μmoles), pyridoxal 5′-phosphate (50

TABLE V: Enzymatic Transamination of Pyruvate Formed by Aldolytic Cleavage of KHB.[a]

	Solvent	Ratio	R_F Values: Glutamic Acid	Homoserine	Alanine	Enzymatic Product
A	2-Propanol–15 N NH_4OH	5:1	0.25	0.75	0.68	0.67
B	Ethanol–water	7:3	0.40	0.45	0.54	0.52
C	Phenol saturated with citrate–phosphate buffer[b]		0.35	0.52	0.56	0.56

[a] Whatman No. 1 filter paper, ascending solvent flow, was used except for solvent A where thin-layer chromatography was employed. Amino acids were visualized by spraying with a 0.25% solution of ninhydrin in pyridine–water (1:1, v/v). [b] Phenol saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% sodium dihydrogen phosphate.

TABLE VI: Incorporation of [^{14}C]Formaldehyde into KHB in the Presence of Pyruvate and KHG-aldolase.[a]

	Solvent	Ratio	R_F Values: Pyruvate	KHB	Radioactive Enzymatic Product
A	*n*-Butyl alcohol–acetic acid–water	12:3:5	0.42	0.36	0.36
B	H_2O–saturated *n*-butyl alcohol–formic acid	95:5	0.36	0.02	0.02
C	*n*-Butyl alcohol–pyridine–water	6:4:3	0.32	0.06	0.07
D	*n*-Butyl alcohol–water–propionic acid	10:7:5	0.38	0.32	0.30

[a] Whatman No. 3MM filter paper was used with descending solvent flow, except for solvent A where Whatman No. 1 filter paper was used with ascending solvent flow. Keto acids were visualized by spraying with a 0.4% solution of 2,4-dinitrophenylhydrazine in 2 N HCl. Radioactive compounds were detected by radioautography.

μg), and 25 μg of highly purified glutamate–alanine transaminase from pig heart. After an additional incubation period of 3 hr at 37°, the reaction mixture was heated for 5 min at 100° and centrifuged. The supernatant fluid was applied to a column (0.5 × 1.8 cm) of Dowex 50 (H^+) resin. The resin column was washed extensively with water, and the amino acids were then removed with 2 N NH_4OH. After the ammonia was evaporated from the eluate by concentrating to dryness *in vacuo* several times, alanine was identified chromatographically as a product arising by transamination of the pyruvate formed initially by aldolytic cleavage of KHB (Table V). No alanine was detected in the appropriate controls.

REVERSIBILITY OF THE REACTION. The reversibility of the KHB-cleavage reaction was demonstrated as follows. An incubation mixture was prepared as described in the preceding section for the identification of pyruvate and formaldehyde as their 2,4-dinitrophenylhydrazone derivatives, except that KHB was replaced by the same concentrations of pyruvate and of formaldehyde. The reaction mixture was incubated for 2.5 hr at 37°. The 2,4-dinitrophenylhydrazone derivatives were prepared, extracted, and subjected to paper chromatography in the usual manner. The dinitrophenylhydrazone derivative of KHB was identified in the following solvent systems using Whatman No. 1 filter paper with ascending solvent flow: ethanol–water (7:3, v/v), R_F 0.72; *n*-butyl alcohol–absolute ethanol–0.5 N NH_4OH (7:1:2, v/v), R_F 0.63; *n*-butyl alcohol–acetic acid–water (15:3:7, v/v), R_F 0.78.

High-pressure catalytic hydrogenation of the 2,4-dinitrophenylhydrazone of enzymatically formed KHB yielded the expected product, DL-homoserine, which was identified by paper chromatography as before: ethanol–water (7:3, v/v), R_F 0.46; pyridine–acetic acid–water (10:7:3, v/v), R_F 0.40; *tert*-butyl alcohol–water–methyl ethyl ketone–diethylamine (10:5:10:1, v/v), R_F 0.35.

Finally, the following experiment was performed. KHG-aldolase (0.29 mg) was incubated for 3 hr at 37° in 2.0 ml of 0.1 M Tris-HCl buffer (pH 8.4) containing 2.5 mM pyruvate and 2.5 mM ^{14}C-labeled formaldehyde (1.4×10^6 cpm per μmole). The reaction was terminated by adding 0.6 ml of acetone, and the precipitated protein was removed by centrifugation. The supernatant fluid was concentrated by lyophilization and then applied to a column (0.5 × 5 cm) of Dowex 1 (formate) resin. The resin was first washed extensively with water until no radioactivity could be detected in the eluate. Acidic compounds were then eluted with 6 N formic acid; those fractions (0.5 ml) containing radioactivity were pooled and repeatedly lyophilized to dryness. Aliquots of the final aqueous solution were examined by paper chromatography and radioautography. The data presented in Table VI show that a radioactive compound was formed which migrated identically with authentic KHB which was cochromatographed in the solvent systems listed.

EQUILIBRIUM CONSTANT. A quantitative study of the equilibrium of the reaction, starting with either KHB or pyruvate plus formaldehyde, was carried out at a substrate concentration of 1.7×10^{-3} M. The approach to equilibrium was followed

TABLE VII: Stoichiometry of the Aldolase Reaction with KHB as Substrate.[a]

Substrate	Substrate Concentration (mM)				Net Change (mM)	
	Initial		Final			
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Cleavage						
KHB	1.46[b]	1.66	1.12	0.38	−0.34	−1.28
HCHO			0.30	1.31	+0.30	+1.31
Pyruvate			0.35	1.25	+0.35	+1.25
Condensation						
KHB			0.43	0.68	+0.43	+0.68
HCHO	1.64	2.38	1.28	1.62	−0.36	−0.76
Pyruvate	1.78	2.32	1.35	1.64	−0.43	−0.68

[a] Experimental conditions are described in the text. The analytical determination of each compound was performed as described in the legend of Figure 3. [b] Incubation time in this instance was 60 min with 0.22 mg of enzyme.

by measuring the appearance or disappearance of formaldehyde (Figure 3). The equilibrium for the aldolase reaction with KHB as substrate favors cleavage of the ketohydroxy acid. Under the conditions specified, the equilibrium mixture contained approximately 75% pyruvate and formaldehyde and 25% KHB, regardless of whether the synthesis or the cleavage of KHB was measured. The average value of the equilibrium constant, K_{equil} (obtained from five determinations), for the reaction, KHB $\rightleftharpoons$ pyruvate + HCHO, is 4.1 $\times 10^{-3}$ M. When higher substrate concentrations (2.7×10^{-3} M; 4.7×10^{-3} M) were used, the average value for K_{equil} remained the same.

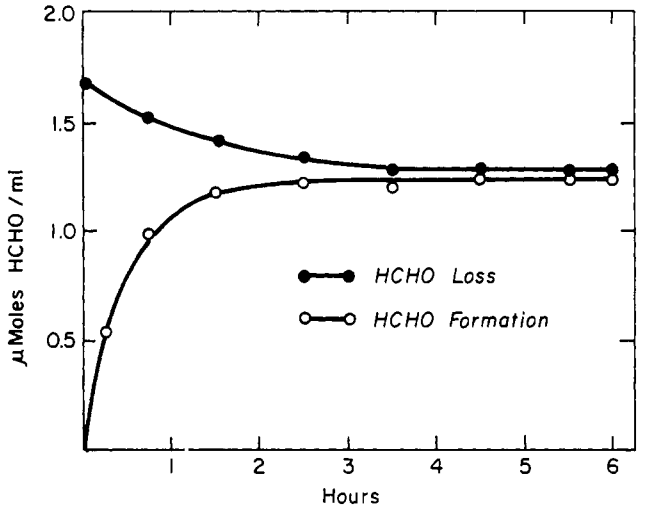


FIGURE 3: Approach to equilibrium of the aldolase-catalyzed reaction with KHB as substrate. The reaction mixture (2.0 ml) contained 400 μmoles of potassium glycylglycine buffer (pH 8.1), 5 μmoles of GSH, 3.4 μmoles of KHB (cleavage reaction), 3.4 μmoles each of pyruvate and formaldehyde (condensatiou reaction), and 0.72 mg of aldolase. Incubation was carried out at 37°. The reaction was followed by removing 0.1-ml aliquots at various time intervals and assaying for formaldehyde as described in the section entitled Methods. The concentration of KHB *plus* pyruvate at equilibrium was determined spectrophotometrically at 340 mμ by measuring the amount of NADH oxidized after complete reaction in the presence of lactate dehydrhgenase (Lane and Dekker, 1969). KHB was analyzed in the equilibrium mixture by a modification of the procedure of Cori and Lipmann (1952) after quantitative lactonization in 0.1 N HCl (Lane and Dekker, 1969). The amount of pyruvate present was calculated by difference. The values shown in the figure were corrected for a nonspecific reaction of formaldehyde with the protein which occurred during prolonged incubation at pH 8.1 and 37°.

STOICHIOMETRY OF THE REACTION. The stoichiometry of the reaction using KHB as substrate was determined by incubating for 4 hr at 37° a reaction mixture (2.0 ml) which contained 200 μmoles of glycylglycine buffer (pH 8.1); 5 μmoles of GSH; 0.72 mg of KHG-aldolase; and KHB or pyruvate plus formaldehyde at the concentrations shown in Table VII. The data presented in Table VII show that 1 mole each of pyruvate and of formaldehyde is formed per mole of KHB cleaved. Similarly, the aldolase catalyzes the condensation of 1 mole of formaldehyde with 1 mole of pyruvate to yield 1 mole of KHB.

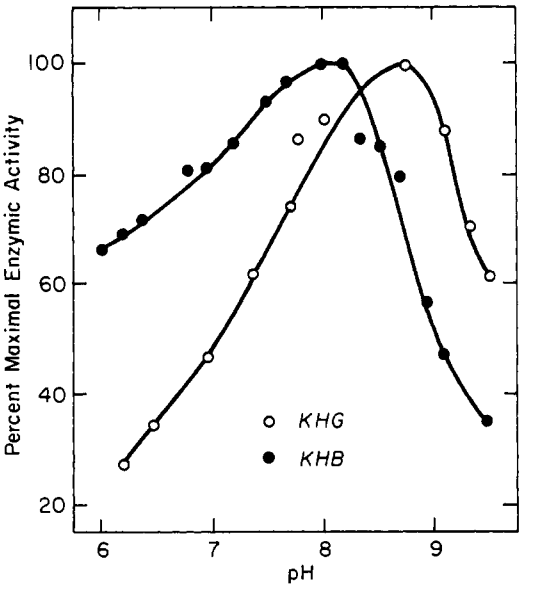


FIGURE 4: Effect of pH on aldolase activity with KHG and KHB as substrates. Enzymatic activities were determined under standard assay conditions except that the following buffers were used: 0.25 M potassium phosphate (pH 6.0 to 7.5); potassium glycylglycine (pH 7.5 to 9.2). The data obtained at pH values above 9.0 are corrected for the spontaneous cleavage of KHG; such corrections were not necessary when KHB was used as substrate.

TABLE VIII: Paper Chromatographic Identification of the Amino Acids Formed by the Reversible Transamination of L-Homoserine as Catalyzed by Aspartate and Alanine Aminotransferases.[a]

	Solvent	Ratio	Reference L-Glutamate R_F	Reference L-Homoserine R_F	Enzymatic Products: Aspartate Aminotransferase, 2-Keto-glutarate-L-homoserine System R_F	Enzymatic Products: Aspartate Aminotransferase, KHB-L-glutamate System R_F	Enzymatic Products: Alanine Aminotransferase, 2-Keto-glutarate-L-homoserine System R_F	Enzymatic Products: Alanine Aminotransferase, KHB-L-glutamate System R_F
A	2-Propanol–15 N NH_4OH	5:1	0.33	0.74	0.32	0.72	0.34	0.76
B	Phenol–water (4:1)–ethanol–15 N NH_4OH	15:4:1	0.11	0.37	0.11	0.38	0.10	0.35
C	Phenol saturated with citrate–phosphate buffer[b]		0.34	0.53	0.36	0.53	0.33	0.52
D	*tert*-Butyl alcohol–water–methyl ethyl ketone–diethylamine	10:5:10:1	0.08	0.33	0.07	0.35	0.07	0.33
E	Ethanol–water–ammonia	18:1:1	0.03	0.14	0.03	0.16	0.03	0.14

[a] Whatman No. 1 filter paper was used with ascending solvent flow, except for solvent A where thin-layer chromatography was employed. Amino acids were visualized by spraying with a 0.25% solution of ninhydrin in pyridine–water (1:1, v/v). [b] Phenol saturated with an aqueous solution containing 6.3% sodium citrate and 3.7% sodium dihydrogen phosphate.

pH Optimum. The effect of pH on aldolase activity with either KHG or KHB as substrate is shown in Figure 4. The pH–activity profile for aldolytic cleavage of KHG exhibits a sharp pH optimum at pH 8.8, in excellent agreement with the previously reported value (Kobes and Dekker, 1969). The plot of KHB cleavage as a function of pH is somewhat broader with KHB being cleaved maximally at pH 8.1. Whereas aldolase activity with KHG as substrate declines rather steeply on both sides of the pH optimum, a gradual decrease in aldolytic cleavage of KHB is observed on the acid side of the pH–activity profile. Thus, while aldolase activity toward KHG is only 35% of the maximal value at pH 6.5 (Figure 4), the rate of KHB cleavage at the same pH is approximately 75% the rate measured at its optimum pH.

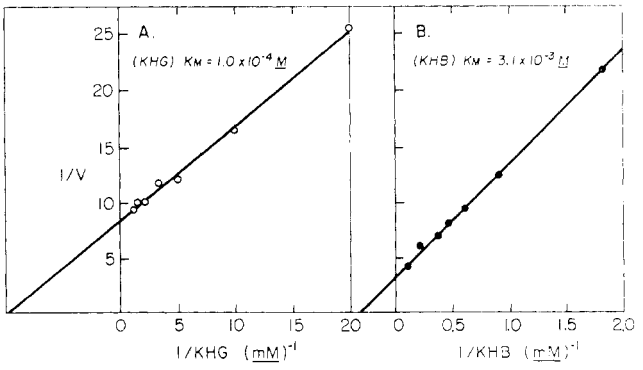


FIGURE 5: Double-reciprocal plots of the rate of aldolase activity as a function of (A) KHG concentration and (B) KHB concentration. Enzymatic activities were determined under normal assay conditions in 0.1 M potassium glycylglycine buffer (pH 8.1). Initial velocities (v) are expressed as the number of micromoles of glyoxylate or of formaldehyde formed in 20 min at 37°.

EFFECT OF SUBSTRATE CONCENTRATION. The effects of KHG and KHB concentration on aldolase activity are shown in Figure 5; initial velocities (v) are expressed as the number of micromoles of glyoxylate or of formaldehyde formed in 20 min at 37°. K_m values were determined from double-reciprocal plots by the method of Lineweaver and Burk (1934). In 0.1 M potassium glycylglycine buffer (pH 8.1) at 37°, the apparent Michaelis constant for DL-KHG is 1.0×10^{-4} M compared to a value of 3.1×10^{-3} M for KHB. Under the same conditions of assay, the V_{max} for dealdolization of KHB is approximately one-third the V_{max} for KHG cleavage. The apparent Michaelis constant for DL-KHG agrees well with the value reported by Rosso and Adams (1967) using KHG-aldolase purified from rat liver.

The affinity of bovine liver KHG-aldolase for the two optical antipodes of KHG was found to be essentially the same. K_m values for L-KHG and D-KHG were determined to be 7.1×10^{-5} M and 1.4×10^{-4} M, respectively.

Functional Role of KHG-aldolase in Homoserine Metabolism. REVERSIBLE TRANSAMINATION OF L-HOMOSERINE BY ASPARTATE AND ALANINE AMINOTRANSFERASES. In 1966, Finkelstein *et al.* (1966) reported that crude extracts of human liver catalyzed the formation of KHB from L-homoserine. A participation of L-homoserine in the glutamine transaminase system was noted in a review article by Meister (1956). Since KHB is structurally similar to OAA, the possibility was considered that homoserine might be reversibly transaminated to form KHB in a reaction catalyzed by highly purified aspartate aminotransferase from pig heart. To test this possibility, an incubation mixture was prepared as follows.

The reaction mixture (0.4 ml) contained 50 μmoles of Tris-HCl buffer (pH 8.4); 5 μmoles of EDTA (pH 7.4); 30 μmoles of L-homoserine; 30 μmoles of 2-ketoglutarate; and 0.50 mg of glutamate–aspartate transaminase. The reversibility of the transamination reaction was tested by replacing L-homoserine

and 2-ketoglutarate by the same concentrations of L-glutamate and KHB, respectively. After the reaction mixture was incubated for 2 hr at 37°, it was applied to a column (0.5 × 2.5 cm) of Dowex 50 (H^+) resin. The resin column was washed with 10 ml of water, and the amino acids were then removed with 4 ml of 2 N NH_4OH solution. The eluate was concentrated to dryness *in vacuo* at 38°; this procedure was repeated three times to eliminate excess ammonia. The final residue was dissolved in 0.2 ml of water and examined by paper chromatographic techniques. The data presented in Table VIII show that a ninhydrin-positive compound was formed in the L-homoserine–2-ketoglutarate system which migrated identically with authentic L-glutamate in the five solvent systems listed. In addition, an amino acid was formed in the L-glutamate-KHB system which exhibited identical paper chromatographic properties with reference L-homoserine. Moreover, it can be seen that identical results were obtained when glutamate–aspartate transaminase was replaced by glutamate–alanine transaminase (0.50 mg) in the incubation mixture.

The reversible transamination of L-homoserine with OAA as amino group acceptor (catalyzed by aspartate aminotransferase) or with pyruvate as amino group acceptor (catalyzed by alanine aminotransferase) could also be demonstrated. The reaction mixture (0.4 ml) contained 50 μmoles of Tris-HCl buffer (pH 8.4), 5 μmoles of EDTA (pH 7.4), 10 μmoles of L-homoserine, 4.5 μmoles of OAA, and 0.49 mg of aspartate aminotransferase. The reversibility of this reaction was tested by replacing L-homoserine and OAA by 30 μmoles of L-aspartate and 28 μmoles of KHB, respectively. After the reaction mixture was incubated for 90 min at 37°, the amino acids were isolated as described before. L-Aspartate was identified as a product of the L-homoserine–OAA system by paper chromatography in the following solvent systems: solvent A (Table VIII), R_F 0.16; solvent C (Table VIII), R_F 0.17; *tert*-amyl alcohol–ethanol–ammonia (1:1:1, v/v), R_F 0.33; ethanol–water (7:3, v/v), R_F 0.29. L-Homoserine was similarly identified as a product of the L-aspartate–KHB system (see Table VIII).

In similar experiments using alanine aminotransferase, L-alanine was identified as a product of the L-homoserine–pyruvate system as follows: solvent B (Table VIII), R_F 0.44; solvent D (Table VIII), R_F 0.24; and solvent E (Table VIII), R_F 0.18. Likewise, L-homoserine was identified as a product of the L-alanine–KHB system by use of the solvent systems listed in Table VIII.

A comparison of the rates of KHB transamination with L-glutamate, as catalyzed by aspartate aminotransferase or by alanine aminotransferase, is shown in Figure 6. The initial rates of reaction at various levels of enzyme were determined by an assay which followed the conversion of [^{14}C]glutamate to [^{14}C]2-ketoglutarate, as described in the section entitled Methods. The rates are expressed as the ethyl acetate extractable radioactivity formed after a 40-min incubation at 37°. Both aminotransferases promote a significant transamination of KHB. Although the rates of both reactions are comparable, aspartate aminotransferase appears to be somewhat more effective in this system than is alanine aminotransferase.

FORMATION OF FORMALDEHYDE AND PYRUVATE FROM HOMOSERINE IN A COUPLED ENZYME SYSTEM. Since the equilibrium for the aldolase-catalyzed reaction with KHB as substrate favors cleavage of the ketohydroxy acid (Figure 3), it has been possible to demonstrate the net formation of either HCHO or pyruvate in a coupled system containing both aspartate aminotransferase and KHG-aldolase. This experiment was performed in the following manner.

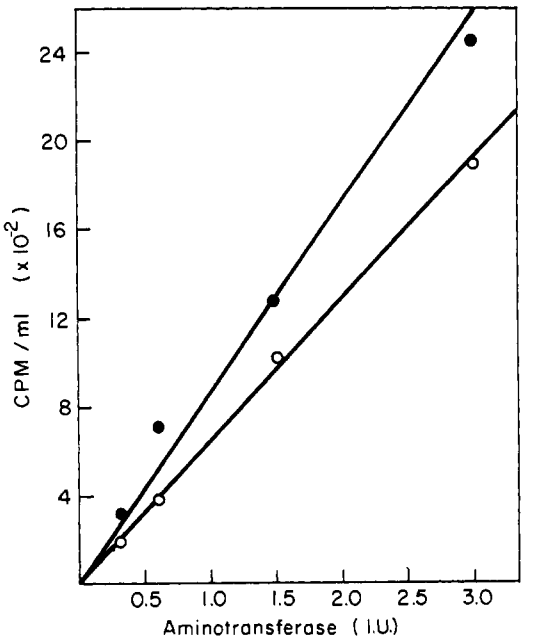


FIGURE 6: Comparison of the rates of transamination of KHB by aspartate and by alanine aminotransferases. Radioactive assays were carried out as described in the section entitled Methods. The reaction mixtures were incubated for 40 min at 37°: (○), alanine aminotransferase; (●), aspartate aminotransferase. I.U. = international unit of enzyme activity.

The reaction mixture (1.8 ml) contained 100 μmoles of potassium glycylglycine buffer (pH 8.2), 10 μmoles of EDTA (pH 7.4), 60 μmoles of L-homoserine, 12 μmoles of 2-ketoglutarate, and 0.98 mg of aspartate aminotransferase. After the mixture was incubated for 90 min at 37°, the reaction was terminated by heating for 2 min at 100° and the precipitated protein was removed by centrifugation. An aliquot of the supernatant fluid was analyzed spectrophotometrically for KHB by measuring the extent of NADH oxidation (at 340 mμ) after complete reaction in the presence of excess lactate dehydrogenase (Lane and Dekker, 1969). The reaction had yielded 0.49 μmole of KHB. No KHB could be detected by this method in incubation mixtures lacking either L-homo-

TABLE IX: Formation of Formaldehyde and Pyruvate from L-Homoserine in a Coupled System Containing Aspartate Aminotransferase and KHG-aldolase.[a]

Additions	Formaldehyde Liberated (μmole)	Pyruvate Liberated (μmole)
Complete system	0.17	0.14
Minus L-homoserine	0	0
Minus 2-ketoglutarate	0	0
Minus aspartate aminotransferase	0	0
Minus KHG-aldolase	0	0
Heat-inactivated enzymes	0	0

[a] Experimental conditions are described in the text.

serine, 2-ketoglutarate, or aspartate aminotransferase or containing heat-denatured aminotransferase.

A portion of this same solution (0.36 μmole of KHB) was then supplemented with 0.14 mg of KHG-aldolase and 7.5 μmoles of GSH in a final volume of 1.5 ml. After an additional incubation period of 60 min at 37°, the reaction was terminated by heating for 2 min at 100°, and the precipitated protein was removed by centrifugation. The supernatant fluid was examined for HCHO content by reaction with chromotropic acid reagent and for the level of pyruvate by reaction with salicylaldehyde in alkaline solution. The results of this experiment are presented in Table IX. It can be seen that HCHO and pyruvate are formed in essentially stoichiometric amounts in a complete system containing both enzymes. Neither of these products could be detected in incubation mixtures lacking either L-homoserine, 2-ketoglutarate, aspartate aminotransferase, or KHG-aldolase, or containing heat-denatured enzymes. Comparable results were obtained when aspartate aminotransferase and KHG-aldolase were incubated together in a complete system.

Discussion

The enzymatic condensation of pyruvate with formaldehyde was first noted by Hift and Mahler (1952); they succeeded in partially purifying (about 60-fold) this condensing enzyme from bovine liver extracts. No detailed study of this enzymatic reaction, however, has appeared and no further information as to the identity of this aldolase or its role in metabolism is presently available.

The data presented in this paper show that 2-keto-4-hydroxyglutarate aldolase catalyzes the reversible cleavage of 2-keto-4-hydroxybutyrate, the α-keto analog of homoserine, yielding stoichiometric amounts of pyruvate and formaldehyde. Several independent lines of evidence provide strong support for the conclusion that the aldolytic cleavage of 2-keto-4-hydroxyglutarate and of 2-keto-4-hydroxybutyrate is catalyzed by the same enzyme. This conclusion is based on the following observations. (1) At all stages of purifying the enzyme to homogeneity (1300-fold purified), the ratio of KHG- to KHB-cleavage activities remains constant; no separation of these two activities could be obtained. (2) Two forms of the aldolase, isolated by polyacrylamide gel electrophoresis, show identical levels of activity with either KHG or KHB as substrate. (3) Partial inactivation of aldolase activity by controlled heat treatment proceeds at the same rate with both substrates. (4) Glycolaldehyde, 2-ketoglutarate, and 2-ketobutyrate competitively inhibit the enzyme in the presence of either KHG or KHB and the dissociation constants of the enzyme–inhibitor complexes (K_i values) are the same for both ketohydroxy acids. (5) The extent of inactivation of the enzyme, when it is incubated with a variety of either substrates or substrate analogs in the presence of $NaBH_4$, is the same regardless of whether KHG or KHB is used as substrate.

KHB is utilized less effectively as substrate than is KHG; this is most likely due to a greater affinity of the aldolase for KHG. Whereas there is about a threefold difference in the maximum velocity with these two substrates, the apparent K_m values (Figure 5) differ by a factor of 30. These results suggest that a cationic group may be at the active site of the enzyme which is involved in binding the carboxylate anion on carbon atom 5 of KHG. In addition, bovine liver KHG-aldolase has essentially the same affinity for the two optical isomers of KHG; the apparent Michaelis constants for L-KHG and for D-KHG are not significantly different. These findings agree with earlier observations (Kobes and Dekker, 1966) which showed that this aldolase catalyzes the cleavage of both isomers with almost equal facility. All indications are, therefore, that the configuration of the hydroxyl group at carbon atom 4 of KHG is not an important contributing factor in substrate binding. It is possible, of course, that the kinetic data reported may be complicated by the presence of more than one active form of the enzyme (*cf.* Table II). When we have, however, on rare occasions obtained samples of KHG-aldolase which showed only one protein-stainable band on disc gel electrophoresis ($R_M \sim 0.5$), the same K_m values for KHB and KHG were obtained.

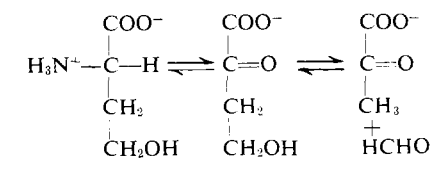
It is of interest that the pH-activity profiles of the aldolase with KHG and KHB as substrates are not identical (Figure 4). A sharp pH optimum is observed for dealdolization of KHG whereas KHB is cleaved over a broader pH range. Moreover, the pH optima for the two reactions are different. The significance of these findings is not immediately apparent. The data may reflect a differential effect of pH on the apparent K_m values for the two substrates; pH-induced conformational changes may be more critical for effective binding of KHG than for KHB. The fact that KHG contains a carboxylate group at carbon atom 5 which is not present in KHB may be a contributing factor. Nonidentical pH optima for different substrates have been observed before. Hexokinase from *Aspergillus parasiticus* (Davidson, 1960) has different pH–activity profiles for the phosphorylation of D-galactose and D-galactosamine and the activity of hexokinase from *Leuconostoc mesenteroides* as a function of pH differs for D-mannose and D-fructose (Sapico and Anderson, 1967). Similar examples have been reported for fructose diphosphatase (Pontremoli *et al.*, 1965), lactate dehydrogenase (Meister, 1950), glutamate dehydrogenase (Bitensky *et al.*, 1965), and L-asparaginase (Campbell and Mashburn, 1969).

L-Homoserine is made biosynthetically in bacteria and in fungi from aspartate. In microorganisms, homoserine functions as a metabolic intermediate in the biosynthesis of threonine (*via* its *O*-phosphoryl derivative); it has a similar role in methionine biosynthesis in bacteria (*via* its *O*-succinyl derivative) and in fungi (*via* its *O*-acetyl derivative). There is no evidence, however, that homoserine is a precursor of threonine or methionine in mammalian metabolism, nor that it is derived from aspartic acid. The most widely accepted metabolic fate of homoserine in mammals is its degradation to 2-ketobutyrate and NH_3 (Matsuo *et al.*, 1956). Homoserine dehydratase, the enzyme which catalyzes this reaction, has been isolated in crystalline form from rat liver extracts by Matsuo and Greenberg (1958). This same enzyme also catalyzes the cleavage of cystathionine yielding cysteine, 2-ketobutyrate, and NH_3.

Direct evidence has now been obtained for an alternate route of L-homoserine degradation in mammals and establishes 2-keto-4-hydroxybutyrate as a key metabolic intermediate in this pathway. The enzymatic reactions involved in this pathway are shown in Scheme I.

The first reaction is a transamination of L-homoserine

SCHEME I



yielding KHB. This enzymatic conversion has been shown to be catalyzed by a highly purified preparation of glutamate–aspartate transaminase from pig heart; either oxaloacetate or 2-ketoglutarate serve as amino group acceptors in this process (eq 3 and 4, respectively).

$$\text{L-homoserine} + \text{OAA} \rightleftharpoons \text{KHB} + \text{L-aspartate} \quad (3)$$

$$\text{L-homoserine} + \text{2-ketoglutarate} \rightleftharpoons \text{KHB} + \text{L-glutamate} \quad (4)$$

Transamination of homoserine was also shown to occur in the presence of pig heart glutamate–alanine transaminase and either 2-ketoglutarate or pyruvate (eq 4 and 5, respectively).

$$\text{L-homoserine} + \text{pyruvate} \rightleftharpoons \text{KHB} + \text{L-alanine} \quad (5)$$

Collectively, these results indicate that L-homoserine is readily transaminated in the mammal. Finkelstein *et al.* (1966) have reported that crude extracts of human liver catalyze the formation of KHB from L-homoserine. Their findings indicated that KHB accounted for 20 to 40% of the total keto acid formed from L-homoserine (the major keto acid product being 2-ketobutyrate formed by deamination of homoserine by homoserine dehydratase).

The second reaction in Scheme I is the subsequent cleavage of KHB forming pyruvate and HCHO. Both of these products may then be further metabolized by well-known enzymatic reactions. On the basis of the evidence now available, dealdolization of KHB appears to be mediated by 2-keto-4-hydroxyglutarate aldolase. Although earlier studies demonstrated that KHG-aldolase catalyzes a terminal step in the degradation of hydroxyproline by mammals, this same enzyme must now be regarded as also playing an important, previously unrecognized role in the metabolism of L-homoserine. A fairly general role of KHG-aldolase in metabolism is suggested by its presence in bacteria (Aronson *et al.*, 1967; Nishihara and Dekker, 1969), in plants (Payes and Laties, 1963), and in several mammalian tissues (Maitra and Dekker, 1964).

Pyruvate and HCHO are formed in equimolar amounts from L-homoserine in a coupled system containing both aspartate aminotransferase and KHG-aldolase (Table IX). Moreover, eq 2, 3, 4, and 5 are all reversible. The metabolic reactions outlined in Scheme I, therefore, may provide a possible route for the biosynthesis of homoserine in mammals. No systematic attempts to demonstrate the net formation of L-homoserine from substrate quantities of pyruvate and HCHO in the coupled enzyme system have been made as yet.

Studies on the mechanism of action of bovine liver KHG-aldolase indicate that it is a Class 1 aldolase (classification according to Rutter, 1964). The enzyme forms azomethine complexes with all three of its substrates (KHG, pyruvate, and glyoxylate) which are stabilized by reduction with $NaBH_4$ and enzymatic activity is destroyed (Kobes and Dekker, 1966; Kobes, 1967) (Table III). A similar loss of aldolase activity occurs with KHB or HCHO in the presence of borohydride as well as with several other carbonyl compounds. It is of interest, therefore, that 2-ketoglutarate, 2-ketobutyrate, and glycolaldehyde, when incubated with the aldolase in the presence of $NaBH_4$, extensively inactivate the enzymatic cleavage of either KHG or KHB since these same three analogs also function as competitive inhibitors of the KHG- and KHB-cleavage reactions (Table IV). It is possible, therefore, that the aldolase activity lost in the presence of $NaBH_4$ is due to reduction of a Schiff base formed between these inhibitors and the same lysyl residue (or residues) in the protein molecule which is involved in the binding of substrates.

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Purification and Properties of an Alkaline Phosphatase of *Bacillus licheniformis**

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ABSTRACT: *Bacillus licheniformis* MC 14 was chosen from 40 thermophilic strains of *Bacillus* for the study of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). This facultatively thermophilic organism was selected on the basis of quantitative production of the enzyme. Investigation revealed that the enzyme was particulate and was most likely bound to the cell membrane. The enzyme was solubilized using a 1 M magnesium extraction procedure. Homogeneity of the purified enzyme was established by sedimentation studies, analytical acrylamide gel electrophoresis, and immunological studies. The purified enzyme had the following properties: specific activity 230 units/mg; molecular activity 2.8×10^4 moles of *p*-nitrophenyl phosphate hydrolyzed per min per mole of alkaline phosphatase; molar extinction coefficient 7.5×10^4 at 278 nm; optimum temperature, 50°; optimum pH, 8.5; and partial specific volume, 0.733 ml/g. The synthesis of the enzyme was repressed by inorganic phosphate. Enzyme activity was competitively inhibited by inorganic phosphate. The enzyme has a K_m of 6.0×10^{-4} M for *p*-nitrophenyl phosphate and an apparent K_I of 0.037 M for inorganic phosphate. A molecular weight of 117,000 was determined from sedimentation–diffusion data. Amino acid analysis showed that the basic nature of the enzyme was largely the result of a high lysine content.

Repressible alkaline phosphatases have been found in a variety of microorganisms including *Escherichia coli* (Garen and Levinthal, 1960; Torriani, 1968), *Pseudomonas fluorescens* (Friedberg and Avigad, 1967), *Aerobacter aerogenes* (Wolfenden and Spence, 1967), *Neurospora crassa* (Kadner *et al.*, 1968), *Bacillus subtilis* (Takeda and Tsugita, 1967), *Staphylococcus aureus* (Shah and Blobel, 1967), and *Aspergillus nidulans* (Dorn, 1968; Dvorak, 1968). The enzyme has been studied most extensively in *E. coli*. In this organism the enzyme is soluble and located in the periplasmic region (Garen and Levinthal, 1960; Malamy and Horecker, 1961; Neu and Heppel, 1965; Brockman and Heppel, 1968). The molecule is a dimer (86,000 molecular weight) composed of two identical subunits (Schlesinger and Barrett, 1965; Applebury and Coleman, 1969), and contains four atoms of zinc per molecule (Simpson and Vallee, 1968). Conformational states of the enzyme and subunits have been studied (Schlesinger, 1965; Reynolds and Schlesinger, 1967, 1968). Evidence for a subunit pool within the cell membrane has been reported (Schlesinger, 1968; Torriani, 1968).

This paper describes the solubilization and purification of a repressible alkaline phosphatase of a facultatively thermophilic strain of *Bacillus licheniformis*. Data are also presented on the amino acid composition, molecular weight, immunological characteristics, and some general properties of the enzyme.

Methods and Materials

Organism. The organism used in this investigation was a facultatively thermophilic strain of *Bacillus licheniformis* MC14 (Hulett-Cowling, 1969). This organism was selected by screening 40 thermophilic strains of *Bacillus* for the production of alkaline phosphatase.

Stock cultures were maintained on slants containing 2% Trypticase (BBL) and 2% agar (Difco), pH 7.2.

Buffers. The composition and abbreviations of buffers used throughout this paper are as follows: TA buffer, 0.01 M Tris acetate (pH 7.3); TAC buffer, TA buffer containing 0.1 mM $CoCl_2$; BG buffer, 0.1 M Bicine [*N,N*-bis(2-hydroxyethyl)glycine]–0.05 glycine (pH 7.3); BGC buffer, BG buffer containing 0.1 mM $CoCl_2$.

Production of Enzyme. The media used for the production of the enzyme included Trypticase broth (2% Trypticase (BBL) and 0.1% fructose, pH 7.2), Trypticase agar medium (Trypticase broth containing 2% agar), and Neopeptone medium (1% Neopeptone (Difco) and 0.1% fructose, pH

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