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PURIFICATION OF RAT-LIVER γ -HYDROXYGLUTAMATE TRANSAMINASE AND ITS PROBABLE IDENTITY WITH GLUTAMATE-ASPARTATE TRANSAMINASE

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SUMMARY

1. An enzyme which catalyzes the transamination of γ -hydroxyglutamate has been purified over 300-fold from rat-liver homogenates.

2. The following observations strongly suggest that γ -hydroxyglutamate transaminase is identical with glutamate-aspartate transaminase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1): a, at all stages of purification from either rat-liver or pig-heart extracts, the glutamate to γ -hydroxyglutamate transaminase ratios are not significantly different; b, transaminase activity for both substrates declines at the same rate during controlled heat denaturation of the purified rat-liver enzyme; c, transamination of γ -hydroxyglutamate is strongly inhibited by either L-glutamate or L-aspartate; d, glutarate and maleate function as competitive inhibitors for either glutamate or γ -hydroxyglutamate and determined K_I values for a given inhibitor are the same for either amino acid; and e, the purified rat-liver enzyme has the same pH-activity curve for both substrates.

3. The *erythro*- and *threo*-isomers of γ -hydroxy-L-glutamate serve as substrates for both isozymes of the rat-liver enzyme as well as for the pig-heart enzyme, although the former isomer is a somewhat better substrate. The corresponding D-diaStereo-isomers are enzymically inactive.

4. With γ -hydroxyglutamate, only α -ketoglutarate and oxaloacetate serve as amino group acceptors; pyruvate, α -ketobutyrate, and β -phenylpyruvate are inactive.

5. Other γ -substituted forms of glutamic acid, including γ -methyleneglutamic acid and γ -hydroxy- γ -methylglutamic acid, are also active with the purified enzymes.

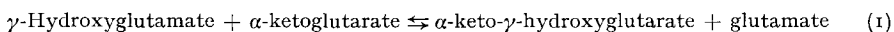
INTRODUCTION

VIRTANEN¹ first stated that plant homogenates catalyzed a transamination reaction between γ -hydroxyglutamate and α -ketoglutarate. In previous reports^{2,3}, we pre-

Abbreviations: γ -HG, γ -hydroxyglutamate; KHG, α -keto- γ -hydroxyglutarate.

* Predoctoral trainee of the United States Public Health Service. These studies are taken from a thesis submitted by U. MAITRA in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Chemistry in the Horace H. Rackham School of Graduate Studies, The University of Michigan.

sented evidence showing that the first enzyme involved in the net conversion of γ -hydroxyglutamate to glyoxylate and alanine by rat-liver extracts is a transaminase. The reaction is as follows:



Oxaloacetate but not pyruvate also served as amino group acceptor. We found this transaminase to be distinct from glutamate-alanine transaminase (EC 2.6.1.2), but it could be replaced by highly-purified pig-heart glutamate-aspartate transaminase.

Two preliminary reports have appeared concerning transamination of γ -hydroxyglutamate by mammalian enzymes. KURATOMI AND FUKUNAGA⁴ reported the presence of a γ -hydroxyglutamate-transaminase in an ammonium sulfate fraction (62–75%) of rat-liver extracts, no mention being made as to its specificity. GOLDSTONE AND ADAMS⁵ indicated that γ -hydroxyglutamate did not serve as substrate for purified glutamate-aspartate transaminase of pig-heart but was readily transaminated by a Mn^{2+} -activated rat-liver enzyme. We noted this apparent contrast with our initial findings in an earlier report². Just recently, a report appeared by GOLDSTONE AND ADAMS⁶ which does not substantiate their initial findings but rather is in accord with some of the results presented here.

The present paper describes the purification and properties of the enzyme from rat-liver extracts that catalyzes the transamination of γ -hydroxyglutamate. Several independent tests establish that this purified enzyme is identical with the well-known glutamate-aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1). Both forms (anionic and cationic) of the enzyme utilize γ -hydroxyglutamate as substrate. Certain features of this work have been reported in abstract form⁷.

MATERIALS AND METHODS

Synthetic *threo*- γ -hydroxy-DL-glutamic acid⁸ was routinely used as substrate in the studies reported unless otherwise indicated. Cryst. *erythro*- γ -hydroxy-DL-glutamic acid was obtained from the supernatant fluid remaining after the *threo*-racemate had been precipitated. The *threo*-L-isomer of γ -hydroxyglutamic acid was isolated from *Phlox decussata* extracts⁹. β,γ -Dihydroxyglutamic acid was prepared by the procedure of TOUSTER AND CARTER¹⁰. γ -Methyl- γ -hydroxyglutamic acid was prepared by converting β -methallylchloride* to β -methylglycerol- α -monochlorohydrin (b.p. 101–106°/13 mm Hg) (ref. 11) which, in turn, was oxidized to β -chloro- α -hydroxyisobutyric acid (m.p. 106–109°) with nitric acid. The acid was then esterified (b.p. 103–107°/30 mm Hg) and acetylated to yield α -acetoxy- α -methyl- β -chloropropionate (b.p. 100–104°/18 mm Hg). Condensation of this product with diethylacetamidomalonate in the usual manner followed by acid hydrolysis liberated the desired product, which was isolated and purified by adsorption and elution from columns of Dowex 1-X10 (acetate phase) resin. The prepared amino acid was chromatographically homogeneous in several different solvent systems. MARCUS AND SHANNON¹² recently reported synthesizing this compound in an analogous manner.

We gratefully acknowledge gift samples of *erythro*- γ -hydroxy-L-glutamic acid from Drs. E. Adams and A. Goldstone, γ -methyleneglutamic acid and γ -methyl- γ -

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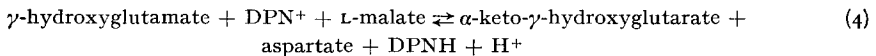
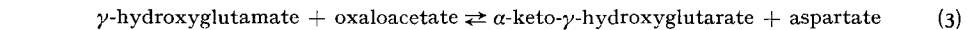
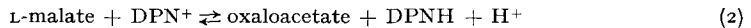
hydroxy- α -ketoglutaric acid from Dr. A. Marcus, *erythro*- β -hydroxy-DL-glutamic acid from Dr. S. Hisada, and isoglutamine from Dr. G. Wolf. All other chemicals were commercial products. Modified celluloses were products of Brown Company, Berlin, New Hampshire (U.S.A.). DPN⁺ and DPNH were purchased from Pabst Laboratories. Malate dehydrogenase (EC 1.1.1.37) was obtained from Calbiochem, Inc. The acidic amino acids and keto acids were neutralized before use by careful addition of KOH soln. Oxaloacetate soln. was prepared fresh each day by dissolving in 0.06 M Tris-HCl buffer (pH 7.4) and then adding solid KHCO₃ until the pH was 7.4.

Rat-liver protein Fraction C, which contains the KHG-cleavage enzyme (α -keto- γ -hydroxyglutarate glyoxalate-lyase) free of γ -HG transaminase, was prepared as described previously³. This preparation is stable at 0° for at least two weeks. Dr. W. G. Robinson of this department kindly supplied us with all the protein fractions obtained in purifying glutamate-aspartate transaminase from pig-heart extracts according to the procedure of JENKINS¹³. The final preparation of Jenkins was described as being 50–80% pure ultracentrifugally. The enzyme preparation used routinely in our studies was approximately 300-fold purified and 65% pure by ultracentrifugal analysis.

Protein was generally measured spectrophotometrically with a correction for nucleic acid content¹⁴. Occasionally, the content in crude extracts was determined nephelometrically¹⁵. Glyoxylic acid was estimated colorimetrically and alanine spectrophotometrically as reported earlier⁸. Absorbancy measurements were made with the use of a Beckman DU Spectrophotometer, 1.0 cm light path.

Enzyme assays

Three independent assays were developed for measuring transaminase activity. *Assay I*: Either L-glutamate or γ -HG served as the amino group donor. Oxaloacetate was generated by a mixture containing an excess of each of L-malate, DPN⁺, and malate dehydrogenase. The dehydrogenase reaction (2), which required about one min to reach equilibrium, was coupled with the transaminase reaction (3) to accomplish reaction (4).



Under the conditions of assay, the equilibrium of the coupled system favors the net reaction. The initial rate of DPNH formation is proportional to the concn. of transaminase over a 4 to 5-fold range of enzyme dilutions.

The reaction mixture contained the following components: 300 μ moles of Tris-HCl buffer (pH 8.1); 0.5 mg of crystalline bovine serum albumin dissolved in 0.2 ml of 0.02 M Tris-HCl buffer (pH 8.1); 50 μ g of pyridoxal 5'-phosphate; and transaminase enzyme in a final vol. of 1.2 ml. This mixture was incubated for 15 min at room temp. Potassium malate (30 μ moles), DPN⁺ (0.8 μ mole), malate dehydrogenase (1 unit, where a unit is defined as the amount of enzyme that catalyzes the reduction of 1 μ mole of oxaloacetate by DPNH), and H₂O to make a final vol. of 2.8 ml were then added and the mixture was stirred for 1 min. The reaction was started by the

final addition to the test cuvette of either 50 μ moles of L-glutamate or 100 μ moles of γ -HG in a vol. of 0.2 ml. The rate of increase in absorbancy at 340 $m\mu$ between 1 and 5 min after initiation of the reaction was used to calculate the activity. Amounts of transaminase were added so as to give an absorbancy change within the range of 0.010–0.040/min. Non-linear rates were obtained above this range.

One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of one μ mole of DPNH/min at 25°, under the conditions specified. Specific activity, in every case, is expressed as units of enzyme/mg protein.

Assay II: So far as we know, this assay is specific for γ -HG. It involves an initial incubation of γ -HG and α -ketoglutarate with the transaminase. The enzyme activity is then destroyed by heating. The amount of KHG formed is determined by a second incubation containing an excess of KHG-cleavage enzyme (rat-liver protein Fraction C). The glyoxylate liberated by KHG cleavage is measured colorimetrically.

The method consisted of subjecting the following mixture to a prior incubation for 15 min at 37°: 200 μ moles of Tris-HCl buffer (pH 8.4); 5 μ moles of EDTA (pH 7.4); 50 μ g of pyridoxal 5'-phosphate; 50 μ moles of γ -HG; and transaminase enzyme in a final vol. of 1.2 ml. Subsequently, 15 μ moles of α -ketoglutarate (in a volume of 0.3 ml) were added to start the reaction. After incubating the mixture at 37° for 30 min, the reaction was stopped by placing the tubes in a boiling water bath for 3.5 min, cooling in ice, and removing the precipitated protein by centrifuging. An aliquot (1 ml) of the supernatant fluid was next incubated with 5 μ moles of GSH and 1.5 mg of rat-liver protein Fraction C in a final vol. of 1.8 ml at 37° for 2 h. The tubes were flushed with N₂ and stoppered before this second incubation. The reaction was terminated by the addition of 0.6 ml of 12% metaphosphoric acid, the precipitated protein was removed by centrifuging, and aliquots of the supernatant fluids were used for the determination of glyoxylate.

In this assay, a unit of transaminase activity is defined as the amount of protein that catalyzes the formation of 1 μ mole of glyoxylate in 1/2 h under the conditions employed.

Assay III: The foregoing assay procedures are coupled enzyme systems and are difficult to use to obtain kinetic constants. Another assay method, therefore, was used which depends upon the disappearance of the absorption at 280 $m\mu$ shown by oxaloacetate-enolate at pH 7.4. Since this method utilizes only the transaminase, saturating concentrations of either substrate can be used. A mixture containing 200 μ moles of Tris-HCl buffer (pH 7.4); 25 μ g of pyridoxal 5'-phosphate and enzyme in a final vol. of 2 ml was incubated for 15 min at room temp. Freshly-prepared oxaloacetate (6.2 μ moles) and H₂O were then added, bringing the vol. to 2.8 ml. The reaction was started by adding 0.2 ml containing 100 μ moles of either L-glutamate or γ -HG. The change in absorbancy at 280 $m\mu$ was measured against a blank lacking the enzyme but containing such an amount of oxaloacetate that the absorbancy of the reaction cuvette at zero time read below 0.700. The average decrease in absorbancy ($-\Delta A$ /min) between 1 and 5 min was used to calculate the enzyme activity. (1 μ mole of oxaloacetate-enolate under the conditions described has an absorbancy of 0.520). A correction was always applied for non-enzymic disappearance of oxaloacetate-enolate. In the linear range of the assay, the average ($-\Delta A$ /min) was between 0.013 and 0.092.

Assay IV: Method of KARMEN¹⁶.

On rare occasions, we used this method to measure transaminase activity. A

mixture containing 200 μ moles of Tris-HCl buffer (pH 8.4); 50 μ g of pyridoxal 5'-phosphate; 0.5 mg crystalline bovine serum albumin dissolved in 0.2 ml of 0.2 *M* Tris-HCl buffer (pH 8.1); 100 μ moles of L-aspartate; and enzyme, in a final vol. of 2.4 ml, were incubated for 10 min at room temp. DPNH (0.3 μ mole) and 1 unit of malate dehydrogenase were then added. The reaction was started by adding 20 μ moles of α -ketoglutarate in a final reaction vol. of 3 ml. The rate of decrease in absorbancy at 340 $m\mu$ during the first 3 min served to measure transaminase activity.

All these assays were checked to determine optimal conditions, and as used, showed linear rates or proportional accumulation of products with increasing concentrations of protein over a defined range.

RESULTS

Purification of γ -HG transaminase from rat-liver extracts

The following operations were carried out between 0 and 4° unless otherwise stated.

Step 1: A dialyzed KCl-ethanol extract of fresh rat-liver* was prepared as described in detail in our earlier paper⁸.

Step 2: The dialyzed extract (510 ml) was chilled to -15° and 220 ml of reagent-grade acetone, previously cooled to -15°, were added dropwise with stirring. The soln. was stirred for an additional 20 min after the complete addition of acetone. The precipitated protein was removed by centrifuging for 30 min at 20 000 $\times g$ at -15° and discarded. More acetone (290 ml) was added dropwise at -15° and the soln. was stirred for an additional 20 min. The precipitated protein, removed by centrifuging as before, was slowly dissolved in 60 ml of 0.1 *M* potassium maleate buffer (pH 6.0), containing 0.001 *M* GSH. This soln. was dialyzed for 8 h against 4 l of 0.05 *M* maleate buffer (pH 6.0), containing 0.001 *M* GSH. The enzyme soln. was turbid at this stage, but was used as such in the next step.

Step 3: Portions (7 ml each) of the resulting dialyzed protein soln. were heated in heavy-walled test tubes in a 75° water bath. The enzyme soln. was stirred gently with a thermometer until the temp. of the soln. reached 45°. At this stage, 200 μ moles of α -ketoglutarate dissolved in 0.5 ml of 0.05 *M* maleate buffer (pH 6.0), and neutralized with KOH to pH 6.0, were added to each 7-ml portion. While the enzyme soln. was continually stirred gently with a thermometer, the temp. of the soln. was raised to 72° and maintained at 72° for 4 min. The tubes were then immediately plunged into an ice bath and the solutions chilled by stirring slowly. The contents of the tubes were pooled and the ppt. of denatured protein was removed by centrifuging and discarded.

Step 4: Cryst. ammonium sulfate was added slowly with stirring to the yellow-colored supernatant fluid (52 ml) obtained in Step 3 until the salt concn. was 277 g/l (0.45 satn.). The mixture was stirred for an additional 45 min and the ppt. obtained by centrifuging in the cold at 23 000 $\times g$ was discarded. The supernatant soln. was brought to a salt concn. of 516 g/l (0.75 satn.) by slow addition with stirring of cryst. ammonium sulfate. The mixture was stirred for an additional 45 min. The ppt. obtained after centrifuging in the cold at 23 000 $\times g$ was dissolved in 12 ml of 0.05 *M* Tris-HCl buffer (pH 7.4), containing 0.001 *M* GSH, and dialyzed for 20 h against 3 l

* The rats used were the gift of the Upjohn Company, Kalamazoo, Mich. (U.S.A.).

of 0.005 M Tris-HCl buffer (pH 7.4), containing 0.001 M GSH. At 5 h intervals, the dialysis soln. was replaced by 3 l of fresh buffer soln. of the same composition. This thorough dialysis completely removed traces of maleate and α -ketoglutarate from the enzyme soln.

Step 5: DEAE-cellulose, previously equilibrated and stored in 0.005 M Tris-HCl buffer (pH 7.4), was transferred as a slurry to a glass column and allowed to settle by gravity to form a resin column 2×34 cm in dimensions. Tris-HCl buffer, (0.005 M), pH 7.4, was passed over the cellulose until the effluent pH also equalled 7.4. The dialyzed ammonium-sulfate fraction was carefully layered on the top of the column and allowed to soak into the column bed. The cellulose column was then washed in turn with 200 ml of 0.005 M, 200 ml of 0.01 M, and 400 ml of 0.02 M Tris-HCl buffer (pH 7.4). The transaminase was then eluted by passing 700 ml of 0.038 M Tris-HCl buffer (pH 7.4), over the cellulose column. The enzyme appeared in the effluent fluid after about 120-140 ml of 0.038 M buffer had passed through the column and continued to be eluted in the following 220 ml of buffer. Throughout this elution process, 10-ml fractions were collected and the flow rate was maintained at 30 ml/h. The fractions with the highest specific activity (5 or 6 tubes) were pooled to give an enzyme preparation with a final specific activity of 26 (Table I). Fractions having lower specific activity than that of the pooled "DEAE eluate" but having at least 3 times as high a specific activity as the ammonium-sulfate fraction were pooled separately.

TABLE I

PURIFICATION OF TRANSAMINASE FROM RAT-LIVER

Enzyme activities were determined by Assay I, using L-glutamate as substrate.

Fraction No.	Protein concn. (mg/ml)	Specific activity	Total units	Recovery (%)
I. KCl-ethanol extract*	11.8	0.21	1204	100
II. Acetone ppt. (30-50%)	45.8	0.34	1019	85
III. Heated at 75°	5.1	1.7	440	37
IV. (NH ₄) ₂ SO ₄ ppt. (45-75% satn.)	7.7	3.6	493	33
V. DEAE-cellulose eluate (pooled)	0.1	26.0	164	14

* The homogenate from which this fraction was prepared had a specific activity of approx. 0.08.

The course of purification of the enzyme is summarized in Table I. At every stage, activity was measured using both γ -HG and glutamate as amino group donors. The specific activity values listed in Table I, however, correspond to those obtained with L-glutamate as substrate. The apparent purification achieved is approximately 125-fold; the single tube of highest specific activity after DEAE-cellulose chromatography was purified about 150-fold over Fraction I. Direct tests showed, however, that the specific activity of Fraction I was approx. 2.0 to 2.5 times higher than centrifuged homogenates of rat-liver. As a result, an overall purification of at least 300-fold has been achieved.

Comparative activities of purified transaminase preparations toward L-glutamate and γ -HG

We previously had found³ that γ -HG served as substrate for the glutamate-aspartate transaminase highly purified from pig-heart extracts. In order to ascertain whether the enzyme purified from rat-liver extracts was one of the known transaminases or a unique transaminase for γ -HG, we determined the specific activities of the protein fractions obtained in various stages of purifying the enzyme both from rat-liver and pig-heart extracts using L-glutamate or γ -HG as substrate. Table II shows the results obtained. Of the two substrates, glutamate is by far the more active (column a) but a measurable reaction is clearly discernible with γ -HG (column b).

TABLE II

ACTIVITY OF TRANSAMINASE FRACTIONS WITH L-GLUTAMATE AND γ -HG

Enzyme activities were determined with the use of Assay I. The enzyme fractions for the rat-liver enzyme refer to those of Table I.

Fraction No.	Pig-heart enzyme		Ratio of specific activities (a/b)
	Specific activity		
	L-Glutamate (a)	γ -HG (b)	
Heated 70°, 20 min	2.47	0.14	17.6
Hydroxylapatite eluate	35.1	2.25	15.6
Acetone ppt. (0-53%)	61.6	4.4	14.0
	Rat-liver enzyme		
I	0.21	0.016	13.4
II	0.34	0.020	17.2
III	1.66	0.123	13.5
IV	3.6	0.266	13.5
V	26.0	2.28	11.4

In spite of this difference in reactivity, the ratios of specific activities for the two amino acids were found to be approximately constant. Similar data were obtained when γ -HG transaminase activity was measured by the colorimetric test for glyoxylate (Table III). Again the ratios of specific activities for the two substrates did not vary widely.

In addition, the tubes obtained in eluting the rat-liver enzyme with 0.038 M Tris-HCl buffer from the DEAE-cellulose column were uniformly checked for activity. Every tube in this elution series, when analyzed for transaminase activity with γ -HG and glutamate as substrates, showed a constant ratio of specific activity. The 0.01 M and 0.02 M Tris-HCl buffer eluates did not have any transaminase activity toward either substrate. Furthermore, after the cellulose column had been eluted with 0.038 M Tris-HCl buffer, extensive washing of the column with buffers of very high ionic strength failed to remove any separate forms of γ -HG or L-glutamate transaminase.

TABLE III

ACTIVITY OF TRANSAMINASE FRACTIONS WITH L-GLUTAMATE AND γ -HG

Enzyme activity with L-glutamate as substrate was determined by Assay I. With γ -HG as substrate, activity was measured by Assay II. The enzyme fractions for the rat-liver enzyme refer to those of Table I.

<i>Pig-heart enzyme</i>			
<i>Fraction No.</i>	<i>Specific activity</i>		<i>Ratio of specific activities (c/a)</i>
	<i>L-Glutamate (a)</i>	<i>γ-HG (c)</i>	
Heated 70°, 20 min	2.47	15.8	6.4
Hydroxylapatite eluate	35.1	224	6.4
Acetone ppt. (0-53%)	61.6	349	5.8
<i>Rat-liver enzyme</i>			
III	1.66	7.5	4.5
IV	3.6	16.5	4.6
V	26.0	137	5.2

Heat denaturation studies

We subjected the purified rat-liver enzyme (DEAE eluate) to heat treatment at 68°, at which temp. enzymic activity was slowly destroyed over a period of 2 to 3 h. For this purpose, 2-ml aliquots of the DEAE eluate were dispensed into each of 6 hard-glass ignition test tubes and heated in a constant-temp. water bath at 70°. The enzyme soln. in each of the tubes was stirred gently with a thermometer until the temp. reached 68°, where it remained constant. At regular time intervals, a tube was removed, immediately plunged into an ice bath, and the contents stirred until chilled to 5° or lower. Enzymic activity was measured after each period of heat treatment and the ratio of specific activities again calculated for the two amino acids. As can be seen in Table IV, the ratios again remained essentially constant.

TABLE IV

HEAT DENATURATION OF RAT-LIVER TRANSAMINASE

Enzyme activities were measured for both L-glutamate and γ -HG by Assay I. The enzyme preparation used was the pooled DEAE-cellulose eluate.

<i>Time heated 68° h</i>	<i>Specific activity</i>		<i>Ratio of specific activities (a/b)</i>
	<i>L-Glutamate (a)</i>	<i>γ-HG (b)</i>	
0	16.4	1.33	12.3
1	9.0	0.71	12.7
1.5	4.6	0.38	12.1
2	1.99	0.16	12.4
3	0.87	0.08	11.0
4	0.71	0.06	11.8

Inhibition studies

Both L-glutamate and L-aspartate acted as inhibitors of γ -HG transamination (Table V). At a given concn., aspartate was found to be a slightly stronger inhibitor than was glutamate. The stronger affinity of aspartate for the enzyme, as suggested by its inhibitory action, is consistent with that indicated by the K_m values determined for these two amino acids (see below).

TABLE V

INHIBITION OF γ -HG TRANSAMINATION BY L-GLUTAMATE AND L-ASPARTATE

The incubation mixtures contained in μ moles: 200, Tris-HCl buffer (pH 7.4); 5, EDTA (pH 7.4); 10, γ -HG; 15, α -ketoglutarate in Expt. A or oxaloacetate in Expt. B; 50 μ g of pyridoxal 5'-phosphate; 1.1 mg of rat-liver protein Fraction C (source of KHG-cleavage enzyme); and 50 μ g of either rat-liver transaminase (Fraction No. V, Table I) or of 300-fold purified pig-heart glutamate-aspartate transaminase. The final vol. was 2 ml. L-Glutamate and L-aspartate were added in the amounts indicated. The mixtures, lacking γ -HG and amino group acceptor, were subjected to a prior incubation at 37° for 20 min. γ -HG and appropriate keto acids were then added and the tubes were incubated for 50 min. at 37°. The reaction was stopped by adding 0.7 ml of 12% metaphosphoric acid, the precipitated protein removed by centrifuging, and aliquots of the supernatant fluids were assayed for glyoxylic acid. Although high levels of oxaloacetate interfere slightly in the colorimetric determination, this was compensated for by appropriate controls.

Expt.	Compound added	Amount added (μ moles/2 ml)	Inhibition (%)
A	L-Aspartate	5	29
		10	43
		25	66
		50	84
		100	94
		200	100
B	L-Glutamate	10	24
		25	45
		50	68
		100	78
		200	100

Several investigators^{17,18} have reported that glutamate-aspartate transaminase activity is inhibited by substrate analogues, particularly aliphatic dicarboxylic acids, that are incapable of transaminating. They showed that certain of these inhibitory compounds are competitive with all four substrates (glutamate, aspartate, α -ketoglutarate, and oxaloacetate) and derived the dissociation constants of the enzyme-inhibitor complexes¹⁸. We found that known glutamate-aspartate transaminase inhibitors like glutarate and maleate also strongly inhibited transamination of γ -HG in a competitive manner. Using Assay III, the dissociation constants of the enzyme inhibitor complexes (K_I 's) were determined with each of the two substrates, L-glutamate and γ -HG. In these experiments, the reaction was initiated by the final addition of 6.2 μ moles of oxaloacetate. Values were determined both by LINEWEAVER-BURK¹⁹ double reciprocal plots and by DIXON²⁰ plots for competitive inhibition. The values found for glutarate were 3.2 and 2.9 mM and for maleate 2.3 and 2.1 mM, in

each case the first value obtained with L-glutamate and the latter with γ -HG as substrate. *p*-Hydroxymercuribenzoate was also found to inhibit the transamination of both amino acids.

γ -HG as substrate for rat-liver transaminase isozymes

Two forms (isozymes) of glutamate-aspartate transaminase are present in rat-liver homogenates. When separated from one another by the use of zone electrophoresis or DEAE-cellulose chromatography, the two forms were found to possess distinctly different substrate affinities, pH-activity relationships, and sensitivity to denaturation by urea²¹. BOYD²² showed that one form of the enzyme is associated with the mitochondria and the other with the 100 000 \times g supernatant fraction. Partial purification of both isozymes has recently been reported by HOOK AND VESTLING²¹. The purified enzymes were found to retain the characteristic electrophoretic and kinetic differences shown by crude preparations.

Since the foregoing data established that γ -HG transamination is catalyzed by well-defined preparations of glutamate-aspartate transaminase, the question remained as to whether γ -HG served as substrate for both isozymes of the rat-liver enzyme. Examination of the transaminase preparation obtained by elution of DEAE-cellulose with 0.038 M Tris-HCl buffer showed that it corresponded to only the "supernatant" form of the enzyme. Evidently during some stage of our purification procedure, the "mitochondrial" form had been preferentially lost.

As can be seen in Table I, the greatest loss of transaminase units in the process of fractionating rat-liver extracts occurred in the heating process. Thinking that this might be the step where the "mitochondrial" form was destroyed, we devised a different heat treatment. Both forms of the enzyme were then obtained. The modified fractionation procedure was as follows. The protein precipitating between 30 to 50% acetone concn., as obtained in Step 2, after dissolving slowly with stirring in 60 ml of 0.1 M Tris-HCl buffer (pH 7.4), containing 0.001 M GSH, was dialyzed against 4 l of 0.05 M Tris-HCl buffer (pH 7.4), containing 0.001 M GSH. Seven-ml portions of the resulting soln. were heated in heavy-walled test tubes in an 80° H₂O bath. The enzyme soln. was stirred gently with a thermometer until the soln. temp. reached 65°. The tubes were then immediately plunged into an ice bath and the contents of the tubes chilled by slow stirring. After centrifuging, the ppt. of denatured protein was discarded and the pooled supernatant solutions were stored at 0°. Fifteen ml of this preparation were dialyzed against 3 l of 0.01 M Tris-HCl buffer (pH 7.4), for 16 h with one change of buffer soln. after 8 h. The resulting soln. was subjected to DEAE-cellulose chromatography. Except for using 0.01 M Tris-HCl buffer (pH 7.4), uniformly in place of 0.005 M buffer, the cellulose column was prepared and the dialyzed protein soln. applied as previously described in Step 5. Subsequently, the cellulose was washed in turn with 300 ml of 0.01 M Tris-HCl buffer (pH 7.4); 300 ml of 0.02 M Tris-HCl buffer (pH 7.4); and 300 ml of 0.05 M Tris-HCl buffer (pH 7.4). Two preparations of glutamate-aspartate transaminase activity were obtained. Prep. I was removed by washing with 0.01 M Tris-HCl buffer, whereas Prep. II was eluted with 0.05 M Tris-HCl buffer. On the basis of reported criteria for the two rat-liver glutamate-aspartate transaminase isozymes^{21,22}, the first preparation corresponded to the "mitochondrial" (cationic) form and the second to the "supernatant" (anionic) form.

Each of the two isozymes was found to catalyze transamination of γ -HG with either α -ketoglutarate or oxaloacetate, yielding KHG (determined by Assay II) and the corresponding amino acid (identified by paper chromatography). The two preparations were next assayed for transaminase activity with either γ -HG or L-glutamate as amino group donor. The results are shown in Table VI. The first isozyme (cationic form), having about twice the specific activity of the anionic form, constituted about 60 to 65% of the total enzyme units applied to the column of DEAE-cellulose.

TABLE VI
ACTIVITY OF RAT-LIVER TRANSAMINASE ISOZYMES WITH L-GLUTAMATE
AND γ -HG

Enzyme activities were determined by Assay I

Preparation	Protein concn. (mg/ml)	Total units*	Recovery (%)	Specific activity		Ratio of specific activities (a/b)
				L-Glutamate (a)	γ -HG (b)	
Dialyzed fraction III Table I						
65° Heat-treated	22	136	100	0.48	0.03	16.0
Mitochondrial (cationic) form	0.57	91	66	3.20	0.20	16.0
Supernatant (anionic) form	0.53	57	41	1.35	0.10	13.5
"Cationic" GAT** (200-fold purified)	1.4	—	—	13.6	0.82	16.5
"Anionic" GAT** (300-fold purified)	0.11	—	—	26.0	2.20	12.0

* Calc. for L-glutamate as substrate.

** GAT = glutamate-aspartate transaminase.

The ratios of specific activities with γ -HG and glutamate as substrates are also listed for purified preparations of the individual rat-liver transaminase isozymes, each isozyme being completely free of the other. The 300 to 400-fold purified anionic form is the preparation obtained by the procedure summarized in Table I. The 200-fold purified cationic form was prepared by R. H. HOOK AND C. S. VESTLING²¹ of the University of Ill., Urbana, and was generously made available to us by them. The ratios of specific activity of both forms are again seen to be very nearly the same, although the values for different preparations of the same isozyme are in better agreement than are the values for the anionic and cationic forms. We did not attempt to determine whether or not the differences noted in this respect were significant.

The order of elution of the two isozymes of rat-liver glutamate-aspartate transaminase from DEAE-cellulose, as described above, was reversed on CM-cellulose. For this purpose, the individual cationic and anionic isozymes separated on DEAE-cellulose were adjusted to the same protein concn. (0.56 mg/ml) and 10–15 ml of each were dialyzed separately against 3 l of 0.005 M Tris-HCl buffer (pH 7.4), for 14 h. CM-cellulose, previously equilibrated with and stored in 0.05 M Tris-HCl buffer (pH 7.4), was transferred as a slurry to two separate glass columns to give cellulose columns 2 × 17 cm in dimensions. Dialyzed Prep. I (cationic form, 10 mg) was added to one column, and the same quantity of Prep. II (anionic form) to the other. After

the protein solutions soaked into the cellulose beds, the columns were washed with 200 ml of 0.005 M Tris-HCl buffer (pH 7.4). The flow rate was kept uniform in both cases (30–35 ml/hr). The transaminase present in Prep. I was adsorbed by CM-cellulose, whereas the form of enzyme in Prep. II, previously adsorbed by DEAE-cellulose, was easily washed from CM-cellulose with 0.005 M Tris-HCl buffer (pH 7.4), as a sharp protein peak. The other isozyme, however, was not eluted from the column of CM-cellulose until 300 ml of 0.05 M Tris-HCl buffer (pH 7.4), were used.

Both preparations obtained after chromatography on CM-cellulose were examined for glutamate-aspartate transaminase activity. Using Assay I, L-glutamate and γ -HG again were active with both forms of the transaminase and in the same ratio as found previously.

pH Dependence of enzyme activity

Boyd²² reported that the two isozymes of glutamate-aspartate transaminase in rat-liver homogenates were quite different in their pH-activity dependencies. This was also found to be true for partially-purified forms of the enzyme²¹. We found that when either γ -HG or L-aspartate was used as substrate with our purified preparations of the two forms of the transaminase, the isozymes again differed significantly in their pH-activity relationships (Fig. 1). However, the pH-activity curve obtained with a given isozyme (either anionic or cationic) and either substrate was essentially the same.

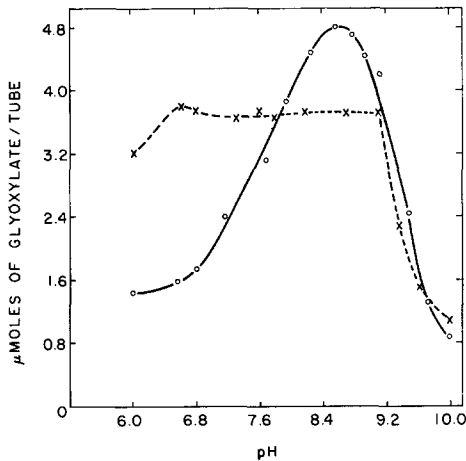


Fig. 1. Activity of the two forms of rat-liver glutamate-aspartate transaminase with γ -HG as substrate, as a function of pH. Transaminase added was 50 μ g of either the anionic (○—○) (pooled DEAE eluate) or cationic form (×—×) (unretarded 0.01 M eluate). Enzyme activities were determined by Assay II. The pH of the supernatant fluids was measured with the glass electrode (points plotted) after the first incubation period. Weak acid or alkali was then added, as required, to adjust the pH to 8.4 prior to the second incubation period. Buffers used to cover the range indicated were: potassium phosphate (pH 6.0 to 7.8); Tris-HCl (pH 7.8 to 9.2); carbonate-bicarbonate (pH 8.9 to 10.0).

Comparison of K_m and V_{max} values for glutamate and γ -HG

The apparent Michaelis constants and relative max. velocities with either L-glutamate or *threo*-L- γ -HG as amino group donors were determined. K_m values were

obtained from LINEWEAVER-BURK¹⁹ plots. The experiments were carried out with purified rat-liver transaminase (Fraction V, Table I) in 0.1 M (final concn.) Tris-HCl buffer (pH 7.4) at 25°. Reaction rates were measured by the use of Assay III, the initial concn. of oxaloacetate being 2 mM in every determination. Under these conditions, L-glutamate was found to have a K_m value of $2.2 \cdot 10^{-2}$ M as compared to a value of $3.0 \cdot 10^{-1}$ M for *threo*-L- γ -HG. Under the same conditions, the V_{max} (defined as μ moles of oxaloacetate consumed/min/mg protein) for L-glutamate was 136 and that for *threo*-L- γ -HG was 35. Using a different assay (Assay IV) with an initial concn. of 6.66 mM α -ketoglutarate, the K_m value for L-aspartate was $5.7 \cdot 10^{-3}$ M.

The K_m values for the keto acids have been reported to be much lower than those for the amino acids^{18,22}. We have found that oxaloacetate under our assay conditions (Assay III) has a K_m value of $1.5 \cdot 10^{-4}$ M, the initial L-glutamate concn. being 0.15 M.

Substrate specificity

Among the various α -keto acids tested with purified transaminase obtained from either rat-liver or pig-heart extracts, only α -ketoglutarate and oxaloacetate readily acted as amino group acceptors with γ -HG. Pyruvate, α -ketobutyrate, and β -phenylpyruvate failed to show any significant amount of transamination. The activity of all these compounds was compared under conditions identical to those described in the legend of Table V, with L-glutamate or L-aspartate being, of course, omitted.

Although *threo*-DL- γ -HG was used routinely in these studies, both isozymes of the rat-liver transaminase as well as pig-heart glutamate-aspartate transaminase are apparently specific for only the L-isomer since the initial rate of transamination was found to be twice as great with the *threo*-L-isomer as with an equimolecular concn. of the *threo*-DL-mixture (Table VII). The same was true of the *erythro*-racemate, where again only the L-isomer was found to be enzymically active. Direct comparison of the rates of transamination with the L-*erythro* and L-*threo* isomers (Table VII) showed that the former was about 1.8 to 1.9 times as effective as substrate as the *threo*-isomer at the concn. tested.

TABLE VII

OPTICAL ISOMERS OF γ -HG AS SUBSTRATES FOR PURIFIED TRANSAMINASE

Purified rat-liver transaminase (Fraction V, Table I) was used in all cases. Reaction rates were measured by the use of Assay I. Essentially identical results were obtained when the enzyme preparation indicated was replaced by either the cationic form of the transaminase prepared from rat-liver extracts (200-fold purified) or by 300-fold purified pig-heart glutamate-aspartate transaminase.

Isomer	Concn. (μ moles/3 ml)	DPNH formed (μ moles/min/mg protein)
DL- <i>Threo</i> - γ -HG	28	0.24
L- <i>Threo</i> - γ -HG	14	0.24
L- <i>Threo</i> - γ -HG	28	0.48
DL- <i>Erythro</i> - γ -HG	28	0.47
L- <i>Erythro</i> - γ -HG	14	0.46
L- <i>Erythro</i> - γ -HG	28	0.93

Several analogs of glutamic acid were tested in two ways as possible amino group donors in the transamination reaction. First, α -ketoglutarate served as the acceptor and the glutamic acid formed was detected with the aid of paper chromatographic methods. The components present in the incubation mixtures were resolved in a variety of different solvents known to separate glutamic acid and the added amino acid substrate. A strong ninhydrin positive spot, identical in R_F value with known glutamate, was obtained only in complete incubation mixtures containing either γ -methylene-DL-glutamic acid or γ -hydroxy- γ -methyl-DL-glutamic acid. A faint but definite spot of glutamic acid was obtained with *erythro*- β -hydroxy-DL-glutamic acid whereas, β,γ -dihydroxyglutamic acid did not appear to react at all. The same results were obtained using purified rat-liver transaminase.

Second, the rates of transamination of these amino acids with oxaloacetate as amino group acceptor were determined directly (Table VIII). At the concn. tested, the rates of transamination measured with all three γ -substituted forms of glutamic acid were not widely different. β -Substituted analogs failed to show any significant rate of reaction under these conditions.

TABLE VIII

ANALOGS OF GLUTAMIC ACID AS SUBSTRATES FOR PURIFIED TRANSAMINASE

The enzyme used was 300-fold purified pig-heart glutamate-aspartate transaminase. Reaction rates were measured by the use of Assay I. In each case, 100 μ moles of amino acid were added in a final reaction vol. of 3 ml.

Amino acid	DPNH formed (μ moles/min/mg protein)
DL-Threo- γ -HG	0.79
DL- γ -Methyleneglutamate	0.71
DL- γ -Hydroxy- γ -methylglutamate	0.73
DL-Erythro- β -hydroxyglutamate	0
DL- β,γ -Dihydroxyglutamate	0

DISCUSSION

The evidence presented in this paper supports the thesis that γ -HG transaminase and glutamate-aspartate transaminase are very likely identical enzymes. This conclusion is based on several independent criteria. First, the ratios of the two activities in different assay systems are not significantly different at various stages in purifying the enzyme. This is true regardless of whether the enzyme is extensively purified from rat-liver or from pig-heart extracts. Furthermore, the two activities have the same elution profile from columns of DEAE-cellulose and the ratio of activities remains essentially constant from one fraction to the next. Second, the activity of the purified rat-liver enzyme declines at exactly the same rate for both substrates under conditions whereby the enzyme is slowly heat-denatured. Third, L-aspartate and L-glutamate strongly inhibit γ -HG transaminase activity. In addition, known inhibitors of glutamate-aspartate transaminase block the transamination of both L-glutamate and γ -HG. Of the inhibitors tested, glutarate and maleate are competitive with either substrate and virtually identical K_I values are obtained for any given inhibitory sub-

stance when tested with γ -HG or glutamate. These findings imply that the same catalytic center is involved in the transamination of the two substrates. We have also shown that γ -HG serves as substrate for both isozymes [mitochondrial (cationic) and cytoplasmic (anionic)] of rat-liver glutamate-aspartate transaminase and that the pH-dependency curve obtained with either L-aspartate or γ -HG is identical when tested with a given isozyme. Finally, examination of the mitochondrial and supernatant fractions of rat-liver homogenates for transaminase activity showed that the ratio of the first form to the second is approx. 7:3 when calculated for either γ -HG or L-glutamate as substrate (Assay I).

γ -HG has a lower affinity for the transaminase than does L-glutamate. This is apparent when the rates of transamination are compared at low concentrations of either substrate (e.g. 0.017 M, calculated for the L-isomer), as seen in Tables II and III. The ratios of the two activities at this concn. indicate that L-glutamate is 13 to 16 times more active. When the relative max. velocities are compared at infinite concentrations of the two substrates, however, we found that L-glutamate is only about 3.8 times more reactive than γ -HG.

The present findings also establish that both the *erythro*- and *threo*-L-isomers of γ -HG readily undergo enzymic transamination, although the former isomer is a somewhat more effective substrate. This finding correlates with the report of other investigators²³ that L-hydroxyproline is converted by mammals to *erythro*-L- γ -HG. Since the corresponding D-isomers are not utilized by the transaminase, the enzyme apparently is specific with respect to the D- and L-forms of the α -amino group but not on the stereochemical relationship of the α -amino to the γ -hydroxy group. The latter factor, however, may be important in determining either the affinity of the substrate for the enzyme or its relative max. velocity or both.

Although crude preparations of the rat-liver enzyme were stimulated slightly by added pyridoxal 5'-phosphate, no effect was demonstrable with purified fractions. Apparently, the transaminase is present in these fractions in combination with its cofactor. Since the presence of pyridoxal 5'-phosphate in glutamate-aspartate transaminase and its role in enzymic transamination are well established^{17,24-27}, no attempt was made in the present study to resolve the holoenzyme. We also were not able to demonstrate any requirement for added metal ions at various stages of purifying the rat-liver enzyme, as originally claimed by GOLDSTONE AND ADAMS⁵.

Comparison of the properties of our 300 to 400-fold purified rat-liver enzyme with those reported by BOYD²² and HOOK AND VESTLING²¹ for the two isozymes of rat-liver glutamate-aspartate transaminase indicates that our preparation is the "anionic" form. In the course of studying the properties and identity of this enzyme, therefore, a high degree of purification of this specific isozyme of rat-liver glutamate-aspartate transaminase has been accomplished.

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