

# Threonine Synthase of *Lemna paucicostata* Hegelm. 6746

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## ABSTRACT

Threonine synthase (TS) was purified approximately 40-fold from *Lemna paucicostata*, and some of its properties determined by use of a sensitive and specific assay. During the course of its purification, TS was separated from cystathionine  $\gamma$ -synthase, establishing the separate identity of these enzymes. Compared to cystathionine  $\gamma$ -synthase, TS is relatively insensitive to irreversible inhibition by propargylglycine (both *in vitro* and *in vivo*) and to gabaculine, vinylglycine, or cysteine *in vitro*. TS is highly specific for *O*-phospho-L-homoserine (OPH) and water (hydroxyl ion). Nucleophilic attack by hydroxyl ion is restricted to carbon-3 of OPH and proceeds stereospecifically to form threonine rather than *allo*-threonine. The  $K_m$  for OPH, determined at saturating *S*-adenosylmethionine (AdoMet), is 2.2 to 6.9 micromolar, two orders of magnitude less than values reported for TS from other plant tissues. AdoMet markedly stimulates the enzyme in a reversible and cooperative manner, consistent with its proposed role in regulation of methionine biosynthesis. Cysteine (1 millimolar) caused a slight (26%) reversible inhibition of the enzyme. Activities of TS isolated from *Lemna* were inversely related to the methionine nutrition of the plants. Down-regulation of TS by methionine may help to limit the overproduction of threonine that could result from allosteric stimulation of the enzyme by AdoMet.

No evidence was obtained for feedback inhibition, repression, or covalent modification of TS by threonine and/or isoleucine.

In plants, the branch point for the threonine and methionine biosynthetic pathways is at OPH<sup>4</sup> (16). TS catalyzes the reaction:



the first committing step in threonine synthesis. In spite of its potential role in regulating threonine synthesis, TS has not been extensively studied in plants. Schnyder *et al.* (22) first reported TS activity in cell-free preparations of plants, using extracts of peas and *Lemna minor* incubated for an extended period (25 h) with OPH and pyridoxal phosphate. Shortly thereafter, the enzyme was unequivocally demonstrated in crude extracts of sugar beet and radish leaves, and shown to be markedly stimulated by AdoMet (19). TS has been purified 14-fold from barley (1) and 10-fold from peas (26), and some of its properties, including

stimulation by AdoMet and localization in chloroplasts (31), have been briefly described. In the present work TS of *Lemna paucicostata* was purified approximately 40-fold and its catalytic and regulatory properties examined in detail. Parts of this work have been presented in a preliminary report (30).

## MATERIALS

**Solvents.** Common solvents for chromatography were: solvent A, 2-propanol:88% HCOOH:H<sub>2</sub>O (7:1:2, v/v); solvent B, 1-butanol:propionic acid:H<sub>2</sub>O (250:124:175, v/v); solvent C, 1-butanol:acetone:diethylamine:H<sub>2</sub>O (10:10:2:5, v/v).

**Buffers.** Buffers used in the purification of TS were: buffer A, 20 mM K Hepes (pH 7.8), 5 mM DTT, 0.1 mM pyridoxal phosphate, and 1 mM PMSF in 0.35 M DMSO; buffer B, 20 mM K Mes (pH 6.0), 5 mM DTT, 0.1 mM pyridoxal phosphate, 15% (v/v) ethylene glycol, and 1 mM PMSF in 0.35 M DMSO.

**Preparation of [U-<sup>14</sup>C]OPH.** L-[U-<sup>14</sup>C]Homoserine (8.066 × 10<sup>8</sup> dpm, 9.32 μmol) (Amersham) was purified by Dowex 50-H<sup>+</sup> chromatography and converted with yeast homoserine kinase to [U-<sup>14</sup>C]OPH as in the synthesis of [<sup>3</sup>H]OPH (9). [<sup>14</sup>C]OPH was further purified by addition of 211 mg of decolorizing charcoal to remove UV-absorbing material (9), followed by paper electrophoresis and chromatography to remove Pi and traces of adenine nucleotides. Electrophoresis for 2 h in 0.47 M HCOOH:1.39 M acetic acid (pH 1.9) resolved [<sup>14</sup>C]OPH (2 cm to anode) from Pi (15 cm to anode), and partially from AMP and ADP (each 4 cm to cathode). Subsequent paper chromatography (solvent A) of the eluant from the electrophorogram resolved [<sup>14</sup>C]OPH ( $R_f$ , 0.25) from ATP ( $R_f$ , 0), and from remaining ADP ( $R_f$ , 0.03) and AMP ( $R_f$ , 0.17). The overall radiochemical yield of [<sup>14</sup>C]OPH from [<sup>14</sup>C]homoserine was 30%. The freshly prepared compound was at least 97% radiopure as judged by electrophoresis in 125 mM triethanolamine-HCl (pH 8.0) for 45 min (9). [<sup>14</sup>C]Homoserine was the only detectable labeled impurity, and did not interfere with assay of TS.

**Other Materials.** Unless specified otherwise, radioactive compounds were from Amersham or New England Nuclear.  $\beta$ -Chloro-L-alanine was from Cyclo (Los Angeles) and BSA from Miles Lab. Unless specified, all other compounds were from Sigma or Calbiochem. Preparation of OPH and other homoserine esters (9) and the source of L-threo- and L-erythro-2-amino-3-mercaptoputyric acid (thiothreonine) (10) have been described.  $\beta$ -Methylanthionine was prepared according to Schoberl *et al.* (23) and was generously provided by the late Dr. Erhard Gross. The major species contains an L-configuration at the cysteine  $\alpha$ -carbon and a D-configuration at the  $\alpha$ -carbon of the four-carbon moiety (23). This preparation is therefore a diastereoisomer of any  $\beta$ -methylanthionine synthesized by TS, which presumably would contain the L-configuration at both  $\alpha$ -carbons.

The iodide salt of AdoMet, 85 to 90% pure, from Sigma was used for most assays. AdoMet, at least 98% pure by the chromatographic criteria described below, was used for detailed stud-

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<sup>4</sup> Abbreviations: OPH, *O*-phospho-L-homoserine; TS, threonine synthase; AdoMet, *S*-adenosylmethionine; PAG, DL-propargylglycine (DL-2-amino-4-pentynoic acid); PMSF, phenylmethylsulfonyl fluoride.

ies on the effects of AdoMet concentrations on TS activity. This *p*-toluenesulfonate salt of AdoMet (BioResearch, Milan), supplied by Dr. G. L. Cantoni, was titrated with KOH to a pH between 3 and 4, and converted to the chloride salt by passage through Dowex 1-Cl<sup>-</sup> at 4°C. AdoMet was determined by UV absorption. No UV or ninhydrin-positive compounds other than AdoMet were detected when this AdoMet chloride was chromatographed in 0.1 M sodium phosphate (pH 6.8; 100 ml) containing 60 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 2 ml 2-propanol, or in solvent B. AdoMet was resolved from possible impurities, as shown by R<sub>f</sub> values in these respective solvents: AdoMet (0.43, 0.10); 5'-methylthioadenosine (0.09, 0.77); adenosine (0.13, 0.51); adenine (0.13, 0.65); homoserine (0.91, 0.23); and methionine (0.81, 0.48).

## METHODS

**General Methods.** Methods for paper electrophoresis and paper chromatography (9), and for protein determination (28) have been described. TS was extracted and purified at 4°C and assayed at 30°C.

**Plants.** *Lemna paucicostata* Hegelm. 6746 was grown mixotrophically in medium 4 with 20 μM inorganic sulfate (11). Supplements were added at concentrations that allowed steady state growth with little or no effects on growth rates. Depletion of supplement did not exceed 20%. Plants were grown in the presence of supplements for at least three generations prior to enzyme assay or amino acid analysis.

**Preparation of Enzyme Extracts.** Although no significant difference was detected in TS activities from fresh control plants and those frozen at -80°C, TS activities were routinely determined with fresh plants homogenized in 20 mM K HEPES, pH 7.8 (28), centrifuged for 30 min at 48,000 g, and the supernatant fraction gel filtered (28) with Sephadex G-25 (medium) equilibrated with the homogenizing buffer. Extracts were assayed immediately since approximately 50% of the activity was lost during storage for 16 h at 4°C. Extracts were stable at least 4 d stored at -80°C.

**Assay of TS.** The standard assay for TS contained: K HEPES (100 mM, pH 7.8), pyridoxal phosphate (0.1 mM), AdoMet (0.05 mM), [<sup>14</sup>C]OPH (5 μM), and enzyme in a final volume of 300 μl. BSA (0.83%, w/v) was added for assay of ammonium sulfate, Sephacryl S-300, and DEAE-Sephacel fractions, but not normally for assays of crude extracts. Incubation was for 15 to 45 min. The reaction was terminated by addition of cold 10% TCA containing 250 nmol each of threonine and homoserine. Radioactive threonine, together with any homoserine formed, was separated from radioactive OPH on a column of Dowex 50-H<sup>+</sup> (9). For determination of low activities, this step was repeated to ensure complete removal of any residual [<sup>14</sup>C]OPH. Radioactivity in threonine was separated from that in homoserine by the method of Madison and Thompson (19), modified to minimize variation that we experienced in recovery of the products of periodate oxidation of [<sup>14</sup>C]threonine. The sample containing [<sup>14</sup>C]threonine and any [<sup>14</sup>C]homoserine was incubated at 4°C for 1 min in a reaction mixture (final volume, 0.675 ml) containing 18.5 mM periodate and 11 mM sodium phosphate, pH 6.9. The reaction was terminated with 13 μl of 2.5 M ethylene glycol, the solution applied to a column (0.9 × 2 cm) of Dowex 50-H<sup>+</sup>, and radioactivity washed through with 4 ml of water into a scintillation vial. This fraction, which contains [<sup>14</sup>C]glyoxylic acid and [<sup>14</sup>C]acetaldehyde formed by the oxidation of [<sup>14</sup>C]threonine, was neutralized to pH 8 with 1 M Tris base (75 μl), and evaporated to dryness with a stream of air at room temperature. [<sup>14</sup>C]Acetaldehyde was completely volatilized, whereas the Tris salt of [<sup>14</sup>C]glyoxylic acid was retained, and its radioactivity determined. With this modified procedure 45% of the radioactivity originally in [<sup>14</sup>C]threonine was routinely recovered, cor-

responding to a recovery from [<sup>14</sup>C]threonine of 90% (2 × 45%). No correction was made in the assays for this loss. An assay mixture incubated without enzyme provided a correction for the small (0.02%) nonenzymic conversion of radioactivity into the [<sup>14</sup>C]glyoxylate fraction. [<sup>14</sup>C]Homoserine could be determined from the radioactivity eluted from the Dowex 50-H<sup>+</sup> column with 3 N NH<sub>4</sub>OH. Separate experiments with authentic [U-<sup>14</sup>C]homoserine showed that 0.6% of radioactivity in this compound was converted to the glyoxylate fraction. This small conversion had no significant effect on the determination of [<sup>14</sup>C]threonine. A Dowex 50-H<sup>+</sup> column was used for one assay only.

**Cystathionine γ-Synthase.** This was assayed as described (28).

**Purification of TS and Separation from Cystathionine γ-Synthase.** Plants (11.5 g) were homogenized with 46 ml buffer A and the supernatant solution obtained by centrifugation was fractionated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Protein precipitating between 43 and 62% saturation was dissolved in 5 ml of buffer A containing 50 mM NaCl and applied to a column (1.5 × 84 cm; void volume 50 ml) of Sephacryl S-300 Superfine. The column was developed with the same buffer at a flow rate of 8.7 ml/h. Fractions (1 ml) eluting between 77 and 88 ml were pooled, and ethylene glycol was added to a final concentration of 15% (v/v).

Ten percent of this pooled fraction was gel filtered through Sephadex G-25 (PD-10 columns, Pharmacia) equilibrated with buffer B containing 100 mM NaCl. The gel-filtered extract (3.25 ml) was applied to a column (1.0 × 10.3 cm) of DEAE-Sephacel (Pharmacia). A gradient of NaCl in buffer B was then applied to the column, commencing at 150 mM and increasing linearly to 250 mM. Fractions (2 ml) were collected at a flow rate of 4 ml/h. An aliquot (0.45 ml) was removed for protein determination. BSA was added to the remaining solution (final concentration, 1.7% [w/v]) to improve recovery of enzyme activity during subsequent gel filtration against buffer A containing 15% (v/v) ethylene glycol, 1 mM EDTA, and 1.7% (w/v) BSA (performed as soon as possible after collection of each fraction). This step minimized inactivation (up to 30% in 26 h at 4°C) of the enzyme at the pH of 6.0 used for chromatography, and avoided introduction of inhibitory concentrations of NaCl into the assay. The gel-filtered enzyme was stable for many months when stored at -80°C.

**Characterization of Product.** A crude extract of *Lemna* was incubated for 30 min with [<sup>14</sup>C]OPH (86,580 dpm/nmol) and other components of the standard assay mixture. A reaction mixture was incubated also in the absence of enzyme. The reaction products were isolated on Dowex 50-H<sup>+</sup> as described for the TS assay, mixed with marker L-[<sup>3</sup>H]threonine and L-[<sup>3</sup>H]homoserine, and chromatographed with solvent C. [<sup>14</sup>C]Threonine formation was calculated by multiplying the ratio of <sup>14</sup>C/<sup>3</sup>H in the eluate of strips 27 to 32 (Fig. 1A) by the amount of [<sup>3</sup>H]threonine added. An estimate of [<sup>14</sup>C]homoserine formation was similarly calculated by elution of strips 15 to 24 in Figure 1A, correcting for nonenzymic synthesis (Fig. 1B).

Since *allo*-threonine may not have resolved completely from homoserine and threonine, a maximal estimate of the amount of *allo*-[<sup>14</sup>C]threonine formed was made: An aliquot of each of the eluates described above and of the eluate of strips 25 and 26 in Figure 1A was rechromatographed with solvent C with marker *allo*-L-threonine. *allo*-Threonine resolved from [<sup>3</sup>H]threonine (Fig. 1C) and [<sup>3</sup>H]homoserine (data not shown). The sum of <sup>14</sup>C that comigrated with marker *allo*-threonine on the three chromatographs provided an upper limit of enzymic synthesis of *allo*-[<sup>14</sup>C]threonine. Corrections were made for corresponding amounts of <sup>14</sup>C present in the reaction mixture incubated in the absence of *Lemna* extract. The same methods were used for determination of the products formed by a Sephacryl S-300 fraction.

**Products Formed from Sulfide and Cysteine.** Formation of

sulfur products was determined with a DEAE-Sephacel fraction and with the same fraction after preincubation with 50  $\mu\text{M}$  PAG in 20 mM K Hepes (pH 7.8) for 10 min at 30°C, and gel filtration before assay. This procedure did not affect TS activity (see "Results"), but inactivated any traces of cystathionine  $\gamma$ -synthase which might contribute to the formation of sulfur products. Homocysteine and  $\beta$ -methylanthionine were the only compounds whose upper limits of synthesis were reduced due to PAG-treatment of the enzyme. The upper limits for synthesis reported for these two sulfur compounds are those obtained with PAG-treated enzyme, and were about 20% of the values for untreated enzyme. The upper limits for synthesis of all other sulfur compounds were obtained with untreated enzyme.

For determination of products formed from sulfide, enzyme was incubated for 1 h in a standard reaction mixture, except for the presence of 30  $\mu\text{M}$  OPH and 5.1 mM [ $^{35}\text{S}$ ]sulfide. [ $^{35}\text{S}$ ] Homocysteine, *threo*-[ $^{35}\text{S}$ ]thiothreonine, and *erythro*-[ $^{35}\text{S}$ ]thiothreonine were isolated by electrophoresis of the oxidized products (10), and further purified by sequential chromatography with 2-propanol:88% formic acid (3:2, v/v), and 1-butanol:acetic acid:water (12:5:3, v/v). The reaction mixture for determination of products formed from cysteine was identical except that [ $^{35}\text{S}$ ]sulfide was replaced by 302  $\mu\text{M}$  [ $^{35}\text{S}$ ]cysteine. Radioactive products were isolated on Dowex 50-H<sup>+</sup>, then mixed with [ $^3\text{H}$ ]cystathionine, and oxidized with performic acid (28). The oxidized products were isolated on Dowex 50-H<sup>+</sup> (28), and electrophoresed for 2.25 h in 0.46 M HCOOH. Cystathionine formation was calculated from the  $^{35}\text{S}/^3\text{H}$  ratio in the cystathionine sulfone peak (migration 29 cm toward the cathode). Authentic  $\beta$ -methylanthionine similarly oxidized with performic acid migrated to two areas (17 and 19 cm toward the cathode). Since the sample of authentic  $\beta$ -methylanthionine was probably a diastereoisomer of any enzymically formed  $\beta$ -methyl-[ $^{35}\text{S}$ ]anthionine (see "Materials"), the area corresponding to any  $\beta$ -methyl-[ $^{35}\text{S}$ ]anthionine was not localized precisely. Accordingly, the total amount of  $^{35}\text{S}$  that migrated 6 to 21 cm toward the cathode was taken as a maximal estimate of  $\beta$ -methylanthionine synthesis. This estimate is therefore larger than those for the other products of TS. All estimates were corrected for any apparent synthesis in the absence of enzyme, and were compared to threonine synthesis measured also at 30  $\mu\text{M}$  OPH.

**Amino Acid Analysis.** External amino acids were removed from the plants by repeated washing in fresh standard growth medium. Soluble amino acids were extracted with 10% TCA containing tracer amounts of [ $^3\text{H}$ ]threonine and [ $^{14}\text{C}$ ]isoleucine, and recovered by Dowex 50-H<sup>+</sup> chromatography as described for the TS assay. Isoleucine was determined from the amount of amino acid that migrated with authentic isoleucine during chromatography with an amino acid analyzer. After preliminary removal of serine by preparative paper chromatography with solvent B, threonine was determined with an amino acid analyzer from the amount of periodate-sensitive amino acid that comigrated with authentic threonine. Amino acid analyses were performed on a Beckman model 118CL, using the procedure described in the Beckman 118/119CL Application Notes (April 1977) for analysis of hydrolysate by a 90-min single column. Recoveries of radioactivity were used to correct for losses of amino acids. Tissue concentrations of threonine and isoleucine were estimated on the basis of a mean value of 509  $\mu\text{g}$  fresh weight/frond (8), i.e. 0.509  $\mu\text{g}/\text{frond}$ .

## RESULTS

**Optimization of the Assay for TS in Crude Extracts.** [ $^{14}\text{C}$ ] Threonine formed was not further metabolized, since authentic L-[U- $^{14}\text{C}$ ]threonine added to the incubation was recovered quantitatively, and addition of 200  $\mu\text{mol}$  carrier L-threonine to the assay did not affect the amount of [ $^{14}\text{C}$ ]threonine recovered.

Approximately 90% of the total activity of the homogenate was recovered in the supernatant fraction. No significant differences in the yields of activity were obtained by extraction of plants with other buffers (K Mes [pH 6.1 and 6.4], K Hepes [pH 6.8 and 8.5], or Na borate [pH 7.8]), nor by incorporation of Tween 80 (0.1%) or diethyldithiocarbamate (10 mM) into the standard extraction buffer. Supplementation of K Mes (pH 6.1) with 1 mM DTT plus 0.1 mM pyridoxal phosphate also did not affect recovery of activity.

The activity of TS in crude extracts assayed in 100 mM K Hepes had a pH optimum near pH 8.0, with activities approximately 25% lower at the lowest pH (7.0) and highest pH (8.5) tested. Replacement of Mes in the standard assay with the following buffers (each at 100 mM, pH 8.0) did not yield higher TS activity; bicine, glycinamide, glycyglycine, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid, imidazole, 3-(*N*-morpholino)propanesulfonic acid, 3-([tris(hydroxymethyl)methyl]amino)propanesulfonic acid, Tes, Tricine, and phosphate. Stimulations of approximately 30% were observed at 0.05 to 1 mM pyridoxal phosphate. DTT at 2 mM did not affect activity in the absence of added pyridoxal phosphate, nor at 2, 5, and 10 mM added to the standard reaction mixture containing 0.1 mM pyridoxal phosphate.

**Characterization of Product.** A single enzyme-dependent  $^{14}\text{C}$  product formed when a crude extract of *Lemna* was incubated with [ $^{14}\text{C}$ ]OPH (Fig. 1, A and B). This product was characterized as [ $^{14}\text{C}$ ]threonine<sup>5</sup> as follows. (a) It cochromatographed with authentic L-[ $^3\text{H}$ ]threonine with solvent C (Fig. 1, A and C), and with isobutyric acid:0.5 M NH<sub>4</sub>OH (5:3, v/v) and solvent B (data not shown). (b)  $^{14}\text{C}$  in the peaks eluted from each of these chromatograms was oxidized by periodate. (c) The product resolved from authentic *allo*-threonine in solvent C (Fig. 1C).

The minor peak of  $^{14}\text{C}$  (approximately 1.7% of the radioactivity originally present in OPH) that cochromatographed with L-[ $^3\text{H}$ ]homoserine was not enzyme-dependent (Fig. 1, A and B).

The rate of [ $^{14}\text{C}$ ]threonine synthesis calculated from the  $^{14}\text{C}/^3\text{H}$  ratio in the major peak in Figure 1A agreed within experimental error with that determined by the standard assay, thereby validating the use of the standard assay for crude extracts. Enzymic synthesis of *allo*-threonine or homoserine was estimated (see "Methods") to be no more than 3% and 1% of threonine synthesis, respectively.

**Purification of TS and Resolution from Cystathionine  $\gamma$ -Synthase.** Steps resulting in a 38-fold purification of TS (39% yield) from a crude extract of *Lemna* are summarized in Table I. Details of gel filtration on Sephacryl S-300 and chromatography on DEAE-Sephacel are shown in Figure 2, A and B. Gel filtration resolved TS and cystathionine  $\gamma$ -synthase, establishing that these activities are catalyzed by different enzymes.

**Properties of TS. Effect of BSA.** [ $^{14}\text{C}$ ]Threonine formation by crude extracts was increased about 25% by addition of BSA to the assay, and larger increases were observed with more purified fractions: BSA (0.83% w/v) increased [ $^{14}\text{C}$ ]threonine formation by the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction by 46%, and by the Sephacryl S-300 fraction by 80%. The effect of BSA on the most purified (DEAE-Sephacel) fraction could not be determined, since this fraction contained added BSA. Approximately the same stimulation of activity of the Sephacryl S-300 fraction was observed over the range of BSA concentrations (0.017–0.83%, w/v) tested.

**Substrate Specificity.** Substrate specificities were determined with partially purified preparations of TS. The affinity of TS (DEAE-Sephacel fraction) for a variety of related compounds

<sup>5</sup> Although not shown definitively, the stereochemical configuration is almost certainly L, rather than D, since the substrate OPH has the L-configuration, and a stereochemical inversion at the  $\alpha$ -carbon would not be expected.

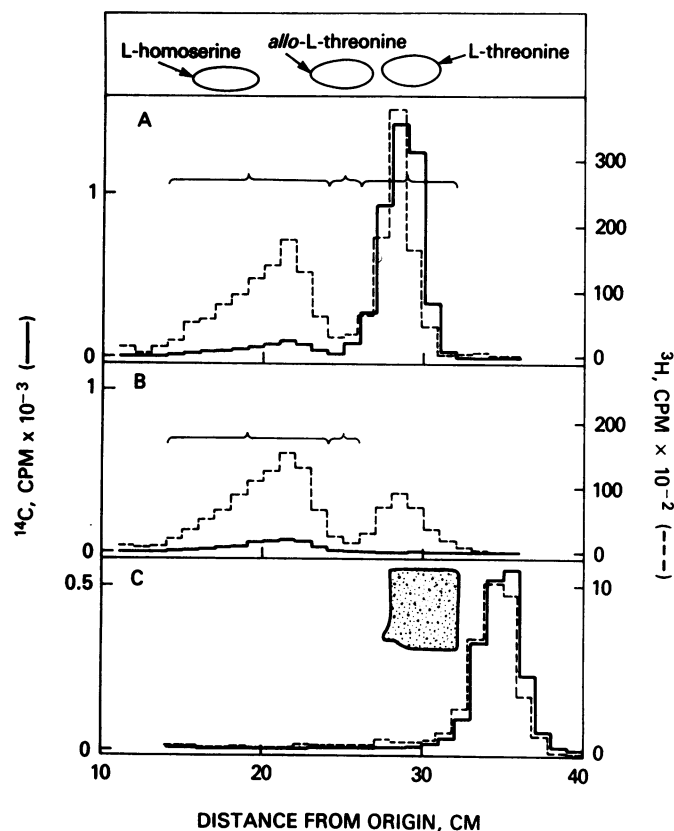


FIG. 1. Products of metabolism of [ $^{14}\text{C}$ ]OPH by a crude extract. A, Products from an incubation mixture containing a crude extract of *Lemna*. B, Products from an incubation mixture from which *Lemna* extract was omitted. [ $^3\text{H}$ ]Threonine added to B was one-third that added to A. C, Rechromatography of an aliquot of the elute of the bracketed threonine peak from chromatogram A in the presence of *allo*-L-threonine and additional [ $^3\text{H}$ ]threonine. Hatching defines the area of migration of *allo*-L-threonine. The areas of migration of L-homoserine, *allo*-L-threonine, and L-threonine which were chromatographed in parallel with the chromatograms of A and B are indicated. Dashed lines show the location of L- $^3\text{H}$ homoserine and L- $^3\text{H}$ threonine added as internal markers. Brackets indicate those strips that were eluted for analyses of [ $^{14}\text{C}$ ] homoserine, *allo*- $^{14}\text{C}$ ]threonine, or [ $^{14}\text{C}$ ]threonine (see "Methods").

Table I. Purification of TS

The apparent increase in yield resulting from Sephacryl S-300 chromatography of the  $(\text{NH}_4)_2\text{SO}_4$  fraction probably resulted from the presence of inhibitory concentrations of  $(\text{NH}_4)_2\text{SO}_4$  in the  $(\text{NH}_4)_2\text{SO}_4$  fraction, which was not gel filtered before assay.

Fraction	Specific Activity Purification		Yield
	$\text{nmol}/\text{min} \cdot \text{mg}$ protein	-fold	
Crude extract	0.68	(1.0)	(100)
$(\text{NH}_4)_2\text{SO}_4$ (43–62% saturation)	0.78	1.1	68
Sephacryl S-300 (77–88 ml)	7.40	10.9	85
DEAE-Sephacel (20–32 ml)	26.1	38	39

ranged from one to three orders of magnitude less than that for OPH (Table II). In agreement with the results reported above for a crude extract, the Sephacryl S-300 fraction<sup>6</sup> was markedly

<sup>6</sup> This fraction was used rather than the most highly purified BSA-containing DEAE-Sephacel fraction, since BSA catalyzed a slow conversion of OPH to homoserine, presumably due to contaminating phosphatase. For this reason, BSA was omitted also from these mixtures.

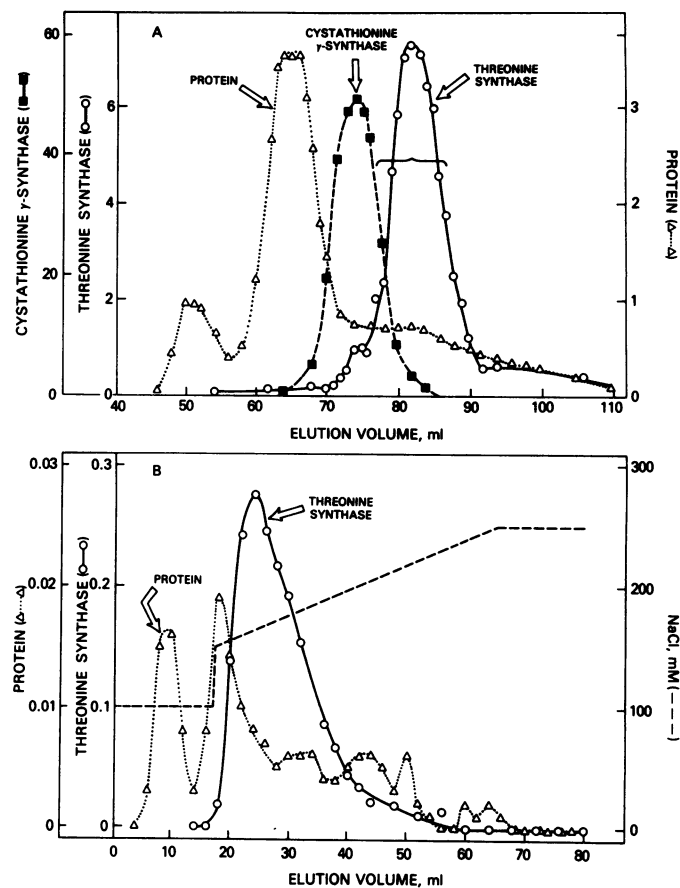


FIG. 2. Purification of TS and separation from cystathionine  $\gamma$ -synthase. Activities of TS and cystathionine  $\gamma$ -synthase are expressed as  $\text{nmol}/\text{min} \cdot \text{fraction}$ , and protein as  $\text{mg}/\text{fraction}$ . A, Gel filtration with Sephacryl S-300 of 43% to 62%  $(\text{NH}_4)_2\text{SO}_4$  fraction, collected in 1-ml fractions. B, DEAE-Sephacel chromatography of pooled fractions (indicated by bracket) from A. The broken line indicates the approximate calculated concentration of NaCl in the fractions (each 2 ml).

specific for synthesis of threonine. Little, if any, synthesis of *allo*-threonine or homoserine occurred. Neither sulfide nor cysteine were effective nucleophiles since a DEAE-Sephacel fraction failed to form detectable homocysteine or thiothreonine from sulfide, or cystathionine or  $\beta$ -methylanthionine from cysteine. Upper limits for synthesis of these amino acids relative to threonine were: homocysteine, 0.3%; *threo*-thiothreonine, 0.2%; *erythro*-thiothreonine, 0.2%; cystathionine, 0.5%; and  $\beta$ -methylanthionine, 3%.

**Reversibility of AdoMet Stimulation.** Crude extracts were preincubated for 10 min at 30°C either in the presence or absence of 0.1 mM AdoMet, then gel filtered and assayed in the absence or presence of AdoMet. Identical activities were obtained following each preincubation, *i.e.* low activities for assays in the absence of AdoMet, and approximately 20-fold stimulations for assay under standard conditions with 0.05 mM AdoMet.

**Activity as a Function of OPH and AdoMet Concentrations.** The effects on TS activity of increasing concentrations of OPH at several fixed concentrations of AdoMet are shown in Figure 3A. In the absence of AdoMet, the rates were too low to define the relationship between velocity and OPH concentration. In the presence of AdoMet, TS activity increased hyperbolically with increasing concentrations of OPH. Eadie-Hofstee plots (Fig. 3B) confirmed the Michaelian relationship between velocity and OPH concentration, and provided no indication of curvature characteristic of cooperativity. The results of Figure 3 further show that increasing concentrations of AdoMet resulted in in-

Table II. Specificity of TS for Carbon Substrate

The extent to which each specified unlabeled compound inhibited conversion by a DEAE-Sephacel enzyme fraction of radioactivity from [<sup>14</sup>C]OPH to threonine was determined. The affinity ( $K_m$  or  $K_i$ ) of the compound for threonine synthase was calculated assuming linear competition with [<sup>14</sup>C]OPH (24), and expressed as a multiple of the  $K_m$  for OPH. The values of  $K_m$  or  $K_i$  for compounds that caused no detectable inhibition were calculated assuming that 10% inhibition could have escaped detection. The values of  $K_m$  for OPH used in these studies were determined in analogous experiments from the inhibition of <sup>14</sup>C conversion to threonine that resulted from addition of unlabeled OPH (24). Except as noted in footnote d, assays were under standard conditions at pH 8.0, and calculations were based on a mean value of 5.1  $\mu$ M ( $n = 7$ ; SE = 0.6) for the  $K_m$  of OPH determined at this pH. Because of their increased instability at higher pH, compounds specified in footnote d were assayed at pH 7.0 (K Hepes), and calculations were based on a mean value of 3.9  $\mu$ M ( $n = 2$ ; SE = 1.0) for the  $K_m$  of OPH determined at pH 7.0. The determinations were not sufficiently accurate to conclude whether the  $K_m$  values determined at pH 7.0 and 8.0 were significantly different.

Compound	Concn.	Inhibition	Relative <sup>a</sup> $K_m$ or $K_i$
	$\mu$ M	%	
O-Phospho-L-homoserine	— <sup>b</sup>	— <sup>b</sup>	(1)
O-Phospho-DL-threonine	360	48	19 <sup>c</sup>
O-Phospho-L-serine	1000	38	160
O-Succinyl-L-homoserine	180	0 (<10)	>160
O-Malonyl-L-homoserine	180	0 (<10)	>160
O-Acetyl-L-homoserine	1000	0 (<10)	>1000 <sup>d</sup>
O-Oxalyl-L-homoserine	1000	0 (<10)	>1000 <sup>d</sup>
L-Homoserine	180	0 (<10)	>160
DL-Vinylglycine	360	0 (<10)	>160 <sup>c</sup>
L-Allylglycine	1000	0 (<10)	>900
O-Acetyl-L-serine	1000	0 (<10)	>1000 <sup>d</sup>
$\beta$ -Chloro-L-alanine	1000	0 (<10)	>900
$\beta$ -Cyano-L-alanine	1000	0 (<10)	>900
L-threo- $\alpha$ -Amino- $\beta$ -chlorobutyric acid	1000	0 (<10)	>900

<sup>a</sup> Expressed as a multiple of the  $K_m$  for OPH.

<sup>b</sup> No single value is shown since the  $K_m$  values for OPH were determined at pH 8.0 and 7.0, and with various concentrations of OPH.

<sup>c</sup> Calculation was based on assumption that only the L-isomer (180  $\mu$ M) was active.

<sup>d</sup> Determined at pH 7.0.

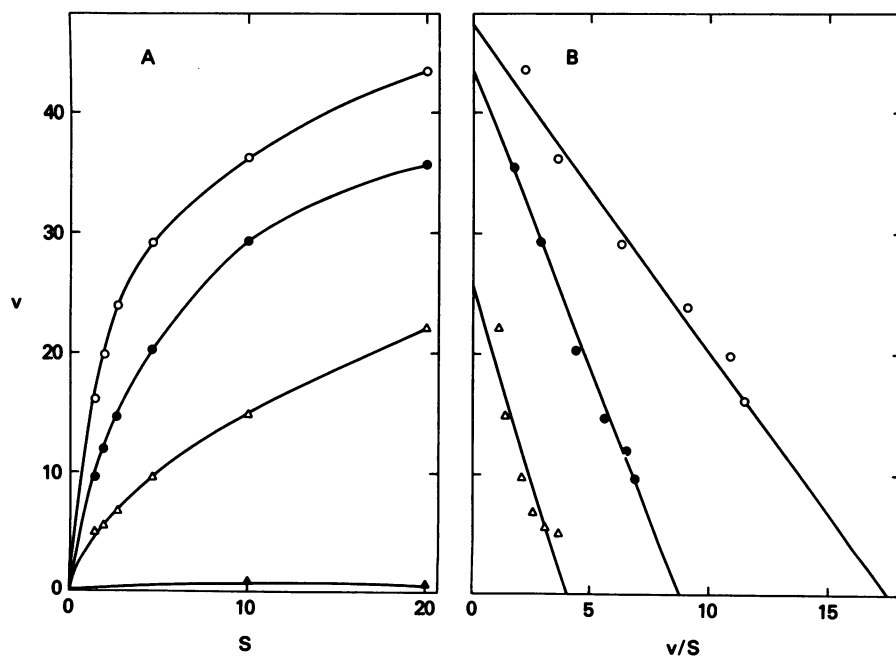


FIG. 3. Effect of increasing concentrations of OPH on TS activity at fixed concentrations of AdoMet. A DEAE-Sephacel fraction was used.  $v$  = velocity of threonine synthesis in pmol/40 min.  $S$  = OPH concentration ( $\mu$ M). AdoMet was added at the following concentrations ( $\mu$ M): ( $\Delta$ ), zero; ( $\Delta$ ), 25; ( $\bullet$ ), 40; ( $\circ$ ), 120. A, Plots of  $v$  versus  $S$ . B, Eadie-Hofstee plots. Values of  $V_{max}$  are given by the intercepts on the  $y$  axis, and of  $K_m$  for OPH by the slopes of the lines. Values of  $V_{max}$  and  $K_m$ , as well as the lines of best fit, were calculated according to Zivin and Waud (33). The following values (SE in parentheses) were calculated at the respective concentrations of 25, 40, and 120  $\mu$ M AdoMet:  $V_{max} = 25.6$  (3.3), 43.6 (1.3), and 47.2 (1.6) pmol threonine/40 min;  $K_m = 6.4$  (1.3), 5.0 (0.3), and 2.6 (0.2)  $\mu$ M.

creasing values of  $V_{max}$ . While the results of Figure 3B suggest that increasing concentrations of AdoMet may result also in decreasing values of  $K_m$  for OPH, the decreases were small and the  $K_m$  values were all within the range (2.2–6.9  $\mu$ M) of nine determinations made at saturating AdoMet.

Figure 4 illustrates the virtually absolute requirement for AdoMet and that this stimulation is cooperative with a Hill coefficient ( $n_H$ ) of 2.5, and a value of 40  $\mu$ M for  $[AdoMet]_{0.5}$ , the concentration of AdoMet supporting one-half  $V_{max}$ . Similar properties were observed with a crude extract (data not shown), but

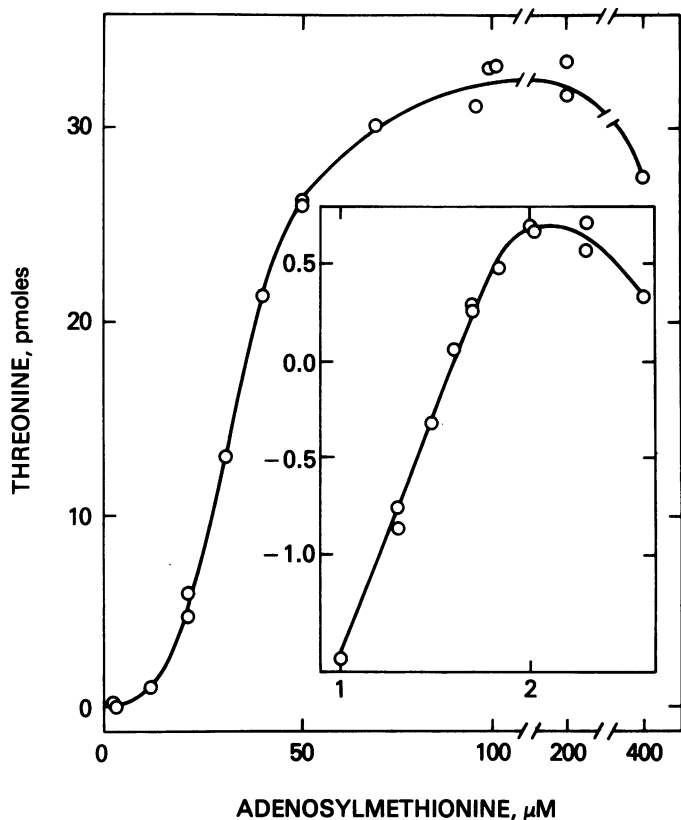


FIG. 4. Stimulation of TS by AdoMet. A DEAE-Sephacel fraction was incubated in the standard assay for 40 min. The insert is a Hill plot of  $\log[v/(V_{max}-v)]$  versus  $\log$  AdoMet.  $V_{max}$  of 40 pmol/40 min was calculated by extrapolation of a plot of  $1/v$  versus  $1/\text{AdoMet}$  to zero on the  $1/\text{AdoMet}$  axis. The line of best fit for the Hill plot was determined for values on the  $x$  axis between 1.0 and 1.845.

the  $n_H$  of 1.2 and  $[\text{AdoMet}]_{0.5}$  of 20  $\mu\text{M}$  were significantly less than those determined with the DEAE-Sephacel fraction.

**Effects of Inhibitors.** TS and cystathionine  $\gamma$ -synthase share the same substrate (OPH) in plants, and are believed to have similar mechanisms (12). The rates of irreversible inhibition of TS activity by the mechanism-based inhibitors PAG, vinylglycine, cysteine, and gabaculine, were therefore determined, and compared to the effects of these compounds on cystathionine  $\gamma$ -synthase. Crude extracts were preincubated in the presence of each compound for 10 min at 30°C in 20 mM K Hepes (pH 7.8), then gel filtered, and assayed. A control extract was treated identically in the absence of the potential inhibitor. No irreversible inhibition of TS was detected after preincubation with 50  $\mu\text{M}$  PAG either in the absence or presence of 0.1 mM AdoMet. TS was similarly insensitive to PAG *in vivo*. TS specific activity from plants growing in 10 nM PAG was not significantly different from that of control plants. Even growth in 300 nM PAG (in medium supplemented with 2  $\mu\text{M}$  methionine to permit steady state growth [7]) did not appreciably reduce TS activity below that of plants growing in 2  $\mu\text{M}$  methionine alone. This insensitivity of TS to PAG is in sharp contrast to the previously reported potent *in vitro* and *in vivo* irreversible inhibition by this compound of *Lemna* cystathionine  $\gamma$ -synthase (27). DL-Vinylglycine and L-cysteine, each at 10 mM, irreversibly inhibited TS activities by only 22% and 17%, respectively, much lower inhibitions than reported for *Lemna* cystathionine  $\gamma$ -synthase under comparable conditions (27). TS was not irreversibly inhibited by preincubation with 50  $\mu\text{M}$  gabaculine (in the presence of 0.2 mM AdoMet), while cystathionine  $\gamma$ -synthase was appreciably inhibited (G. A. Thompson, in preparation).

Table III. TS Activities and Amino Acid Contents of Plants Growing in Steady States in the Presence of Amino Acids

Plants were grown in the presence of supplements for at least three generations before analysis. TS activities on a protein basis are expressed as percentages of the mean value for 13 control cultures (range in parenthesis). TS of plants supplemented with 2  $\mu\text{M}$  threonine and/or isoleucine were each determined on one culture. All other values for TS extracted from supplemented plants are means of three cultures (individual values shown in parentheses). Values for threonine and isoleucine contents are means determined on duplicate cultures used also for TS assays. Each of the duplicate determinations of amino acid content deviated from the mean value by less than 20%.

Supplement ( $\mu\text{M}$ )	TS	Amino Acid Content	
		Threonine	Isoleucine
	%	<i>mM</i>	
None	100 (87–120)	0.18	0.03
Threonine (2)	117		
Threonine (3)	98 (94, 99, 102)	4.5	0.09
Isoleucine (2)	89		
Isoleucine (3)	97 (72, 106, 112)		2.8
Threonine (2) + isoleucine (2)	78		
Threonine (3) + isoleucine (3)	87 (72, 92, 97)	6.7	2.8
Methionine (0.67)	67 (59, 69, 72)		
Methionine (2)	45 (42, 43, 50)		

**Regulation of TS.** Table III summarizes TS activities and amino acid contents of *Lemna* grown in the presence of various amino acids of the aspartate pathway compared to the corresponding values for control plants. Supplementation with threonine and/or isoleucine did not significantly affect TS activities, in spite of the large increases within the plant of the amino acid used for supplementation. The most pronounced effects on TS were observed with methionine supplementation, which caused a dose-dependent decrease in TS activity.

TS activity of a DEAE-Sephacel fraction was unaffected by addition of 10 mM threonine or methionine to the assay. The following amino acids (each 10 mM) inhibited less than 15%: isoleucine; threonine plus isoleucine; valine; threonine plus isoleucine plus valine. Cysteine (1 mM) inhibited 26%.

## DISCUSSION

The enzymes for threonine and cystathionine synthesis are similar in plants, each catalyzing a nucleophilic attack on OPH with elimination of Pi. For threonine synthesis (reaction 1), the nucleophile is water (hydroxyl ion); for cystathionine synthesis (reaction 2), it is cysteine:



During gel filtration on Sephacryl S-300, TS and cystathionine  $\gamma$ -synthase activities were separated in good yields, establishing for the first time that these reactions are catalyzed by separate enzymes<sup>7</sup> in plants.

TS from *Lemna* is highly specific for both the four-carbon and nucleophilic substrates. The importance of the phosphate ester

<sup>7</sup> Several factors could explain the slight irregularity in elution profile of TS around 74 ml (Fig. 2B): (a) experimental error in assaying the low TS activities eluting near 74 ml; (b) a separate minor species of TS which would comprise no more than 5% of the total TS activity; or (c) a relatively low rate of synthesis of threonine by cystathionine  $\gamma$ -synthase.

group of OPH is demonstrated (Table II) by the findings that the affinities of TS for a variety of *O*-acyl-homoserine esters, and homoserine itself, were each at least two orders of magnitude less than that for OPH. The poor binding of *O*-phosphothreonine and *O*-phosphoserine suggests that optimally the phosphate is attached to a  $\gamma$ -carbon. The importance of the phosphate group is additionally supported by the findings that the low affinities for *O*-phosphothreonine and *O*-phosphoserine were decreased further by replacement of the phosphate groups of these compounds by other substituents, as in  $\alpha$ -amino- $\beta$ -chlorobutyric acid, *O*-acetylserine,  $\beta$ -chloroalanine, and  $\beta$ -cyanoalanine. Similar patterns to those described in Table II for partially purified TS were observed with crude extracts (data not shown). The  $K_m$  for OPH determined with a DEAE-Sephacel fraction at 100  $\mu\text{M}$  AdoMet, a concentration approaching saturation, ranged from 2.6 to 6.9  $\mu\text{M}$  (nine determinations). Similar values were obtained during limited studies with crude extracts. These  $K_m$  values are at least two orders of magnitude less than those reported for the TS of a crude extract of sugar beet leaves (19) or a partially purified preparation from peas (26). While this difference in  $K_m$  values may indeed be real, contributions by factors such as the two described below have not been excluded. (a) The results of the assays of TS may be affected by the presence of other enzymes that catalyze metabolism of OPH and/or threonine. That this is not the case in the present work is indicated by the findings that products other than threonine were not detected, and that [ $^{14}\text{C}$ ] threonine was recovered quantitatively. (b) The apparent  $K_m$  for OPH would be increased in the presence of an inhibitor that is competitive with OPH. Pi is a potent (30) and competitive (J. Giovanelli, unpublished) inhibitor of TS synthase from *Lemna*. Pi was reported to have no effect on pea TS (26), but this anion was tested in the presence of a high concentration of OPH, so that potential competitive inhibition might have been missed. Thus, the  $K_m$  reported for OPH may have been increased by the unspecified amount of Pi added to the assay because the enzyme was dissolved in phosphate buffer (26).

TS from *Lemna* also showed marked specificity for the hydroxyl ion. Nucleophilic attack by hydroxyl ion is restricted to carbon-3 of OPH, proceeds stereo-specifically yielding threonine, rather than *allo*-threonine. Neither sulfide nor cysteine were effective substrates for TS, even at concentrations 5100- and 300-fold greater, respectively, than hydroxyl ion. Consistent with previous studies with crude extracts of *L. paucicostata* (10), no synthesis of thiothreonine was detected with partially purified TS in the presence of sulfide. The mechanism by which crude extracts of peas catalyze synthesis of thiothreonine (22) therefore remains undefined.

Although the catalytic mechanisms of TS and cystathionine  $\gamma$ -synthase have not been examined directly in plants, in microorganisms (14, 15, 32) each of these enzymes requires removal of a  $\beta$ -proton of the substrate, followed by formation of the intermediate vinylglycine. The effectiveness of PAG as a suicide inhibitor of pyridoxal phosphate enzymes is based on the ability of the target enzyme to abstract a  $\beta$ -proton from its natural substrate (32). In contrast to the potent inhibition by PAG of *Lemna* cystathionine  $\gamma$ -synthase, no effect was observed on TS either *in vitro* or *in vivo*. Gabaculine, which appears also to be specific for pyridoxal phosphate enzymes that abstract  $\beta$ -protons (25), had little effect on TS at a concentration that caused appreciable irreversible inhibition of cystathionine  $\gamma$ -synthase. The poor inactivation of TS by vinylglycine is consistent with this compound showing little or no affinity for the enzyme (Table II). It would be premature to suggest from these combined data that the mechanism of plant TS is different to that in microorganisms. For example, failure to observe appreciable inactivation by PAG or gabaculine could occur if a  $\beta$ -carbanion species failed to react further with a site on the enzyme required for activity.

Previous studies have provided examples of pyridoxal phosphate enzymes that proceed via a vinylglycine intermediate, but which exhibit  $K_m$  values for vinylglycine that are much greater than those for the natural substrates (13, 18).

In agreement with previous reports of TS isolated from other plants sources (1, 19, 26), the enzyme from *Lemna* showed a marked dependence on AdoMet. Stimulation by AdoMet was reversible and, at 5  $\mu\text{M}$  OPH<sup>8</sup> (approximating the concentration occurring naturally in *Lemna* [10]), showed cooperative kinetics, yielding an  $[\text{AdoMet}]_{0.5}$  of 40  $\mu\text{M}$  and an  $n_H$  of 2.5 for the partially purified enzyme. These values were lower (20  $\mu\text{M}$  and 1.2, respectively) for a crude extract, suggesting that during purification the enzyme may have undergone modification which reduced the affinity and increased the cooperativity for AdoMet. Our results are consistent with those of others (19, 26) in that AdoMet stimulated TS by increasing the  $V_{max}$ , but do not determine whether AdoMet also decreased the  $K_m$  for OPH, as reported for pea seedlings (26). We plan to examine the structural specificity of the activation in detail, but preliminary studies confirm the report (19) of a high specificity for AdoMet. Thus, a crude extract of TS from *Lemna* was not activated by 120  $\mu\text{M}$  methionine, *S*-methylmethionine sulfonium ion, or *S*-adenosylhomocysteine, nor was activation by 120  $\mu\text{M}$  AdoMet affected by the addition of 120  $\mu\text{M}$  *S*-adenosylhomocysteine.

The present work supports and extends the suggestion of Madison and Thompson (19) of the central role of TS in the regulation of methionine biosynthesis. In this scheme, the primary signal of overproduction of methionine is an increased concentration of AdoMet, which allosterically stimulates TS and thereby diverts OPH into the threonine rather than the methionine branch. *Lemna* growing under standard conditions has an AdoMet concentration of approximately 15  $\mu\text{M}$  (8, 16). TS activity would be limited by this concentration of AdoMet to about 7% of  $V_{max}$  (determined with a DEAE-Sephacel fraction) or to about 30% of  $V_{max}$  (determined with a crude extract). When *Lemna* is supplemented with 0.67 or 2.0  $\mu\text{M}$  methionine, the steady state concentration of AdoMet increases 6-fold or more (8, 16) and net biosynthesis of methionine is reduced by approximately 80% (16, 17). From the cooperative relationships described, these increased concentrations of AdoMet will result in large increases in TS activity to values approaching  $V_{max}$ . This initial increase in threonine synthesis, if allowed to continue, would present the plant with the problem of threonine overproduction. Two schemes have been proposed to avoid this problem (see 16). First, the increased concentration of threonine may decrease the flux between aspartate and OPH by feedback inhibition of homoserine dehydrogenase by threonine, and of aspartokinase by threonine and/or lysine. A recent report (2) suggests that feedback inhibition of homoserine kinase by threonine, isoleucine, and AdoMet may also help to reduce this flux. Second, the initial diversion of OPH into threonine rather than into cystathionine (methionine) biosynthesis may cause an accumulation of cysteine which, in turn, may inhibit TS. Inhibition of TS in this way does not appear to be physiologically important. As previously reported for TS from other plant tissues (1, 19, 26), cysteine was not a potent inhibitor of the *Lemna* enzyme. The concentration of 1 mM cysteine required for 26% reversible inhibition greatly exceeds the concentration of 20  $\mu\text{M}$  (8, 16) in control plants, or the increased concentrations (up to 70  $\mu\text{M}$ ) resulting from methionine supplementation (8). One additional factor may now be suggested to limit the overproduction of threonine, namely, down-regulation of TS by methionine (Table III). Further tentative support for regulation of TS by methionine is provided by a single experiment with plants growing with

<sup>8</sup> Estimates of the physiological concentrations of this and other compounds in *Lemna* assume uniform distribution throughout the plant.

lysine (36  $\mu\text{M}$ ) plus threonine (3  $\mu\text{M}$ ). The methionine content was reduced to approximately 50% of that in control cultures (8), and the specific activity of TS was increased to 130%.

Although it has not been established in *L. paucicostata* whether or not threonine regulates specifically its own synthesis *in vivo*, there is evidence for such regulation in other plant tissues (see 4). For example, Bright *et al.* (3) reported that in wheat seedlings threonine almost completely inhibits its own synthesis without affecting methionine biosynthesis. This observation cannot be explained only by the feedback inhibitions described above for enzymes (aspartokinase, homoserine dehydrogenase, and homoserine kinase) that are common to the methionine and threonine branches. Bright *et al.* (3) proposed that a controlling site "would have to be at TS as it is the only known enzyme after the branch to threonine". The present studies fail to establish any regulatory property of TS that would allow threonine to regulate specifically its own synthesis. That control of TS by feedback inhibition is probably not of major importance is indicated by the finding that neither threonine, isoleucine, threonine plus isoleucine, valine, threonine plus isoleucine plus valine, nor methionine appreciably inhibited TS at 10 mM concentrations of each amino acid. Similar findings have been reported for TS isolated from other plants (1, 26). The concentration of 10 mM far exceeds the physiological concentrations of these amino acids in *Lemna* growing under standard conditions (Table III; 8, 16; J. Giovanelli, unpublished), and is slightly greater than the concentrations of threonine and isoleucine (Table III) and of methionine (8, 16) accumulating in plants growing in supplemented medium. The results of Table III further argue against major down-regulation of TS by repression or covalent modification due to exogenous threonine and/or isoleucine. No appreciable decreases in TS were detected in plants supplemented with amounts of these amino acids sufficient to bring about large increases in their tissue concentrations, and close to the maxima which could be tolerated without causing severe growth inhibition.

These findings suggest a need to seek for mechanisms other than feedback inhibition or down-regulation of TS to control the flux into the threonine biosynthetic branch. One possibility is that regulation may proceed predominantly at threonine-sensitive aspartokinase (20, 21), with the products of this step being selectively channeled into the threonine branch. Two crucial elements would be required for this proposal to be tenable: (a) the presence of at least two aspartokinases, one of which is controlled by threonine; (b) synthesis of threonine predominantly from the products of the threonine-sensitive aspartokinase. In certain enterobacteria, both the first (29) and second (5, 6) requirements have been satisfied, and there is evidence to suggest that feedback regulation of threonine-sensitive aspartokinase is indeed the most important factor in regulating threonine biosynthesis (for review, see 29). In plants, while the first requirement has been met (4, 21), the question of whether threonine synthesis is normally dependent predominantly on the products of the threonine-sensitive aspartokinase has yet to be examined.

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