METABOLIC PATHWAYS OF HOMOSERINE IN THE MAMMAL*

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The importance of homoserine in the metabolism of amino acids in a variety of living forms is now well established. This amino acid was found to be the common precursor of methionine and threonine in Neurospora crassa (1). Enzymes involved in the conversion of aspartic acid to threonine via homoserine have been studied in Escherichia coli (2-5) and in bakers’ yeast (6-8). The presence of homoserine (9) and its derivatives (10, 11) has been demonstrated in various plants. In intact rats at least a part of the reported de novo synthesis of methionine seems to utilize the carbon skeleton of homoserine lactone (12). The metabolic formation of homoserine in mammals from methionine has been suggested from the study of the cleavage of cystathionine (13), and evidence for such conversion has been reported from this laboratory (14). In continuation of the studies on the metabolic fate of the main carbon chain of methionine, the investigation reported in this paper was undertaken in order to clarify the course of homoserine metabolism.

Using D,L-homoserine-2-C14, we have been able to isolate and identify α-ketobutyric, α-hydroxybutyric, α-amino-n-butyric, and propionic acids by chromatographic means (cf. Figs. 1 and 2, and Table I).

That homoserine is converted into α-ketobutyric acid in a rat liver system has been reported previously by Carroll, Stacy, and du Vigneaud (13), who isolated and identified the compound. The present study confirms this finding and also demonstrates that α-ketobutyric acid is amminated to α-aminobutyric acid, probably by transamination, and that glutamic acid is probably the principal, if not the sole, amino donor in the transamination. The effects of additions of glutamic acid and α-ketoglutaric acid to dialyzed and non-dialyzed rat liver enzyme systems are presented in Table I. This and our previous finding that homoserine is metabolically formed from methionine explain the observation of Dent (15) of an increased ex-

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cretion in the urine of α-aminobutyric acid in a human subject following the oral administration of methionine. Such an explanation has been suggested previously by Carroll et al. (13).

No ammonia was formed when O-acetyl-DL-homoserine was incubated with a rat liver extract which converts free homoserine to α-ketobutyric acid and releases ammonia into the reaction mixture. This is analogous to the reported inactivity of serine and threonine dehydrogenases upon the derivatives of these β-hydroxyamino acids in which the β-hydroxyl hydrogen was substituted (16). It should be pointed out that the substitution of the hydroxyl hydrogen of homoserine with an acetyl group increases the rate of oxidative deamination of homoserine by an ophio-L-amino acid oxidase. This fact indicates that the acetyl substitution interferes with the conversion of homoserine to α-ketobutyric acid and suggests that dehydration is involved in the deamination mechanism.

The formation of α-hydroxybutyric acid can be explained by the action of certain dehydrogenases and the tendency toward equilibrium, in the incubation mixture, between α-ketobutyric acid and this compound. Thus,

<table>
<thead>
<tr>
<th>Product</th>
<th>Non-dialyzed</th>
<th>Dialyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control + α-ketobutyric acid</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>α-Ketobutyric acid</td>
<td>1.7</td>
<td>16.6</td>
</tr>
<tr>
<td>α-Hydroxy-α-butyric acid</td>
<td>11.5</td>
<td>18.2</td>
</tr>
<tr>
<td>α-Amino-α-butyric acid</td>
<td>40.7</td>
<td>20.8</td>
</tr>
<tr>
<td>Homoserine</td>
<td>14.3</td>
<td>38.3</td>
</tr>
</tbody>
</table>

1 Unpublished observations of the authors with L-amino acid oxidase of moccasin venom (Agkistrodon piscivorus). DL-Homoserine is a very poor substrate for this enzyme. Substitution of the hydroxyl hydrogen by an acetyl group raised the $Q_{O_2}$ value at 37°C approximately 10-fold. ($Q_{O_2}$ values for homoserine, O-acetylhomoserine, and leucine, determined on DL compounds and calculated for the L isomers of the enantiomorphs, are 13, 140, and 444, respectively.)
the L-amino acid oxidase of liver isolated by Blanchard et al. (17) has a higher activity on L-α-hydroxy acids than on L-amino acids. Also, lactic dehydrogenase of muscle has been reported to be active in reducing α-ketobutyric acid (18). We have observed that incubation of radioactive α-ketobutyric acid\(^2\) with the rat liver extracts used in the present investigation yielded radioactive α-hydroxybutyric acid.

On the basis of the above findings we propose the metabolic pathway for homoserine, in the rat liver system studied, presented in Diagram 1. That α-ketobutyric acid is the first product in the pathway of metabolism was demonstrated by an experiment in which radioactive homoserine was incubated with an enzyme fraction precipitated between 33 to 66 per cent ammonium sulfate saturation. This fraction virtually yielded only radioactive α-ketobutyrate and no other radioactive products.

No significant amount of radioactivity was incorporated into homocysteine, methionine, threonine, or alanine. These compounds, if formed, should easily have been detected by our chromatographic procedure. Participation of homoserine in an oxidative deamination or a transamination reaction could not be demonstrated, because we could isolate neither radioactive α-keto-γ-hydroxybutyric nor α-keto butyrolactone from the reaction mixtures. Since no labeling of pyruvate, β-hydroxypropionate, or alanine occurred, metabolism of homoserine through α-keto-γ-hydroxybutyric acid does not seem to proceed to any significant extent under the conditions of the experiments. If α-keto-γ-hydroxybutyric acid were formed, it would be expected to give β-hydroxypropionic acid by decarboxylation, or to form

\[
\text{HO-CH} = \text{CH} = \text{CHNH} = \text{COOH} \\
\downarrow \\
\text{CH}_2 = \text{CH} = \text{CHOH-} \text{COOH} \\
\text{α-Hydroxybutyric acid} \\
\downarrow \\
\text{CH}_2 = \text{CH} = \text{COOH} \\
\text{Propionic acid} \\
\downarrow \\
\text{Tricarboxylic acid cycle}
\]

**Diagram 1.** Sequence of reactions in the catabolism of homoserine-2-C\(^4\) illustrating the expected appearance of radioactive carbon-containing intermediates.

\(^2\) Isolated by silica gel chromatography from incubation mixtures containing labeled homoserine.
formaldehyde and pyruvic acid, if the reverse of the reaction described by Hift and Mahler (19) is operative. Formation of radioactive pyruvic acid would, in turn, lead to the formation of radioactive alanine. This negative finding is in accord with the observation of Patwardhan (20).

EXPERIMENTAL

Preparation of Rat Liver Extract—Livers of adult, Long-Evans, male rats (300 to 350 gm.), fasted for 18 hours, were used in the experiments. The liver was homogenized in a Waring blender at half speed for 1 minute with 2 volumes of cold 0.05 M phosphate buffer, pH 7.4. The homogenate was centrifuged at 300 × g for 15 minutes to remove the coarse debris, and the supernatant liquid was centrifuged at 4 × 10⁴ × g for 30 minutes in the cold. The supernatant liquid was used, with or without dialysis against two portions of 100 volumes of 0.01 M phosphate buffer, pH 7.4, in the cold for approximately 18 hours. Similar results were obtained by using the crude homogenate without the higher speed centrifugation, but centrifugation of the homogenate is desirable because it makes it easier to remove proteins after the incubation.

Incubations in Vitro—0.1 ml. of the DL-homoserine-2-C¹⁴ solution, containing 1.7 μmoles of homoserine (3.7 × 10⁶ c.p.m. or 0.6 μC. of radioactivity) was incubated anaerobically with 5 ml. of the liver extract at 37° for 1 hour. After the incubation period the incubation vessels were immersed in a boiling water bath for 45 to 50 seconds and quickly chilled in running cold water. The coagulated proteins were centrifuged and washed twice with 2 ml. of water. The combined supernatant fluids were brought to pH 8 and dried in vacuo over calcium chloride and phosphorus pentoxide at room temperature. The dried material was redissolved in 0.5 ml. of water and acidified to pH 2 with 6 N HCl; 10 ml. of absolute ethanol were added to this solution, and the precipitate was removed by centrifugation. This procedure was repeated once, and the combined supernatant solutions were divided into two equal fractions and were dried in vacuo after adjusting the pH to 8. One of the processed incubation mixtures was assayed for the labeled nitrogenous compounds by chromatographing it on an acid Dowex 50 column (Fig. 2), and the other was chromatographed on a silica gel column to isolate non-nitrogenous carboxylic acids (Fig. 1).

Chromatography on Dowex 50-X8 Column—The procedure of Stein and Moore (21) was employed in this work. A resin bed of 0.9 × 110 cm. was used and the chromatograms were developed with 130 ml. of water, 200 ml. of 1.5 N HCl, and 250 ml. of 2.5 N HCl in the order mentioned. The flow rate was adjusted to 4 ml. per hour, and fractions were collected in 1 ml. volumes in polyethylene planchets and dried with infra red lamps.

Chromatography on Silica Gel Column—Preparation of the silica gel column and construction of the solvent dispenser were done according to the
procedure of Kinnory, Takeda, and Greenberg (22). The benzene-ether and the chloroform-tert-amyl alcohol solvent systems described by these authors were used. The effluent was collected with a Technicon fraction collector in 80 drop fractions. This number of drops, under the particular set of experimental conditions, with the benzene-ether solvent system, gave the initial fraction volumes of 3 ml. The size of the fractions decreases as the concentration of ether in the solvent system increases during the course of the development of the chromatogram.

Standard chromatograms had been constructed previously with the two solvent systems by chromatographing authentic samples of compounds related to the present investigation singly and in combinations. It was learned that there is a striking parallelism between the order of elution of carboxylic acids in the benzene-ether mixture and their partition coefficients between ether and water. Collander gives the partition coefficients of about 300 organic compounds (23) in an ether-water system.

Approximately 5 μmoles of each of the known non-radioactive acids under study were added to the sample to be analyzed before chromatography in order to serve as carriers.
Further Identification of Intermediates—The radioactive intermediates of homoserine metabolism, isolated by column chromatography, were further identified as described below.

α-Ketobutyric Acid—Aliquots of the isolated α-ketobutyric acid were chromatographed on paper with an n-butanol-propionic acid mixture (95 parts of water-saturated n-butanol and 5 parts of propionic acid), together with aliquots of a standard preparation of the acid. The keto acid spot was revealed under ultraviolet light after spraying the chromatogram with 0.1 per cent semicarbazide hydrochloride and 0.15 per cent sodium acetate and heating at 110°. The radioactivity was located in the area of the ultraviolet-absorbing spot.

The 2,4-dinitrophenylhydrazone was prepared from the radioactive α-ketobutyric acid isolated from the incubation mixture and from an authentic preparation. Both preparations were chromatographed together on paper with the tert-amyl alcohol-ethanol-water mixture (5:1:4). Radioactivity was detected only on the yellow spots of the 2,4-dinitrophenylhydrazone.

Propionic and α-Hydroxybutyric Acids—These compounds were isolated by silica gel chromatography and were rechromatographed on paper with n-butanol saturated with 1.5 N ammonium hydroxide according to the technique of Reid and Lederer (24). Spots of the acids were detected by spraying the paper with an indicator solution, and the radioactivity was located on the chromatogram by counting sections of the paper chromatograms with a thin mica window counter. Precise correspondence of the radioactivity and the colored spots was obtained.

α-Aminobutyric Acid—α-Aminobutyric acid was isolated by Dowex 50 column chromatography and was rechromatographed on paper with three different solvents. In all cases the radioactivity coincided with the ninhydrin-positive spot of the α-aminobutyric acid. The three solvents used were phenol-water (80:20), n-butanol-acetic acid-water (3:2:1), and colidine-isoamyl alcohol-water (35:35:25).

Isolation of Alanine from Homoserine Incubations—In order to prove that alanine was not labeled, it was isolated from the homoserine incubations. As seen in Fig. 2, homoserine is eluted from the Dowex 50 column as an unusually wide peak, which overlaps with that of alanine.³ By rechromatographing the combined fractions, which contained the alanine on a 0.9 × 55 cm. column of ammonium Dowex 50 with 0.2 M ammonium formate buffer, pH 3.1, containing 40 per cent ethanol, alanine was eluted at 110 to 130 ml. of the effluent, while homoserine was eluted at 75 to 88 ml.;⁴ the alanine thus isolated had no detectable amount of radioactivity.

³ Alanine is eluted from the Dowex 50 column at about the 265th fraction (see Fig. 2).
⁴ A larger portion of homoserine was retained by the column in the form of α-amino-γ-butyrolactone.
Synthesis of DL-Homoserine-2-C¹⁴

Synthesis—The lactone of homoserine-2-C¹⁴ was prepared by modifying the procedure of Painter (25).

To a solution prepared by warming 0.5 gm. of ethyl acetamidomalonate-2-C¹⁴ (3 μc. per mg.) in 5 ml. of absolute ethanol containing 0.056 gm. of sodium, 0.507 gm. of β-phenoxyethyl bromide and 12 mg. of dry potassium iodide were added. The mixture was refluxed for 24 hours and cooled, and an additional 0.250 gm. of phenoxyethyl bromide in 2 ml. of absolute ethanol containing 26 mg. of sodium was added. Refluxing was continued for 18 hours; the solution was cooled, neutralized with acetic acid, and taken to dryness in vacuo. The residue was extracted repeatedly with dry ether. The ether was removed from the filtered extracts and the residue was saponified by refluxing with 2.5 ml. of 10 per cent NaOH for 2.5 hours. The resulting solution was distilled to dryness in vacuo, water was added, and the distillation was repeated. The residue was refluxed for 24 hours with 7 ml. of 6 N HCl and taken to dryness in vacuo three times, as above. The final residue was dissolved in water, treated with Norit, filtered, and concentrated to 3 ml. Pyridine was added to precipitate 280 mg. of O-phenylhomoserine-2-C¹⁴ (62 per cent). The mother liquors contained glycine-2-C¹⁴ and additional O-phenylhomoserine-2-C¹⁴ as shown by paper chromatograms.

Fig. 2. Dowex 50-X8 column chromatogram of the deproteinized extract obtained from the incubation of DL-homoserine-2-C¹⁴ with a dialyzed rat liver enzyme system, without the addition of glutamic acid (cf. Table I, fourth column). The peaks of radioactivity eluted by water in the early fractions are due to carboxylic acids, most of which are lost by evaporation in drying the fractions.
The product was refluxed for 19 hours with 2.5 ml. of 48 per cent hydrobromic acid and evaporated to dryness three times as above. The residue was dissolved in water, treated with Norit, filtered, concentrated, and dried \textit{in vacuo} over phosphorous pentoxide. The homoserine-2-C\textsuperscript{14} lactone hydrobromide contained approximately 0.6 per cent glycine-2-C\textsuperscript{14} and was purified finally as described below on a Dowex 50 exchange resin. The over-all yield of pure homoserine-2-C\textsuperscript{14} lactone, exclusive of the O-phenyl derivative remaining in the original mother liquors, was 38.2 per cent, based upon ethyl acetamidomalonate-2-C\textsuperscript{14}.

\textit{Purification—} A chromatographic procedure described previously (14) was applied to isolate \textit{dL}-homoserine-2-C\textsuperscript{14} from contaminating radioactive glycine. Approximately 150 mg. of the lactone of homoserine-2-C\textsuperscript{14} contaminated with glycine were chromatographed on a relatively short column (1.8 × 16 cm.) of ammonium Dowex 50, with 0.2 M ammonium formate buffer, pH 3.1, containing 40 per cent ethanol. Glycine was eluted from the column with some free homoserine in a discrete peak, and the lactone was eluted later, free from glycine. The portion of the chromatogram between the two peaks showed some radioactivity; this is due presumably to the equilibrium between the free acid and the lactone of homoserine in acid solution (26). The fractions containing both glycine and homoserine were combined and recycled through the Dowex 50 column to improve the yield of glycine-free homoserine lactone. The fractions containing glycine-free \textit{α}-amino-\textit{γ}-butyrolactone were combined and dried on a steam bath, and the ammonium formate was removed from the dried material by sublimation \textit{in vacuo} at 35°. The residue from the sublimation was dissolved in a small amount of water and its pH was adjusted to 7.6 with sodium hydroxide. This solution was heated in a sealed Pyrex tube, together with 10 ml. of 15 M ammonium hydroxide at 110° for 4 hours. The ammonia was removed by drying the hydrolysate \textit{in vacuo} over concentrated sulfuric acid.

Purity of the final solution of \textit{dL}-homoserine-2-C\textsuperscript{14} was tested by paper chromatography and radioautography.

\textit{Other Preparations—} \textit{O}-Acetyl-\textit{dL}-homoserine was prepared according to the method employed by Sakami and Toennies (27) for the synthesis of \textit{O}-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline. \textit{α}-Ketobutyric and \textit{β}-hydroxypropionic acids were kindly prepared by Mr.

\footnote{A paper chromatogram of an aliquot of the material obtained after the sublimation of the buffer salt, developed with \textit{n}-butanol-acetic acid-water (3:2:1) as solvent, revealed four spots (\textit{R}_p values, 0.20, 0.37, 0.41, and 0.52) of comparable radioactivities. Of these spots, two (\textit{R}_p 0.20 and 0.52) were ninhydrin-positive. \textit{R}_p values of homoserine and its lactone on the same chromatogram were 0.20 and 0.35, respectively. Upon hydrolysis with concentrated ammonium hydroxide only the spot of homoserine was detected on a paper chromatogram, and no other area of the chromatogram was radioactive.}
D. C. Morrison of this laboratory. α-Hydroxybutyric acid was prepared in our laboratory by the action of potassium cyanide on the bisulfite addition product of propionaldehyde, followed by hydrolysis of the resulting cyanohydrin with hydrochloric acid.

SUMMARY

To establish the metabolic pattern of the main carbon chain of methionine, the enzymatically formed reaction products of dl-homoserine-2-C14 were determined upon incubation of the amino acid with rat liver homogenates. The radioactive products isolated and identified were α-ketobutyric, α-hydroxy-n-butyric, α-amino-n-butyric, and propionic acids. It is concluded that the primary initiating reaction in the catabolism of homoserine is the deamination to α-ketobutyric acid.

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