Aminomalonic acid, an amino acid which has not yet been found in nature, has nevertheless been of interest to biochemists because (a) its occurrence in the conversion of serine to glycine (consideration of this symmetrical compound as a possible intermediate led to the Ogston hypothesis (1)); (b) enzyme activity capable of decarboxylating aminomalonic acid to glycine, as indicated by the enzyme decarboxylates this amino acid in a nonspecific manner; this result is in contrast to that previously observed with aspartate β-decarboxylase which acts on a specific carboxyl group of aminomalonic acid. In contrast to cytoplasmic serine hydroxymethylase, mitochondrial serine hydroxymethylase does not catalyze the aldol cleavage of allothreonine or the decarboxylation of aminomalonate; this indicates that there is a significant difference between the active sites of these enzymes.

SUMMARY

Studies on the aminomalonate decarboxylase of rat liver indicate that this activity is a property of cytoplasmic serine hydroxymethylase. Thus, throughout purification of the enzyme, aminomalonate decarboxylase, serine hydroxymethylase and allothreonine aldolase exhibited the same relative activities. Competition between the several substrates was observed and resolution of the enzyme by treatment with D-alanine led to loss of all three activities. Cleavage of allothreonine by the enzyme in tritiated water gave S-glycine-2-t (in accord with earlier data which indicate retention of configuration during conversion of L-serine to glycine), but decarboxylation of aminomalonate by the enzyme in tritiated water gave both S- and R-glycine-2-t. Studies with specifically carboxyl-labeled [14C]aminomalonate confirmed the conclusion that the enzyme decarboxylates this amino acid in a nonspecific manner; this result is in contrast to that previously observed with aspartate β-decarboxylase which acts on a specific carboxyl group of aminomalonate. In contrast to cytoplasmic serine hydroxymethylase, mitochondrial serine hydroxymethylase does not catalyze the aldol cleavage of allothreonine or the decarboxylation of aminomalonate; this indicates that there is a significant difference between the active sites of these enzymes.

IDENTITY WITH CYTOPLASMIC SERINE HYDROXYMETHYLASE AND ALLOTHREONINE ALDOLASE*

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Materials

Unlabeled and [2-14C]- and [3-14C]aminomalonic acid were synthesized as described previously (2, 3, 6). L-Threonine and DL-allothreonine were obtained from Calbiochem. Pyridoxal 5'-phosphate, DPNH, TPN, and tetrahydrofolic acid were obtained from Sigma Chemical Co. Yeast alcohol dehydrogenase was obtained from Worthington Biochemical Corp. The analogs of pyridoxal 5'-phosphate were obtained as noted previously (7). L-Allothreonine was prepared by the method of Elliott (8) as described by Greenstein and Winitz (9). The N-oxide of pyridoxal 5'-phosphate was kindly donated by Dr. S. Fukui, Kyoto, Japan. We are indebted to Dr. Daniel Wellner of this department for a generous supply of hog kidney L-amino acid oxidase.

Methods

Determination of Aminomalonic Decarboxylase Activity

The assay mixtures (final volume, 0.1 ml) contained potassium phosphate buffer (6 μmoles; pH 6.0), [2-14C]aminomalonate (2.5 μmoles; 40,000 cpm per μmole), pyridoxal 5'-phosphate
(1 umole), dithiothreitol (10 nmoles), and enzyme. After incubation for 30 min at 37°, aliquots (50 μl) were withdrawn and applied to small columns of Dowex 1 X-8 (acetate form) prepared in Pasteur pipettes. The radioactive glycine formed in the reaction was eluted with 1 ml of water and the eluate was mixed with 10 ml of liquid scintillation medium (10); radioactivity was determined in a liquid scintillation counter. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmole of glycine per min at 37°.

**Determination of L-Serine Hydroxymethylase Activity**

**Assay 1**—This activity was determined essentially as described by Schirch (11) in reaction mixtures containing 5,10-methylene tetrahydrofolate dehydrogenase (prepared from chicken liver (12)) and TPN. The formation of TPNH was determined from the increase in absorbance at 340 nm. The assay mixture (final volume, 1 ml) contained potassium phosphate buffer (100 μmoles; pH 7.5), dl-l-tetrahydrofolate (0.25 μmole), dithiothreitol (1 μmole), pyridoxal 5'-phosphate (10 μmoles), TPN (0.4 μmole), l-serine (5 μmoles), 5,10-methylene tetrahydrofolate dehydrogenase, and serine hydroxymethylase. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmole of TPNH per min at 37° under these conditions.

**Assay 2**—This activity was also determined by measuring the rate of disappearance of formaldehyde in the presence of glycine (13). The assay mixtures (final volume, 0.1 ml) contained in addition to the enzyme, potassium phosphate buffer (10 μmoles; pH 7.5), dl-l-tetrahydrofolate (0.1 μmole), pyridoxal 5'-phosphate (1 μmole), formaldehyde (0.5 μmole), dithiothreitol (50 nmoles), and glycine (5 μmoles). After incubation at 37° for 10 min the reaction was stopped by adding 0.1 ml of 10% trichloroacetic acid. Controls lacking enzyme were carried out. The remaining formaldehyde was determined by a modification of methods previously described for the determination of bisulfite (14, 15). Aliquots (0.1 ml) were made to 4.5 ml with water and then treated with 0.5 ml of a 1:1 (v/v) mixture of 0.01 NaHSO3 and 0.04% p-rosaniline HCl. The solutions were mixed and incubated at 37° for 45 min; the colors were then compared at 560 nm. Under these conditions, 1 μmole of formaldehyde gave an absorbance of 0.5. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the disappearance of 1 nmole of formaldehyde per min at 37° under these conditions. The results obtained by this assay procedure were, within experimental error, the same as those obtained by Assay 1. Assay 2 was more convenient in experiments requiring a large number of analyses.

**Determination of L-Allothreonine (and L-Threonine) Aldolase Activity**

The method employed is a modification of that of Malkin and Greenberg (16). The reaction mixture (final volume, 1 ml) contained potassium phosphate buffer (100 μmoles; pH 7.3), dl-allothreonine (10 μmoles), or L-threonine (40 μmoles), pyridoxal 5'-phosphate (10 nmoles), dithiothreitol (1 μmole), DPNH (150 nmoles), yeast alcohol dehydrogenase (35 units), and the enzyme. The assay mixture was equilibrated at 37° in a water jacketed cuvette compartment of a Cary model 15 spectrophotometer, and the reaction was initiated by adding the enzyme. The decrease in absorbance at 340 nm was recorded. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmole of acetaldehyde per min.

Protein was determined as described by Lowry et al. (17). Specific enzymatic activities are given in terms of units per mg of protein.

**RESULTS**

**Initial Studies on Purification of Rat Liver Aminomalonate Decarboxylase Activity**

Thanassi and Fruton (5) first observed the aminomalonate decarboxylase of rat liver and obtained about a 2-fold purification of this activity from rat liver acetone powder. In the present work we pursued the purification of aminomalonate decarboxylase activity from rat liver acetone powder and obtained, after chromatography on hydroxyapatite and DEAE-cellulose and gel filtration, preparations of relatively high specific activity, i.e. in the range of 10,000 units per mg. Since there seems to be no evidence indicating that aminomalonate is a metabolite, we considered the possibility that the purified enzyme preparations obtained might contain another pyridoxal 5'-phosphate enzymatic activity. The highly purified aminomalonate decarboxylase preparations were therefore tested for the following enzyme activities: l-serine dehydratase, l-allothreonine aldolase, l-threonine aldolase, l-threonine dehydratase, L-serine hydroxymethylase, l-cysteine desulfhydrase, cystathionase, l-aspartate β-decarboxylase, l-aspartate α-decarboxylase, l-cysteine sulfinate decarboxylase, kynureninase, sphingosine synthetase, alanine racemase, glutamate-aspartate transaminase, and glutamate-alanine transaminase. The preparation was also tested for ability to catalyze transamination between α-ketoglutарате and L-leucine, l-serine, L-threonine, and glycine, and between glyoxylate and aminomalonate, L-asparagine, L-glutamine, L-aspartate, and L-alanine. The only catalytic activities detected were serine hydroxymethylase, allothreonine aldolase, and threonine aldolase. In addition, when the various fractions obtained during purification were examined for serine hydroxymethylase and allothreonine aldolase activities, it was found that these activities had been purified to about the same extent as aminomalonate decarboxylase. A number of such studies were carried out on several highly purified aminomalonate decarboxylase preparations with similar results. We then decided to use a method of purification that is substantially the same as that described by Nakano et al. (18) for the isolation of cytoplasmic rat liver serine hydroxymethylase. In the present studies, the procedure of Nakano et al. (18) was modified in certain respects; thus, dithiothreitol was included in all of the buffers used. In our hands, dithiothreitol was found to yield a more stable enzyme preparation. In addition, a new step involving chromatography on Sephadex G-150 was included, facilitating the isolation of an apparently homogeneous enzyme as described below.

**Purification of Aminomalonate Decarboxylase of Rat Liver and Its Apparent Identity with Cytoplasmic L-Serine Hydroxymethylase and l-Allothreonine Aldolase**

Male Sprague-Dawley rats, weighing approximately 300 g, were fasted for 24 hours prior to death by decapitation and removal of the livers. All subsequent steps were carried out at 4°.

**Step 1**—The livers (450 g) were minced and homogenized in a Potter-Elvehjem homogenizer with 900 ml of 0.25 M sucrose containing 5 mM potassium phosphate buffer (pH 7.2) and 1 mM EDTA (sucrose-phosphate buffer); 2250 ml of sucrose-phosphate
buffer were added to this homogenate and the mixture was stirred for 15 min. The solution was then centrifuged at 15,000 \( \times g \) for 30 min. Assays for aminomalonate decarboxylase activity showed that more than 90% of the activity of the homogenate was present in the supernatant solution after centrifugation. (The pellet was used for the preparation of mitochondrial L-serine hydroxymethylase; see below.)

**Step 2**—The supernatant solution obtained in Step 1 was brought to 43% of ammonium sulfate saturation by adding solid ammonium sulfate; the suspension was then centrifuged at 15,000 \( \times g \) for 20 min. The precipitate was discarded and the supernatant solution was brought to 53% of ammonium sulfate saturation by adding solid ammonium sulfate. The precipitated protein was centrifuged, dissolved in 30 ml of 0.02 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 0.2 mM dithiothreitol and 10\(^{-5}\) M pyridoxal 5'-phosphate, and then this solution was thoroughly dialyzed against the same buffer. Of all the buffers used in the subsequent steps of purification contained 1 mM EDTA, 0.2 mM dithiothreitol and 10\(^{-5}\) M pyridoxal 5'-phosphate.

**Step 3**—The dialyzed solution obtained in Step 2 was diluted with 0.02 M potassium phosphate buffer to give a protein concentration of 20 mg per ml. To this solution were added, 0.2 volume of 0.5 M potassium phosphate buffer (pH 6.5) and solid L-serine (final concentration, 10 mM). The solution was then brought to 70°C and kept at this temperature for 3 min; it was then rapidly cooled and the denatured protein was removed by centrifugation.

**Step 4**—Solid ammonium sulfate was added to the supernatant solution obtained in Step 3 to obtain 53% of ammonium sulfate saturation. The precipitate which formed was dissolved in 30 ml of 0.05 M potassium phosphate buffer (pH 6.5), and this solution was dialyzed against 100 volumes of the same buffer.

**Step 5**—The dialyzed solution obtained in the preceding step was applied to the top of a DEAE-cellulose column (Whatman DE-52 microgranular, preswollen; 3.2 \( \times \) 10.8 cm), which had been equilibrated with 0.05 M potassium phosphate buffer (pH 6.5). The column was washed with the same buffer until protein no longer emerged. Further elution was then carried out with a linear gradient established between 1 liter each of 0.05 M and 0.25 M potassium phosphate buffer (pH 6.5). The fractions exhibiting high enzyme activity were pooled.

**Step 6**—The pooled enzyme solution obtained in the previous step was brought to 53% of ammonium sulfate saturation by adding solid ammonium sulfate. The precipitate which formed was collected by centrifugation and dissolved in 1 ml of 0.05 M potassium phosphate buffer (pH 6.5). This solution was applied to a Sephadex G-150 column (3 \( \times \) 55 cm), and elution was carried out with the same buffer. The fractions (2.2 ml each) exhibiting high enzyme activity (Numbers 34 to 40) were pooled (Table I, Fig. 1).

**Step 7**—Sufficient L-serine was added to the pooled enzyme solution obtained in Step 6 to give a final concentration of 1 mM. This solution was applied to a DEAE-cellulose column (2.5 \( \times \) 10 cm) which had been equilibrated with 0.05 M potassium phosphate buffer (pH 6.5). The dialyzed enzyme solution was throughly dialyzed against the same buffer. All of the buffers used in the subsequent steps of purification contained 1 M EDTA, 0.2 mM dithiothreitol and 10\(^{-5}\) M pyridoxal 5'-phosphate.

**Table I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume</th>
<th>Concentration</th>
<th>Protein</th>
<th>Activity*</th>
<th>Ratio of specific activities</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>Total mg</td>
<td>Total units</td>
<td>Aminomalonate decarboxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Specific</td>
<td>to L-threonine aldolase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Specific</td>
<td>to L-serine hydroxymethylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Specific</td>
<td>to L-allo-threonine aldolase</td>
<td></td>
</tr>
<tr>
<td>1. Rat liver extract (soluble fraction)</td>
<td>3,110</td>
<td>21.0</td>
<td>65,300</td>
<td>643,000</td>
<td>2.70</td>
<td>2.68</td>
</tr>
<tr>
<td>2. Ammonium sulfate fractionation</td>
<td>750</td>
<td>34.6</td>
<td>12,100</td>
<td>490,000</td>
<td>3.21</td>
<td>3.15</td>
</tr>
<tr>
<td>3. Heat denaturation of impurities</td>
<td>620</td>
<td>3.16</td>
<td>1,960</td>
<td>440,000</td>
<td>3.21</td>
<td>3.38</td>
</tr>
<tr>
<td>4. Ammonium sulfate fractionation</td>
<td>34</td>
<td>26.1</td>
<td>807</td>
<td>364,000</td>
<td>3.10</td>
<td>3.14</td>
</tr>
<tr>
<td>5. DEAE-cellulose chromatography</td>
<td>72</td>
<td>0.21</td>
<td>15.1</td>
<td>115,000</td>
<td>3.10</td>
<td>3.10</td>
</tr>
<tr>
<td>6. Sephadex G-150 chromatography</td>
<td>17.5</td>
<td>0.40</td>
<td>7.0</td>
<td>92,500</td>
<td>3.08</td>
<td>3.16</td>
</tr>
<tr>
<td>7. DEAE-cellulose chromatography</td>
<td>2.0</td>
<td>0.88</td>
<td>1.5</td>
<td>23,200</td>
<td>3.04</td>
<td>3.07</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured as described in the text.
phate buffer (pH 6.5) containing 1 mM L-serine. The column was washed with the same buffer until protein no longer emerged. Further elution was carried out with a linear gradient established between 400 ml each of 0.05 M and 0.2 M potassium phosphate buffer (pH 6.5) containing 1 mM L-serine. Fractions of 6 ml were collected. Fractions 46 to 49 (Fig. 1B), which exhibited the highest specific activity, were concentrated by ultrafiltration. The enzyme preparation exhibited aminomalonate decarboxylase, L-serine hydroxymethylase, and L-allothreonine aldolase activities with respective specific activities of 15,000, 5,050 and 1,000. The aldolase activity was determined with D-allothreonine and with L-allothreonine; the rates of aldol cleavage were the same with nonsaturating concentrations of D-allothreonine that were twice that of L-allothreonine, indicating that the enzyme is probably active only towards L-allothreonine. Addition of tetrahydrofolic acid did not affect the aminomalonate decarboxylase or allotheonine aldolase activities. A summary of the purification procedure is given in Table I.

As stated above, the aminomalonate decarboxylase activity was found only in fractions which also contained L-serine hydroxymethylase and L-allothreonine aldolase activities. Furthermore, the ratios of these activities remained constant throughout purification (Table I); thus, the ratios of decarboxylase to hydroxymethylase and of decarboxylase to aldolase were close to three for all fractions. The elution patterns shown in Fig. 1 also indicate that the three activities emerge together from the Sephadex and DEAE-cellulose columns.

Further evidence that the three activities are associated with the same protein was obtained from the finding of a single protein band on polyacrylamide gel electrophoresis at pH values of 7.5, 8.0, and 8.5. The electrophoreses were performed in 5% polyacrylamide gels according to the procedure of Davis (19); the buffers used were 0.05 M potassium phosphate (pH 7.5), and 0.05 M Tris-acetate (pH 8.0 and 8.5). Fig. 2B shows the pattern obtained at pH 8 with the fraction obtained from the second DEAE-cellulose column (Step 7, Table I); Fig. 2A shows that more than one protein is present in the eluate from the Sephadex G-150 column (Step 6, Table I).

Gel electrophoresis was also performed according to Weber and Osborn (20) on 6% and 7.5% gels in a continuous buffer system consisting of 0.05 M Tris-acetate (pH 8.5) containing 0.1% (w/v) sodium dodecyl sulfate. The gels were stained with 0.25% Coomassie blue in methanol-acetic acid-water (5:7.5:87.5, v/v). Chymotrypsinogen, ovalbumin monomer and dimer, bovine serum albumin (monomer, dimer, trimer), and L-aspartate β-decarboxylase monomer (21) were used as standards.

It is notable that the ratio of L-allothreonine aldolase activity to L-threonine aldolase activity increased considerably during purification (Table I). Indeed, the final preparation exhibited no activity towards L-threonine. This seems to indicate that the cleavage of L-threonine is probably catalyzed by a different enzyme.

Since catalysis of the interconversion of L-serine and glycine is the only known physiological function of the enzyme isolated in the present studies, we refer to it as serine hydroxymethylase in the following text.

**Studies on Mitochondrial L-Serine Hydroxymethylase**

Mitochondrial L-serine hydroxymethylase was purified as described by Nakano et al. (18) except that chromatography on brushite was omitted. Aminomalonate decarboxylase, L-allothreonine aldolase, and threonine aldolase activities were not detected in the mitochondrial extract, the initial fractions, or a preparation purified by passage through DEAE-cellulose. The mitochondrial enzyme therefore differs markedly in its substrate specificity from the cytoplasmic enzyme. We also found that the mitochondrial enzyme exhibits a substantially lower mobility than does the cytoplasmic enzyme on polyacrylamide gel electrophoresis; thus, the two enzymes were completely separated under the conditions given in Fig. 2B.

**Fig. 2.** Polyacrylamide gel electrophoresis of aminomalonate decarboxylase (L-serine hydroxymethylase). Electrophoresis was carried out on 6% gels prepared in 0.05 M Tris-acetate buffer (pH 8.0). A, the eluate from Sephadex G-150 (Step 6, Table I); 20 μg of protein. The gel was run for 2 hours; 5 mA/gel. B, the eluate from the second DEAE-cellulose column (Step 7, Table I); 15 μg of protein. The gel was run for 3 hours.

**Fig. 3.** Molecular weight calibration curve for 6% (Curve 1) and 7.5% (Curve 2) polyacrylamide gels run in 0.1% sodium dodecyl sulfate. The protein standards were: chymotrypsinogen (CH); ovalbumin monomer and dimer (OV-1 and OV-2); bovine serum albumin (BSA-1, -2, -5); L-aspartate β-decarboxylase monomer (21); bovine serum albumin from Alcaligenes faecalis (ASPD; mol wt of monomer, 27,000). Prior to electrophoresis the proteins were incubated for 3 hours at 25° in Tris-acetate buffer (pH 8.5) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. The latter was omitted in the studies on ovalbumin and serum albumin. AMAD, aminomalonate decarboxylase (L-serine hydroxymethylase).
Properties of Cytoplasmic Serine Hydroxymethylase

The effect of varying pH on the three activities catalyzed by cytoplasmic serine hydroxymethylase is described in Fig. 4. The aminomalonate decarboxylase activity was maximal at values of pH about 5.5 to 6.0; studies were not carried out at lower values of pH at which the substrate undergoes spontaneous decarboxylation. Allothreonine aldolase activity exhibited a broad pH optimum between about pH 6.5 and 7.5. Serine hydroxymethylase activity was maximal between pH 7.5 and 8.0. Nakano et al. (18) reported a pH optimum of 8.0 for cytoplasmic serine hydroxymethylase; this enzyme form exhibits no serine hydroxymethylase activity. In the present studies, the purified enzyme was found to have an optimal pH between about pH 6.5 and 7.5. Serine decarboxylation. Allothreonine aldolase activity exhibited a broad pH optimum between about pH 5.5 and 6.0; studies were not carried out at lower values of pH.

The absorption spectrum of the purified cytoplasmic serine hydroxymethylase exhibited maxima at 380 and 430 nm (Fig. 5, Curve 1). Determination of the pyridoxal 5'-phosphate content of the isolated holoenzyme (after dialysis against 0.05 M potassium phosphate buffer (pH 6.5) containing 0.1 mM dithiothreitol) by the phenylhydrazone method of Wada and Snell (22) gave a value of 1 mole of pyridoxal 5'-phosphate per 59,000 g of protein. Treatment of the enzyme with sodium borohydride led to disappearance of the maximum at 430 nm and the appearance of a new peak at 330 nm (Fig. 5, Curve 2); the reduced enzyme exhibited none of the enzyme activity.

Schirch and Jenkins (24) reported that serine hydroxymethylase catalyzes a relatively slow transamination reaction between d-alanine and enzyme-bound pyridoxal 5'-phosphate to yield pyruvate and the pyridoxamine 5'-phosphate form of the enzyme; this enzyme form exhibits no serine hydroxymethylase activity. In the present studies, the purified enzyme was incubated with d-alanine and the transamination reaction was followed by measuring the disappearance of the absorbance maximum at 430 nm; simultaneous determinations of serine hydroxymethylase and aminomalonate decarboxylase activities were performed. As indicated in Fig. 6, the decrease in absorbance at 430 nm was associated with simultaneous decreases in the serine hydroxymethylase and aminomalonate decarboxylase activities. However, when these activities were determined in the presence of added pyridoxal 5'-phosphate restoration of both activities was observed.

The purified serine hydroxymethylase was resolved from its vitamin B6 cofactor by incubation of the enzyme with d-alanine for 2 hours at 37°C; the solution was then dialyzed against 0.05 M potassium phosphate buffer (pH 6.5) for 18 hours. The apoenzyme solution exhibited no characteristic absorbance at either 430 or 330 nm, nor did it have any of the catalytic activities exhibited by the holoenzyme. When the apoenzyme was incubated with pyridoxal 5'-phosphate, each of the three enzyme activities was restored. The apoenzyme was also incubated with a variety of pyridoxal phosphate analogs as indicated in Table II. Relatively similar activity values were obtained with 2-norpyridoxal 5'-phosphate and pyridoxal 5'-phosphate N-oxide; however, the activities observed with the other pyridoxal analogs, while in most cases similar for serine hydroxymethylase and allothreonine aldolase, were substantially lower than aminomalonate decarboxylase. These findings may be a reflection of the fact that the enzyme exhibits a lower affinity for aminomalonate than for the other substrates (see below).

It seems relevant to note that somewhat similar results have been obtained in studies on the effect of various vitamin B6 phosphate analogs on the activities catalyzed by aspartate β-decarboxylase. These results are described under "Methods."
bovylase. Thus, the relative decarboxylase, cysteine sulfinate desulfinase, and transaminase activities exhibited by this enzyme vary considerably depending upon the pyridoxal 5'-phosphate analog attached to the enzyme (7). It is also notable that the optical specificity of aspartate β-decarboxylase can be substantially altered in this way; thus, the N-methylpyridoxal 5'-phosphate enzyme catalyzes the transamination and the β-decarboxylation of D-aspartate, while the pyridoxal 5'-phosphate form of the enzyme is L-specific (7).

The effect of L-allothreonine on aminomalonate decarboxylase activity and the effect of aminomalonate on L-allothreonine aldolase activity are described in Fig. 7. The data indicate that each substrate competitively inhibits the activity of the enzyme toward the other substrate. The $K_i$ values obtained from these studies are similar to the respective $K_m$ values (Table III). Thus, the $K_i$ value for L-allothreonine was 1.5 mM and the $K_m$ value for L-allothreonine as a substrate was 1.0 mM; similarly, the $K_m$ value for aminomalonate as a substrate (12.8 mM) is close to that for aminomalonate as an inhibitor of L-allothreonine aldolase activity ($K_i$, 10.0 mM). As indicated in Fig. 8,

**TABLE II**

<table>
<thead>
<tr>
<th>Cofactor or analog</th>
<th>L-Serine hydroyxymethylase</th>
<th>Aminomalonate decarboxylase</th>
<th>L-Allothreonine aldolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal 5'-phosphate</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2-Norpyridoxal 5'-phosphate</td>
<td>53.4</td>
<td>54.5</td>
<td>55</td>
</tr>
<tr>
<td>2-Nor-2-ethylpyridoxal 5'-phosphate</td>
<td>68.3</td>
<td>29.4</td>
<td>40</td>
</tr>
<tr>
<td>2-Nor-2-butylypyridoxal 5'-phosphate</td>
<td>1.9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3-O-Methylpyridoxal 5'-phosphate</td>
<td>8.1</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>N-Methylpyridoxal 5'-phosphate</td>
<td>4.7</td>
<td>0</td>
<td>5.1</td>
</tr>
<tr>
<td>Pyridoxal 5'-phosphate N-oxide</td>
<td>90.1</td>
<td>81.5</td>
<td>88.7</td>
</tr>
<tr>
<td>Pyridoxal 5'-sulfate</td>
<td>14.9</td>
<td>1</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* The apoenzyme (25 μg in 0.05 ml of 0.05 M potassium phosphate buffer, pH 6.5, containing 10-4 M dithiothreitol and 1 mM EDTA) was incubated with 5 nmoles of cofactor or analog for 30 min at 25° and then for 16 hours at 4°. The enzyme activities were determined as described under "Methods."

**FIG. 8.** The effect of L-serine on aminomalonate decarboxylase activity. The concentration of DL-allothreonine was varied from 0.2 mM to 4 mM in the absence (Curve 1) and in the presence (Curve 2) of 1 mM L-serine; the reaction mixture also contained 0.1 M potassium phosphate buffer (pH 7.3), 10-4 M pyridoxal 5'-phosphate, 10-4 M dithiothreitol, 1.5 × 10-4 M DPNH, yeast alcohol dehydrogenase (55 units), and the enzyme (2 μg).

**TABLE IV**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Allothreonine decarboxylase</th>
<th>Aminomalonate decarboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Serine</td>
<td>31</td>
<td>15</td>
</tr>
<tr>
<td>D-Serine</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>D-Cysteine</td>
<td>72</td>
<td>20</td>
</tr>
</tbody>
</table>

L-serine is a competitive inhibitor of L-allothreonine aldolase activity; the $K_i$ value (0.33 mM) is not far from that of the $K_m$ value for L-serine (0.45 mM). These observations are in accord with other data presented above, indicating that these three enzyme activities are catalyzed by the same enzyme protein.

The observation that L-serine hydroxymethylase can interact with D-alanine indicates that this enzyme exhibits some affinity for D-amino acids. Other studies have shown that D-serine and L-cysteine are competitive inhibitors of serine hydroxymethylase (25). Furthermore, as indicated in Table IV, both optical isomers of serine and cysteine inhibited the L-allothreonine aldolase activity.
and aminomalonate decarboxylase activities exhibited by the purified serine hydroxymethylase preparation.

**Enzymatic Synthesis of Allothreonine**

The enzyme-catalyzed condensation of glycine and acetaldehyde has been studied with liver preparations from rat (16), sheep (26, 27), and rabbit (28); the products formed were reported to be threonine (27), allothreonine (16), and both threonine and allothreonine (26). In the present studies when the purified serine hydroxymethylase preparation was incubated with labeled glycine and acetaldehyde, more than 97% of the product was found to be allothreonine. Thus, a reaction mixture (0.1 ml) containing 0.1 M potassium phosphate buffer (pH 7.3), 20 mM [1-14C]glycine (180,000 cpm per μmole), 20 mM acetaldehyde, 10 μM pyridoxal 5'-phosphate, 0.1 mM dithiothreitol, and the enzyme (6 μg), was incubated at 37° for 30 min. The mixture was deproteinized by addition of 0.1 ml of ethanol and, after centrifugation, an aliquot of the supernatant solution was subjected to thin layer chromatography on Cellulose MN 300 (Eastman Kodak) precoated sheets in a solvent system consisting of t-butanol-methyl ethyl ketone-H2O-NH2OH (40:30:20:10, v/v) (26). The Rf values in this system for glycine, allothreonine, and threonine are, respectively, 0.41, 0.58, and 0.73. The areas of the sheets corresponding to threonine and allothreonine were removed by scraping and the compounds were eluted with 2 ml of water. Aliquots of the eluates were taken for determinations of radioactivity. In this representative experiment, 222 and 5.5 nmoles of allothreonine and threonine, respectively, were formed.

**Studies on Stereospecificity of Aminomalonate Decarboxylase and l-Allothreonine Aldolase Reactions**

It has been shown that in the oxidation of glycine to glyoxylate by hog kidney n-amino acid oxidase, the hydrogen atom liberated in the course of oxidation is that which corresponds in position to the α-hydrogen atom of n-amino acids (29). Thus, n-amino acid oxidase is useful for the determination of the stereospecificity of reactions in which glycine is formed or utilized (2, 29-31). For example, when glycine-2-t was prepared either from L-serine in a reaction catalyzed by L-serine hydroxymethylase in tritiated water (29), or by decarboxylation of aminomalonate catalyzed by L-serine phosphate β-decarboxylase in tritiated water (22), virtually all of the tritium in the glycine was released into water during its conversion to glyoxylate by n-amino acid oxidase; these findings led to the conclusion that the glycine-2-t prepared by these reactions is S-glycine-2-t. In a similar manner it was shown that R-glycine-2-t is produced by transamination in tritiated water between the pyridoxamine 5'-phosphate form of L-aspartate β-decarboxylase and glyoxylate (2), or between L-aspartate and glyoxylate in a reaction catalyzed by a rat liver transaminase (20).

In the present studies glycine-2-t was isolated from experiments in which the cleavage of L-allothreonine and the decarboxylation of aminomalonate by rat liver cytoplasmic hydroxymethylase were carried out in tritiated water (Table V). The samples of glycine-2-t thus obtained were then oxidized to glyoxylate with hog kidney n-amino acid oxidase. The data obtained indicate that virtually all of the tritium obtained in the oxidation of glycine-2-t derived by cleavage of allothreonine was released into water (Experiment 2, Table V). On the other hand, only about half of the tritium in the glycine-2-t obtained by aminomalonate decarboxylation was released into water.

---

**Table V**

<table>
<thead>
<tr>
<th>Experiment and procedure</th>
<th>Total glycine formed</th>
<th>Incorporation of tritium into glycine</th>
<th>Glycine taken for DOX reaction</th>
<th>Glyoxylate formed</th>
<th>H2O-t formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles</td>
<td>cpm/μmole</td>
<td>pmoles</td>
<td>pmoles</td>
<td>Counts/μl</td>
</tr>
<tr>
<td>1. Enzymatic decarboxylation of aminomalonate</td>
<td>3.00</td>
<td>90,000</td>
<td>1.6</td>
<td>1.4</td>
<td>59,700</td>
</tr>
<tr>
<td>2. Enzymatic cleavage of allothreonine</td>
<td>2.93</td>
<td>85,600</td>
<td>1.1</td>
<td>1.0</td>
<td>41,000</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>92,000</td>
<td>0.4</td>
<td>0.30</td>
<td>32,300</td>
</tr>
</tbody>
</table>

*The conditions used for the oxidation of glycine by n-amino acid oxidase (DOX) were as follows. A sample (0.1 ml) containing glycine-t was treated with 0.2 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3), 0.15 ml of FAD (10 μg), 0.3 ml of a solution containing n-amino acid oxidase (1.5 mg) and bovine serum albumin (3 mg), and 10 μl of catalase (6900 units); the mixture was then incubated at 37° for 8 hours in a stopped tube. The solution was then lyophilized and the distillate was quantitatively trapped in a Dry Ice-acetone bath. The volume of the condensate was measured and an aliquot was taken for determination of radioactivity. The lyophilized residue was dissolved in water and an aliquot was taken for determination of glyoxylate (34).*
labeled \([3-^{14}C]\)aminomalonate (i.e. prepared from \(L-[3-^{14}C]\)serine) the incorporation of tritium into glycine was only 246 cpm per pmole. A greater incorporation of tritium into glycine occurs at higher pH values (7.4, 7.5) and with higher concentrations of water under the same conditions used in this experiment. Gly-

cine formed was close to 50% of that of the \([3-^{14}C]\)amino-
malonate and that about 50% of the radioactivity was released (3) was decarboxylated to glycine by serine hydroxymethylase. This conclusion is supported by the studies described above, which include the effects on the several activities of resolution of the enzyme by threonine cleavage. This conclusion is probably catalyzed by a separate enzyme; this is in accord with the conclusions of Riario-Sforza et al. (40). It is possible of course that the rat liver enzyme undergoes structural change during purification which alters the active site in such a way as to decrease its affinity for \(L\)-threonine. The contradictory reports in the literature concerning the cleavage of threonine and allothreonine may in part be influenced by the fact that some commercially available samples of threonine and allothreonine may be contaminated with each other. In addition, species differences apparently exist; thus, the ratio of serine hydroxy-
methylase to allothreonine aldolase activities for the rat liver enzyme studied here is close to one, while this ratio is about four for the rabbit liver enzyme (28). Furthermore, the reported specific activity (serine hydroxymethylase) of the rabbit liver enzyme is about twice that of the \(L\)-threonine enzyme, and as stated above the rabbit liver enzyme was reported to catalyze the aldol cleavage of \(L\)-threonine, whereas our preparation from rat liver did not. It should also be noted that the monomer molecular weight of the rat liver serine hydroxymethylase as determined by sodium dodecyl sulfate gel electrophoresis is 61,000 ± 2,000, while similar study of the rabbit liver enzyme gave a value of 59,000 ± 5,000 (41).

A most interesting observation made in the present study is that rat liver mitochondrial serine hydroxymethylase purified by the method of Nakano et al. (18) exhibited no activity toward aminomalonate, allothreonine, or threonine. This striking difference between the specificities shown by the cytoplasmic and mitochondrial hydroxymethylases indicates that the structures of the active sites of these enzymes must be quite different; indeed other differences between the two enzymes have been reported (18). It is conceivable that the mitochondrial serine hydroxymethylase is part of the mitochondrial glycine synthesis system, which catalyzes the formation of 2 moles of glycine from 1 mole each of serine, ammonia, and carbon dioxide (42-45).

There is substantial evidence that the serine hydroxymeth-

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Aminomalonate taken (nmoles)</th>
<th>Products (cpm)</th>
<th>Glycine (cpm)</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sentinel</td>
<td>170</td>
<td>13,000</td>
<td>5,700</td>
<td>5,440</td>
</tr>
</tbody>
</table>

\* Prepared from \(L-[3-^{14}C]\)serine; see Palekar et al. (3).

\† The reaction mixture (0.2 ml) contained potassium phosphate buffer (12 \(\mu\)moles; pH 6.5), \([3-^{14}C]\)aminomalonate (170 nmoles; 13,000 cpm), pyridoxal 5'-phosphate (2 nmoles), dithiothreitol (2 nmoles), and the enzyme (5 \(\mu\)g). After incubation for 30 min at 25\(^\circ\), the formation of \(^{14}CO_2\) and \([^{14}C]\)glycine was determined as described (3).

\‡ Determined by the ninhydrin method (32).

(Experiment 1, Table V). As a control, unlabeled glycine (5 \(\mu\)moles) was incubated with the hydroxymethylase in tritiated water under the same conditions used in this experiment. Glycine was then purified from the reaction mixture in the same way; the incorporation of tritium into glycine was only 246 cpm per \(\mu\)mole. A greater incorporation of tritium into glycine occurs at higher pH values (7.4, 7.5) and with higher concentrations of glycine (24, 30); such incorporation is increased about 500-fold when tetrahydrofolate is added (24).

Table VI summarizes the results of studies in which specifically labeled \([3-^{14}C]\)aminomalonate (i.e. prepared from \(L-[3-^{14}C]\)serine (3)) was decarboxylated to glycine by serine hydroxymethylase. The findings indicate that the specific radioactivity of the \([^{14}C]\)glycine formed was close to 50% of that of the \([3-^{14}C]\)amino-
malonate and that about 50% of the radioactivity was released as carbon dioxide. This shows that the enzyme does not catalyze the removal of a specific carboxyl group of amino-
malonate.

**DISCUSSION**

The present studies indicate that the decarboxylation of aminomalonate (Reaction 1), the aldol cleavage of \(L\)-allothreonine (Reaction 2), and the tetrahydrofolate-dependent interconversion of serine and glycine (Reaction 3) are catalyzed by the same enzyme protein of rat liver, i.e. cytoplasmic \(L\)-serine hydroxy-
methylase. The isolated enzyme, which is homogeneous by the criterion of polyacrylamide gel electrophoresis, exhibits these three activities in the same ratios as found for all of the partially purified fractions obtained.

\[
\text{Aminomalonate} \rightarrow \text{glycine} + \text{CO}_2 \tag{1}
\]

\[
\text{L-Allothreonine} \rightleftharpoons \text{glycine} + \text{acetaldehyde} \tag{2}
\]

\[
\text{L-Serine + tetrahydrofolate} \rightleftharpoons \text{glycine} + 5,10\text{-methylene tetrahydrofolate} \tag{3}
\]

It seems probable that virtually all of the aminomalonate decar-
boxylase activity found in rat liver homogenate may be ascribed to cytoplasmic serine hydroxymethylase. This conclusion is supported by the studies described above, which include the effects on the several activities of resolution of the enzyme by reaction with \(\alpha\)-alanine and the studies which indicate com-

petition between the several substrates. The present and earlier studies on cytoplasmic serine hydroxymethylase thus indicate that this enzyme, like certain other vitamin \(B_6\) enzymes (35-39), is capable of catalyzing a number of chemical reactions. It is of interest that, while the hydroxymethylation reaction requires tetrahydrofolate, the aldol cleavage of allothreonine and the decarboxylation of aminomalonate do not; thus, the structure of the substrate appears to determine the reaction pathway.

There are apparently conflicting reports in the literature about the possible identity of serine hydroxymethylase, threonine aldolase, and allothreonine aldolase. Thus, Karsek and Greenberg (26) reported evidence for the presence of two distinct enzymes in sheep liver that catalyze, respectively, the cleavage of \(L\)-threonine and \(L\)-allothreonine. Malkin and Greenberg (16) reported evidence that a single enzyme in rat liver catalyzes the aldol cleavage of both \(L\)-threonine and \(L\)-allothreonine. The latter finding was contradicted by Riario-Sforza et al. (40), who presented evidence that threonine and allothreonine are cleaved by separate enzymes in rat liver. Schirch and Gross (28) have published evidence that a single enzyme in rabbit liver catalyzes serine-glycine interconversion, threonine cleavage, and allo-
threonine cleavage. While our results indicate that rat liver cytoplasmic serine hydroxymethylase catalyzes the aldol cleavage of \(L\)-allothreonine and the decarboxylation of aminomalonate, our most purified enzyme preparation exhibited no activity toward \(L\)-threonine. Indeed, the ratio of allothreonine aldolase activity to threonine aldolase activity increased considerably during purification, indicating that the cleavage of threonine is probably catalyzed by a separate enzyme; this is in accord with the conclusions of Riario-Sforza et al. (40). It is possible of course that the rat liver enzyme undergoes structural change during purification which alters the active site in such a way as to decrease its affinity for \(L\)-threonine. The contradictory reports in the literature concerning the cleavage of threonine and allothreonine may in part be influenced by the fact that some commercially available samples of threonine and allothreonine may be contaminated with each other. In addition, species differences apparently exist; thus, the ratio of serine hydroxy-
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when the reaction is carried out in tritiated water, S-glycine-2-t is formed since all of the tritium is released from glycine by \( \alpha \)-amino acid oxidase (29). It may therefore be concluded that the tritium incorporated into glycine is in a position analogous to the \( \alpha \)-CH_2OH group of L-serine. The data given in Table V on the cleavage of L-allothreonine lead to an analogous conclusion. However, the decarboxylation of aminomalonate in tritiated water yields glycine-2-t only after half of whose tritium can be released by \( \alpha \)-amino acid oxidase. This indicates that the product is a mixture of \( R \)-glycine-2-t and \( S \)-glycine-2-t. This lack of stereospecificity is confirmed by the studies with specifically carboxyl-labeled \([14C] \)aminomalonate (Table VI). The present data (Table IV and earlier findings (24, 25) indicate that serine-hydroxymethylase can interact with several \( \alpha \)-amino acids. It thus appears that aminomalonate can react with the enzyme both as a \( \alpha \)-amino acid and as an \( \alpha \)-amino acid (as indicated in Fig. 9) so that either one of the carboxyl groups may be removed in the reaction, which thus leads to the formation of both \( R \) and \( S \)-glycine-2-t. This result is in striking contrast to that which obtains with l-aspartate \( \beta \)-decarboxylase, which decarboxylates aminomalonate in tritiated water stereospecifically to yield glycine-2-t (2) it is notable that this enzyme exhibits strict \( \alpha \)-amino acidity (37). Thus, the decarboxylation of aminomalonate to glycine occurs in a specific manner when catalyzed by one \( \alpha \) enzyme (aspartate \( \beta \)-decarboxylase) and in a nonspecific manner when catalyzed by another (cytoplasmic serine hydroxymethylase).

In early investigations on the serine-glycine interconversion, aminomalic acid was considered as a possible intermediate (29–50), but this idea was discarded on the basis of studies with labeled compounds and because aminomalic acid is a symmetrical compound (51). The very considerable amount of information now available about the serine-glycine interconversion and the participation of tetrahydrofolate in this reaction (52), as well as the absence of evidence for the formation of aminomalonate from serine, render unlikely the possibility that aminomalonate is an intermediate between serine and glycine. The present studies, which indicate that the decarboxylation of aminomalonate in liver does not occur in a specific manner, would seem, in retrospect, to support Shemin’s (51) original conclusion (inadequate though it was on logicalgrounds, as pointed out by Ogston (1)) that aminomalonate could not be an intermediate in the conversion of serine to glycine.

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Rat Liver Aminomalonate Decarboxylase: IDENTITY WITH CYTOPLASMIC SERINE HYDROXYMETHYLASE AND ALLOTHREONINE ALDOLASE
Anil G. Palekar, Suresh S. Tate and Alton Meister


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