Homocysteine Biosynthesis in Green Plants

O-PHOSPHORYLHOMOSERINE AS THE PHYSIOLOGICAL SUBSTRATE FOR CYSTATHIONINE \(\gamma\)-SYNTHASE

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SUMMARY

Optimal conditions for biosynthesis of cystathionine with crude extracts of green plants were determined. The specific activities observed under these conditions are considerably higher than those initially detected (GIOVANELLI, J., AND MUDD, S. H. (1966) Biochem. Biophys. Res. Commun. 25, 366), and compare favorably with those of bacterial extracts. At near saturating concentrations of homoserine esters, crude extracts of tissues representing most of the major phylogenetic divisions of green plants generally catalyze the highest rates of cystathionine synthesis in the presence of O-malonylhomoserine, intermediate rates in the presence of O-oxalyl-, O-succinyl-, and O-phosphorylhomoserine, and very low rates with O-acetylhomoserine. Green plants are unique among the organisms studied by us or other workers in their capacity to use O-phosphorylhomoserine in cystathionine synthesis.

Enzymic assay systems permitting the detection of compounds capable of serving as \(\alpha\)-aminobutyryl donors in the synthesis of cystathionine were developed, taking advantage of the fact that crude preparations of plant cystathionine \(\gamma\)-synthase are active with a wide range of compounds. An analogous system designed to detect O-acetylhomoserine with greater sensitivity was developed through the use of an enzyme preparation from Bacillus subtilis. An \(\alpha\)-aminobutyryl donor was purified by ion exchange chromatography and paper electrophoresis from tissues of green plants ranging from the green alga Chlorella to the higher flowering plants. Only a single \(\alpha\)-aminobutyryl donor was detected among these green plants. This compound was characterized as O-phosphorylhomoserine on the basis of its base stability, its behavior during ion exchange chromatography and paper electrophoresis, and a rate of hydrolysis by highly purified alkaline phosphatase that was indistinguishable from that of authentic O-phosphorylhomoserine.

It is proposed that O-phosphorylhomoserine is the dominant physiological precursor of cystathionine in green plants. Some of the implications of this finding are discussed.

During the course of this work, studies on the relative activity of cystathionine \(\gamma\)-synthase with various substrates were extended to a number of lower organisms. The patterns of substrate activity with crude extracts of a blue-green alga and Escherichia coli are very similar, and resemble the pattern for cystathionine \(\gamma\)-synthase of Salmonella typhi-murium (KAPLAN, M. M., AND FLAVIN, M. (1966) J. Biol. Chem. 241, 4463). O-Succinylhomoserine is the most active substrate for all three organisms. Extracts of B. subtilis are most active with O-acetylhomoserine, a pattern not previously observed with bacterial cystathionine \(\gamma\)-synthases.

Over the past several years accumulating evidence has indicated that there is wide diversity in the means whereby different biological forms synthesize the methionine precursor, homocysteine. In this laboratory, we have concerned ourselves with the pathway for homocysteine formation in green plants. Among the reasons for this investigation is the fact that green plants are a major source of the methionine required by humans and most other mammals. As emphasized by Allaway (1), “the need for supplying the essential sulfur amino acids will occupy an increasingly prominent position in the world food problem.”

Our studies (2-4) have shown that crude extracts of spinach catalyze Reactions 1 to 3.

In Reaction 1, homocysteine is formed directly by a sulfhydrase

\[
\text{H}_{2}\text{~S} + \text{X(CH}_{2}\text{CH(NH}_{2}\text{)COOH} \xrightarrow{\text{sulfhydrase}} \text{SH(CH}_{2}\text{CH(NH}_{2}\text{)COOH} + \text{XH} \quad (1)}
\]

\(\alpha\)-aminobutyryl donor

In Reaction 2, cystathionine is formed from cysteine and homocysteine

\[
\text{HOOC(CH}_{2}\text{CH(NH}_{2}\text{)COOH} + \text{X(CH}_{2}\text{CH(NH}_{2}\text{)COOH} \xrightarrow{\text{cystathionine } \gamma\text{-synthase}} \text{HOOC(CH}_{2}\text{CH(NH}_{2}\text{)COOH} + \text{XH} \quad (2)}
\]

\(\alpha\)-aminobutyryl donor

In Reaction 3, homocysteine is formed from cystathionine and water

\[
\text{HOOC(CH}_{2}\text{CH(NH}_{2}\text{)COOH} + \text{water} \xrightarrow{\beta\text{-cystathionase}} \text{H}_{2}\text{~S} + \text{X(CH}_{2}\text{CH(NH}_{2}\text{)COOH} \quad (3)}
\]

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tion reaction in which an α-aminobutyryl moiety is transferred to H₂S. In Reactions 2 and 3, which together comprise the trans-sulphuration pathway, homocysteine is formed by way of the intermediary thioether, cystathionine. In this pathway the initial step also involves α-aminobutyryl transfer, but in this instance cysteine is the acceptor.

The presence of these enzymes suggests that green plants may synthesize homocysteine by either of two alternative pathways, or by a combination of the two. Little additional evidence is available concerning the direct sulphhydration pathway. The following additional evidence supports the occurrence of the transsulphuration pathway. (a) Cystathionine accumulates in a few plants in relatively high concentrations (5-7). Recent application of a highly sensitive enzymic assay for cystathionase has indicated that low concentrations of this compound may be widespread in plants.  
(b) β-Cystathionase, the enzyme catalyzing the cleavage of cystathionine to homocysteine (Reaction 3), has not only been obtained in partially purified form from spinach, but the activity has been demonstrated in all other plant tissues in which it was sought (4). (c) The naturally occurring compound, rhizobitoxine, inhibits plant β-cystathionase both in vivo (8) and in vivo (9). Corn seedlings treated with this toxin and fed SO₄²⁻ accumulated up to 22 times as much radioactivity in cystathionine as did control seedlings, virtually proving that the transsulphuration pathway contributes to homocysteine formation in this plant (9).

These results are compatible with the operation of both the direct sulphhydration and transsulphuration reactions, but leave open the question: Under physiological conditions what are the contributions of these two pathways to homocysteine biosynthesis in green plants? The same question has arisen in the study of Neurospora crassa, Salmonella typhimurium, and Saccharomyces sp. Although there are indications that both pathways may contribute in each organism, it has been suggested that transsulphuration predominates in Neurospora (11, 12) and Salmonella (13), whereas sulphhydration may predominate in Saccharomyces (14-16).

For each pathway, the direct sulphhydration, and the transsulphuration, a further question may be posed: What is the chemical identity of the α-aminobutyryl donor? Early experiments with radioactive compounds established that the carbon skeleton of methionine is derived from homoserine (17, 18). More recently, many lines of evidence have shown that homoserine must be converted to an activated form prior to its participation in the α-aminobutyryl transfer reactions in question. In all cases studied heretofore this activation is brought about by esterification (more specifically, acylation) of the hydroxyl group of homoserine. Once again biological diversity exists, since genetic and biochemical evidence concur in demonstrating that in Escherichia coli and S. typhimurium the important activated form of homoserine is the O-succinyl derivative (19, 21), whereas in N. crassa and Bacillus subtilis it is the corresponding O-acetyl compound (24-28). As for green plants, our preliminary studies have shown that the sulfhydrase of crude spinach extracts is active with O-acetylhomoserine, but much less active with O-succinyl- or O-ethylhomoserine (3). These same extracts could synthesize cystathionine in the presence of either O-succinyl-, O-acetyl- (2), or O-ethylhomoserine (3).

This brief review makes it evident that in order to define the pathway for homocysteine biosynthesis in green plants two major questions must be settled. The relative contributions of the direct sulphhydration and the transsulphuration pathways must be elucidated, and for each pathway, the nature of the activated homoserine derivative which serves as α-aminobutyryl donor must be determined. In an effort to gain further understanding of these questions, we have investigated cystathionine γ-synthase activities in crude extracts of a variety of green plants. Studies of the properties of cystathionine γ-synthase led to unexpected findings with regard to potential α-aminobutyryl donors, and made it possible to obtain definitive evidence as to the functional α-aminobutyryl donor for cystathionine synthesis in green plants. A preliminary report has been presented (29).

**EXPERIMENTAL PROCEDURE**

**Materials**

Source of Chemicals The following chemicals were obtained from the sources indicated: DTTP, L-cysteine, O-succinyl-L-homoserine, L-cystathionine, pyridoxal-P, Aquacide II, and HEPES from Calbiochem; Sephadex G-25 (medium) and G-50 (fine) from Pharmacia Fine Chemicals; MOPS from Schwarz-Mann. Ammonium sulphate was Mann Special Enzyme Grade. "Baker Analyzed" (J.T. Baker Chemical Co.) formic acid was used exclusively. Dowex 50 and Dowex 1 were obtained from Bio-Rad Laboratories under the respective designations of AG 50W-X4, 200 to 400 mesh and AG 1-X10, 100 to 200 mesh. Alkaline phosphatase, type III (E. coli), was obtained from Sigma Chemical Co.

Radiochemicals included: L-[G-3H]methionine, L-[G,3'-3H]cystine, and L-[U-¹⁴C]homoserine (Amersham-Searle); L-[G-3H]-homoserine (Calbiochem); L-[G,3'-3H]serine (New England Nuclear); and L-[U-¹⁴C]cystine (New England Nuclear and Schwarz-Mann).

Preparation of [H]Cystathionine—L-[G,3'-3H]Cystathionine was prepared from L-[G-3H]serine and L-homoserine by the method of Mudd et al. (30).

Preparation of Radioactive Cysteine—Up to 2 μmoles (1 mCi) of radioactive L-cysteine was reduced to radioactive L-cysteine by incubation at pH 9 for 10 min at 24° with 80 μmoles of DTTP. The solution was diluted to 20 ml and applied to a column (0.9 x 5.5 cm) of Dowex 50-H⁺. After a wash with 30 ml of water, the column was eluted with 4-ml portions of 0.4 N HCl. Those fractions collected between 13 and 28 ml contained at least 85% of the radioactivity; they were pooled and lyophilized. The residue was dissolved in water and stored at -65° under N₂.

**Chemical Determination of Cysteine**—The accurate measurement of cysteine required for determination of the stoichiometry of cystathionine synthesis was performed by the method of Ellman (31). It was independently demonstrated by means of automated amino acid analysis that the preparation of cysteine contained no detectable cysteine.

Radioactive Homoserine—L-[G,3'-3H]Homoserine and L-[U-¹⁴C]homoserine were obtained commercially and further purified by chromatography on Dowex 50-H⁺ as described below. L-[G-3H]Homoserine was also prepared from L-[G-3H]methionine by a modification of the method of Flavin and Slaughter (32).

The abbreviations used are: DTTP, dithiothreitol; pyridoxal-P, pyridoxal 5'-phosphate; MOPS, morpholinopropanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
1 ml of 18 N H2SO4 and 0.1 ml of methanol. The solution was diluted with water to 100 ml, and its pH adjusted to 7.1 by addition of 3 N NH4OH. Potassium phosphate, 1 ml, pH 7.1 (1 ml) was added to the solution, which was then diluted to 1 liter and applied to a column (3.3 x 4.2 cm) of Dowex 50-NH4+. The column was washed with 100 ml of water, and S-3H)methylmethionine eluted with 100 ml of 0.6 N NH4OH. The eluate was evaporated to dryness, then refluxed for 2 hours with 0.85 ml of 0.053 M potassium borate, pH 8.3. The solution was diluted with water to 10 ml, and applied to a column (0.9 x 2.9 cm) of Dowex 50-NH4+. [3H]Homoserine was recovered in the combined effluent and water wash (10 ml), and applied to a column (0.9 x 2.9 cm) of Dowex 50-H+. The column was washed with 10 ml of water, and [3H]homoserine eluted with 5 ml of 3 N NH4OH. This eluate was taken to dryness, and the residue dissolved in a minimal amount of water. The product contained 85% of the radioactivity originally present in methionine.

Electrophoresis in a solution of 0.46 M formic acid-1.39 M acetic acid, and chromatography in 2-propanol-88% formic acid-water (7:1:2) (Solvent A) failed to demonstrate any radioim-purity in any preparation of radioactive homoserine, and radio-purity was estimated to be greater than 90%.

Preparation of O-Phosphorylhomoserine—Two preparations of O-phosphoryl-L-homoserine were synthesized. The first preparation, designated O-phosphorylhomoserine (I), contained only a trace amount of [3H] which was incorporated chiefly to provide a convenient method of monitoring the course of the reaction and to facilitate subsequent purification. The amount of O-phosphorylhomoserine (I) prepared could also be readily calculated from the specific activity of the L-[3H]homoserine used and a determination of the [3H] content of O-phosphorylhomoserine (I). The second preparation, designated O-phosphoryl-[G-3H]homoserine (II), was prepared from [3H]homoserine of high specific activity.

O-Phosphorylhomoserine (I) was synthesized by yeast homoserine kinase in the reaction mixture described by Flavin and Slaughter (33). L-[G-3H]Homoserine (2.67 x 107 dpm, 0.11 µmole) was added to the reaction mixture containing 32 mmoles of L-homoserine. The time course of synthesis of O-phosphorylhomoserine was followed by determination of the amount of radioactivity that was not retained on Dowex 50-H+ (see below). Separate experiments demonstrated that no significant radioactivity appeared in this fraction when ATP was omitted from the reaction mixture. After 3.55 hours incubation at 30°, when 89% of the original [3H]homoserine was no longer retained on Dowex 50-H+, the reaction mixture was heated on a steam bath for 25 min, and filtered.

The following steps in the isolation and purification of O-phosphorylhomoserine (I) were performed at room temperature. The filtrate was diluted with water to a final volume of 20 liters and applied to a column (2.5 x 60 cm) of Dowex 1 Cr-. The column was washed with 600 ml of water. The effluent and water washings were discarded. The column was eluted with 7.5 mm HCl, and the radioactive peak eluting between 3250 ml and 5090 ml collected. Location of this radioactive peak was aided by the fact that it preceded the first ultraviolet-absorbing material to be eluted from the column. This material had a maximum A259 at 5570 ml, and overlapped slightly with the trailing edge of the radioactive peak. Ultraviolet-absorbing material in the radioactive fraction was removed by addition of decolorizing carbon (Norit A) prepared according to Smith (24). A solution (18.4 ml) containing 5.1 g of Norit A was added to 1840 ml of the radioactive eluate, the slurry stirred continuously for 30 min, then filtered. The Norit A was washed with 600 ml of water, and the combined filtrate lyophilized to yield a glassy residue. Water was added to the residue to a final volume of 500 ml, the solution applied to a column (0.9 x 12 cm) of Dowex 50-H+, and eluted with water. The radioactive material that eluted between 80 ml and 780 ml was lyophilized to dryness. The glassy residue was dissolved in water and the pH of the solution adjusted with KOH to 6 to 7. The solution contained 34% of the original radioactivity in [3H]homoserine, representing a yield of 5.4 mmoles of O-phosphorylhomoserine (I).

O-Phosphoryl-[G-3H]homoserine (II) was synthesized from L-[G-3H]homoserine (3.0 x 106 dpm, 16.1 µmoles) in a reaction containing the same components at the same concentrations as described for the synthesis of O-phosphorylhomoserine (I), except that NaCN was omitted. The reaction mixture contained only the L isomer of homoserine at the same concentration of L-homoserine used for synthesis of O-phosphorylhomoserine (I). After incubation for 3 hours at 30°, the reaction mixture was heated in a steam bath for 2 min. Insoluble material was removed by centrifugation, and the supernatant solution decanted. The pellet was suspended in 1 ml of water, and the slurry centrifuged. Both centrifugations were performed at 4° for 10 min at 200 x g. The supernatant solutions obtained from each centrifugation were combined and applied to a column (0.9 x 2.9 cm) of Dowex 50-H+. The column was washed with 7 ml of water, the combined effluent and wash solutions evaporated to dryness, and the residue dissolved in 0.42 ml of water. The yield of O-phosphoryl-[G-3H]homoserine (II) from [3H]homoserine was 54%.

O-Oxalylhomoserine—The preparation of O-oxalyl-L-homoserine was described previously (35).

O-Oxalyl-L-[G-3H]homoserine was prepared by incubation of L-[G-3H]homoserine and oxalyl-CoA with a crude extract of homoserine oxalyltransferase prepared from Lathyrus sativus seedlings. Oxalyl-CoA was prepared by the method of Quayle (36). The complete reaction mixture contained L-[G-3H]homoserine (6.7 x 107 dpm, 0.2 µmole), oxalyl-CoA (4 µmoles), an extract of L. sativus seedlings containing 4 mg of protein, and HEPES buffer, pH 7.7 (100 µmoles) in a final volume of 1.0 ml. After 30 min at 30°, the reaction was stopped by addition of 0.4 ml of cold 0.3 M trichloroacetic acid. Precipitated material was removed by centrifugation and the supernatant solution extracted with 2 ml of water-saturated ether. The extraction was repeated three times and the aqueous phase aerated with N2 to remove residual ether. The aqueous phase was applied to a column (0.9 x 2.9 cm) of Dowex 50-H+, which was then washed with 5 ml of water. O-Oxalyl-[G-3H]homoserine was recovered in the combined effluent and washings in a yield of 55% of [3H]homoserine. The compound conigrated with authentic O-oxalyl-L-homoserine when subjected to electrophoresis in 82.8 mm pyridine acetate (pH 5.3) for 1.3 hours, or chromatographed in 1-butanol-acetic acid-water (21:6:10) (Solvent B) or ethanol-water (77:23). In the presence of base, O-oxalylhomoserine undergoes a loss of ninhydrin reactivity (35) which has been ascribed to an O to N acyl transfer. O-Oxalyl-[G-3H]homoserine was mixed with authentic carrier O-oxalylhomoserine and incubated in 1 N KOH for 10 min at 30°. Radioactivity was found in a single peak of changed mobility during electrophoresis or chromatography in the above systems. The peak of radioactivity coincided with a compound which no longer reacted with ninhydrin, but could be visualized as a yellow spot after staining the chromatogram or electrophoretogram in an autoclave for 10 min to remove residual solvent, then dipping in a solution of 0.1% bromoresol green in...
ethanol. No radioimpurity could be detected in the preparation of O-oxalyl-[3H]homoserine, and radiopurity was estimated to be greater than 90%.

**O-Malonylhomoserine**—The preparation of O-malonyl-L-homoserine was described in (35). To obtain an estimate of the concentration of this compound for the stoichiometry experiment, the hydroxylamine assay was modified from that previously used (35). Sodium sulfate was decreased from 30 to 10 μmole, and the absorbance was determined 10 min after addition of FeCl3. An em of 780 at 540 nm for authentic malonylhydroxamate was obtained under these conditions.

**O-Malonyl-L-[U-14C]homoserine was prepared from [14C]homoserine and malonyl dichloride by a modification of the method of Hendrickson et al. (35).** L-[U-14C]Homoserine (6.25 pmoles, 4.44 × 10⁸ dpm), purified by elution from Dowex 50-H⁺ as described above, was evaporated to dryness in the bottom of a conical 12-
mL centrifuge tube. A mixture was prepared by cooling 37.5 ml of acetone almost to solidification in a Dry Ice-ethanol bath, and adding 1.25 ml of 60% perchloric acid, followed by 0.5 ml of malonyl dichloride. The mixture was maintained in the Dry Ice-ethanol bath during its preparation. To the [14C]homoserine were added 50 μl of the cold mixture, and the reaction incubated at 25° for 10 min. The following operations were performed at 4°. Water (25 μl) was added to the reaction mixture and it was extracted two times with 150 μl of ether. The aqueous phase was titrated with 0.5 M KHCO₃ until bromoresol green indicator was a light blue (pH approximately 4.5). The precipitate of potassium perchlorate was removed by centrifugation, and the supernatant solution retained. The precipitate was washed once with 0.5 ml of water, and the wash and supernatant solutions combined. This solution was subjected to electrophoresis for 1.25 hours in 0.02 M potassium phosphate, pH 6.6. The peak of radioactivity that migrated toward the anode between 14 and 16 cm was eluted and subjected to further electrophoresis for 2.5 hours in 1 M acetic acid (pH 2.5). The peak of radioactivity that migrated toward the cathode between 14 and 16 cm was eluted to yield a preparation of O-malonyl-L-[U-14C]homoserine with an over-all recovery of 57% of the radioactivity added as [14C]homoserine. As judged by electrophoresis for 1 hour in 0.02 M potassium phosphate, pH 6.6, in the presence of authentic carrier O-malonylhomoserine, the freshly prepared solution had a radiopurity of over 99%. The only detectable impurity was [14C]homoserine.

**O-Succinyl and O Acetylhomoserine**—O-Succinyl L homoserine was prepared according to the method of Flavin and Slaughter (37), and O-acetyl-L-homoserine essentially by the method of Nagai and Flavin (24).

**O-Acetyl-L-[α-3H]homoserine was prepared as follows.** A solution of L-[α-3H]homoserine (0.30 μmole, 1.21 × 10⁶ dpm) was lyopholized to dryness in a 300-μl Reactivial (Pierce Chemical Co.), then incubated at 25° for 1.25 hours with 50 μl of a freshly prepared solution containing acetic anhydride (1.0 ml), acetic acid (3.7 ml), and 60% perchloric acid (0.29 ml). Unless stated otherwise, the following procedures were performed at 4°. Water (100 μl) was added to the reaction mixture, which was then extracted with 200 μl of ether; this extraction was repeated twice. The aqueous phase was titrated with 0.5 M KHCO₃ in the presence of bromoresol green until the indicator was light blue. The precipitate of potassium perchlorate was removed by centrifugation, and the supernatant solution subjected to electrophoresis in 0.02 M potassium phosphate, pH 6.6, at 3000 volts for 35 min. [3H]Homoserine lactone migrated approximately 10 cm toward the cathode, while the major peak of radioactivity, a mixture of O-acetyl-[3H]homoserine and [3H]homoserine, remained at the origin. This peak was eluted with water and the eluate desalted by passage through a column (0.9 × 2.9 cm) of Dowex 50-H⁺. The resin was washed with 5 ml of water and then eluted with 10 ml of 1.3 M pyridine acetate, pH 5.2. The eluate was lyopholized to dryness, dissolved in a minimal volume of water and chromatographed at 25° in Solvent B for 24 hours. The major peak of radioactivity at 10 cm was effectively resolved from [3H]homoserine, and was eluted to yield a preparation of O-acetyl-[3H]homoserine with an over-all recovery of 15% of the radioactivity. O-Acetyl-[3H]homoserine was characterized by its cochromatography in Solvent B with authentic O-acetylhomoserine, and with authentic N-acetylhomoserine after incubation in 3 N NH₄OH for 10 min at 30°. No radioinactive impurity was detected during chromatography in Solvent B, and radiopurity was estimated to be at least 95%.

**O-Sulfurylhomoserine**—O-Sulfurylhomoserine was prepared by incubation of L-homoserine with H₂SO₄ (specific gravity 1.84) essentially as described by Dodgson et al. (38) for synthesis of O-sulfuryl derivatives of serine, threonine and hydroxyproline. Except for the substitution of homoserine for serine, the procedure for preparation of O-sulfurylhomoserine was followed through Dowex 50-H⁺ chromatography, which was performed at 4°. The combined effluent and washings were lyopholized to dryness. The white residue was dissolved in water, neutralized, and subjected to electrophoresis for 20 min in 33 mM pyridine acetate, pH 5.3. A single ninhydrin-positive band migrated toward the anode; this material, which moved 5.5 cm, was eluted to yield O-sulfurylhomoserine. The low over-all yield was probably due to the rapid lactonization of homoserine in H₂SO₄.

Evidence that the product was O-sulfurylhomoserine is as follows. (a) The method used is known to produce the O-sulfuryl derivatives of hydroxy amino acids (38). (b) The product exhibited chromatographic and electrophoretic properties during its purification that are consistent with the highly acidic nature of O-sulfurylhomoserine. (c) The product was quantitatively converted to homoserine by incubation in 0.5 M KOH at 100° for 15 min.

**Tissues**—Spinach (Spinacea oleracea L cv Dixie Market), parsley (Petroselinum crispum Nym. "Plain"), pea (Pisum sativum L. var Alaska) and Lathyrus sativus were grown in a greenhouse. In summer months spinach was also grown in a growth chamber at 18°, 98% relative humidity and irradiance of 220 microeinsteins m⁻² s⁻¹. Irradiance was measured with a LI-185 Quantum Meter (Lambda Instruments, Lincoln, Nebraska) equipped with a quantum sensor No. LI-190S designed to measure only photons in the 400 to 700 nm waveband. Corn (Zea Mays L. var U.S. No. 13) seedlings were grown at 30°, 58% relative humidity and irradiance of 220 microeinsteins m⁻² s⁻¹. Irradiance was measured with a LI-185 Quantum Meter (Lambda Instruments, Lincoln, Nebraska) equipped with a quantum sensor No. LI-190S designed to measure only photons in the 400 to 700 nm waveband. Barley (Hordeum vulgare L. var Wong) seedlings were grown in the same way, except the temperature was 20°. These seedlings were harvested after 4 days growth. Spruce (Picea Abies Karst.) seedlings were grown as described by Romberger et al. (39) and harvested at 15 days.

**Chlorella sorokiniana**—Shihira and Krauss, strain 7-11-05, was grown phototrophically in Roux bottles containing 500 ml of minimal medium consisting of the macronutrients described in (40) and the micronutrients described in (41). The cells were stirred and aerated with a stream of 1% CO₂ in air, at a temperature of 31° and an irradiance of 200 microeinsteins m⁻² s⁻¹. Cells were harvested late in the exponential phase of growth. The possible presence of contaminating microorganisms in each bottle was checked just prior to harvesting. One milliliter of cell sus-
pension was inoculated into 4 ml of minimal medium enriched with 0.1% yeast extract, 0.1% proteose peptone, and 0.5% glucose. A loop of cell suspension was also streaked onto agar (1.5%) plates of the same nutrient composition. Plates and tubes were incubated in the dark at 34°C for 48 hours to confirm the absence of contaminants before the Chlorella cells were utilized. No contamination was ever detected.

Axenic barley seedlings were obtained in the following way. Seeds of Hordeum vulgare var Betzes were soaked for 3 hours in 10% Clorox to which one drop of detergent was added. They were rinsed twice in sterile distilled water and the embryos were dissected aseptically from the seeds. The embryos were rinsed briefly in 10% Clorox and then rinsed twice in sterile distilled water. Five embryos were planted in each 50-ml flask containing 22 ml of medium containing lactate, glucose, yeast extract, and salts, and harvested by centrifugation after 7 days growth at 27°C. All manipulations were at 4°C.

Frozen pastes of E. coli, Strain B, ATCC 11303, and B. subtilis, ATCC 6633, were obtained from Miles Laboratories. E. coli was cultured on a yeast extract-glucose-salt medium and harvested in the mid log phase. B. subtilis was cultured on a medium containing lactate, glucose, yeast extract, and salts, and was harvested in the late log phase. Frozen cells of Anabaena flos-aquae (Lyngb.) delbr. were provided by Dr. L. D. Owens, United States Department of Agriculture, Beltsville, Md. All other plant tissues were obtained from actively growing specimens collected locally. Tissues were frozen at -65°C until used.

**Methods**

**General Methods**—Unless stated otherwise, the following methods were used. Paper electrophoresis was performed at 2500 volts at 4°C on Whatman No. 3MM paper with a model FP-22A Flat Plate Electrophoresis System (Savant Instruments). Paper chromatography was performed at room temperature on Whatman No. 1 paper with solvent descending. Two commonly used solvents were Solvent A, 2-propanol-88% formic acid-water (7:1:2) and Solvent B, 1-butanol-acetic acid-water (24:6:10). After radioactivity was determined on the 1-cm strips from chromatograms and electrophoretograms, the strips were removed from the phosphor and washed in ether before elution of radioactivity or visualization of amino acids with ninhydrin. Details of the determination of radioactivity have been described (9). Evaporation was carried out in vacuo at 25°C with a Rotary Evaporimix (Buchler Instruments).

Protein was determined by the method of Lowry et al. (45). Solutions of O-acylhomoserine esters were prepared by mixing sufficient crystals of the ester and a solution of 1 mM K$_2$HPO$_4$ to yield finally a solution which is 0.2 M with respect to the L isomer of the ester, in 0.05 M phosphate. The pH of this mixture was adjusted carefully with 1 M KOH to pH 6.5, and the final volume adjusted by addition of water.

**Preparation of Enzyme Extracts**—Crude extracts were prepared from all tissues, with the exceptions noted below, by the following standard procedure. Tissue was ground in a mortar with sand and 0.1 M potassium phosphate, pH 7.2, (2 to 2.4 ml per g of tissue). The brei was passed through cheesecloth and the filtrate centrifuged 10 min at 48,000 × g. The supernatant solution was subjected to gel filtration with Sephadex G-25 equilibrated with 0.1 M potassium phosphate, pH 7.2, then mixed with Tris-HCl (final concentration 0.2 M, pH 8.7) and pyridoxal-P (final concentration 0.025 M). Unbound pyridoxal-P was removed from the mixture by subjecting it to gel filtration with Sephadex G-25 equilibrated with 0.01 M Tris-HCl, pH 8.0. All manipulations were at 4°C.

Fern and conifer leaves treated by this standard method yielded extracts containing virtually no protein, presumably because the high concentration of phenolic compounds in these tissues rendered proteins nonextractable (46). Extraction in the presence of polyvinylpyrrolidone (46) or Dowex 1-Cl$^-$ (47) did not result in active extracts from fern fronds. However, active extracts were obtained by using the following modifications of the standard procedure. Fresh fern fronds were ground in liquid N$_2$, extracted in 0.2 M sodium borate, pH 7.6 (8 ml per g of tissue), and subjected to gel filtration with Sephadex G-50 (48). Active preparations from a conifer were obtained by extraction of spruce seedlings with 0.2 M sodium borate, pH 7.6 (5 ml per g of tissue).

Unicellular organisms were disrupted in a French press. E. coli cells were suspended in 0.05 M potassium phosphate, pH 7.2, containing 20 μM pyridoxal-P and 1 mM β-mercaptoethanol. Cells of the following organisms were suspended in 0.1 M potassium phosphate, pH 7.2: B. subtilis (4 ml per g of cells), Anabaena (3 ml per g of cells), and Chlorella (7 ml per g of cells). Each suspension was passed through a French press at 24,000 p.s.i. at 4°C. Centrifugation, gel filtrations, and pyridoxal-P treatment were the same as in the standard procedure.

**Assay of Cystathionine γ-Synthase**—In the standard procedure, the following (in micromoles) were incubated at 30°C in a final volume of 50 μl: L-cystathionine (0.007); DTT (0.032); L-[14C]cysteine (0.015) containing 4.4 × 10$^3$ dpm; MOIS, pH 7.7 (30); homoserine derivative (1.0); and crude extract containing 0.025 to 0.15 ng of protein. Incubation was in a stopped centrifuge tube flushed with N$_2$. The reaction was stopped by addition of 0.5 ml of a cold solution containing trichloroacetic acid (68.8%), L-cysteine (126 μmoles) and L-cystathionine (0.34 μmole). Insoluble material was removed by centrifugation and cystathionine isolated by chromatography on a column of Dowex 50-H$^+$ as previously described (30). The NH$_4$Cl eluate from the column was taken to dryness and dissolved in 50 μl of 88% formic acid. An aliquot (usually 35 or 40 μl) was spotted on paper and oxidized in the following manner. The spot was rinsed with a solution of 0.02% ammonium molybdate (40) and dried. It was then ringed with performic acid (9) and kept wet with performic acid for 1 hour. It was dried and subjected to electrophoresis in 0.46 M formic acid for 1.75 hours. The paper was dried for 1 hour at 70°C, cut into 1-cm strips, and the radioactivity determined. Separate experiments showed that 50 to 80% of [H, 14C]cystathionine added to the trichloroacetic acid solution used to stop the reaction could be recovered as cystathionine sulfone (9). Rates reported for cystathionine γ-synthase determined by the standard procedure have not been corrected for this loss.

**Assay of α-Aminobutyryl Donors in Plants**—Compounds capable of serving as α-aminobutyryl donors in the synthesis of cystathionine were assayed by two methods (A and B). Method A is based on the fact that the presence of additional α-aminobutyryl donor lowers the $^{14}$C ratio in cystathionine synthesized enzymically from O-phosphoryl-[PH]homoserine and [14C]cysteine.

The control reaction mixture for assay by Method A contained the following (in micromoles) in a final volume of 60 μl: L-cysta-
tion of a reasonable amount of cystathionine, minimizing the thionine (0.007); DTT (0.126); L-[14C]cysteine (0.054) containing 4 \times 10^6 dpm; O-phosphoryl-[3H]homoserine (0.003) containing 2.5 \times 10^6 dpm; MOPS, pH 7.25 (30); and spinach extract containing 0.35 to 0.5 mg of protein. Each experimental reaction mixture contained, in addition, an unknown, the \( \alpha \)-aminobutyryl donor content of which was to be determined. Incubation was for 1 hour at 30°. Radioactive cystathionine was isolated as cystathionine sulfone in the standard way, and the ratio of \( ^{3}H: ^{14}C \) was determined. Any \( \alpha \)-aminobutyryl donor present in the unknown sample would lower the specific activity of the \( ^{3}H: ^{14}C \) was determined. Any \( \alpha \)-aminobutyryl donor present in the unknown sample would lower the specific activity of the \( ^{3}H: ^{14}C \) ratio lower than that observed in cystathionine formed in the control vessel. The specific conditions in the control incubation were chosen chiefly on the basis of three considerations. First, the O-phosphoryl-[\( ^{3}H \)]homoserine content was kept as low as possible consistent with the production of a reasonable amount of cystathionine, minimizing the amount of unlabeled \( \alpha \)-aminobutyryl donor required in the experimental vessel to bring about a detectable change in the \( ^{3}H: ^{14}C \) ratio of the cystathionine. Second, the [\( ^{14}C \)]cysteine concentration was kept high to minimize the effect of any unlabeled cyst(e)ine added as a contaminant with samples to be assayed. Third, the specific radioactivities of the two substrates were chosen so that the \( ^{3}H: ^{14}C \) ratio of the cystathionine would be in the range permitting accurate determination of each isotope under our counting conditions.

Results of a representative experiment with authentic esters are shown in Fig. 1. As increasing amounts of each homoserine ester were added to experimental reaction mixtures the \( ^{3}H: ^{14}C \) ratio of the cystathionine formed was progressively lowered. Different amounts of each ester were required to lower the ratio by one-half, as would be expected since the effectiveness of a given \( \alpha \)-aminobutyryl donor in lowering this ratio will be a function of its \( K_m \) and \( V_{max} \) for cystathionine synthesis.

To provide a means of expressing the results of an assay of a preparation containing an unknown \( \alpha \)-aminobutyryl donor or a mixture of such donors, a unit of activity was defined as the amount of donor required to lower the \( ^{3}H: ^{14}C \) ratio to one-half the control value. The number of units added to a reaction mixture could then be calculated from the relationship:

\[
\frac{R_I}{R_0} = \frac{1 + U}{1}
\]

where, \( R_I = ^{3}H: ^{14}C \) ratio of cystathionine synthesized in the control vessel;

\( R_0 = ^{3}H: ^{14}C \) ratio of cystathionine synthesized in the experimental vessel;

\( U = \) number of units of \( \alpha \)-aminobutyryl donor added to the experimental vessel. Upon rearrangement,

\[
U = \frac{R_I - R_0}{R_0}
\]

Thus, after experimental determination of \( R_I \) and \( R_0 \), the number of units in an unknown sample could be calculated. The number of nanomoles required to contribute a unit of activity differs for each homoserine ester. Once the identity of an \( \alpha \)-aminobutyryl donor has been established, the number of nanomoles can be calculated from the units by use of the conversion factors specified in the legend to Fig. 1.

Assay with Method B is based on the observation that the rate of enzymic synthesis of [\( ^{14}C \)]cystathionine from [\( ^{14}C \)]cysteine is a linear function of low concentrations of \( \alpha \)-aminobutyryl donors. Method B was more sensitive than Method A, but had the disadvantage that it required additional reaction mixtures (see below) to correct for any inhibition of cystathionine synthesis caused by the extract to be assayed.

Extracts of cystathionine \( \gamma \)-synthase from both spinach and \textit{B. subtilis} were used to assay donor activity by Method B. The reaction mixture utilizing the spinach preparation contained the following (in micromoles) in a final volume of 60 \( \mu l: \) L-cystathionine (0.007); DTT (0.126); [\( ^{14}C \)]cysteine (0.0097) containing 3.4 \times 10^6 dpm; MOPS, pH 7.25 (30); and extract containing 0.3 to 0.5 mg of protein. Incubation was for 15 min at 30°. This mixture, with O-phosphorylhomoserine as standard (see below), was used for assays when unknown donors, or mixtures of donors, might be present. Once the identity of a donor was established, the appropriate ester was used as standard. Cystathionine \( \gamma \)-synthase from \textit{B. subtilis} was used to assay \( O \)-acyethylhomoserine, since this extract is some 50 times more active with this ester than in spinach extract. The reaction mixture for assay of \( O \)-acyethylhomoserine by Method B contained the following (in micromoles) in a final volume of 50 \( \mu l: \) DTT (0.126); [\( ^{14}C \)]cysteine (0.0106) containing 3.7 \times 10^6 dpm; MOPS, pH 7.25 (30); and \textit{B. subtilis} extract containing 0.62 mg of protein. Incubation was for 20 min at 30°. Fig. 2 illustrates that the incorporation of [\( ^{14}C \)]cysteine into [\( ^{14}C \)]cystathionine in the presence of O-phosphoryl-, \( O \)-oxalyl-, and \( O \)-acetylhomoserine at low concentrations is indeed linear under the assay conditions used for Method B.

The \( \alpha \)-aminobutyryl donor content of an unknown sample, assayed by Method B using either spinach or \textit{B. subtilis} cystathionine \( \gamma \)-synthase, was expressed as the rate of synthesis of
activity in cystathionine sulfone was at least 50%.

yielded cystathionine sulfone with a $^{3}\text{H} : ^{14}\text{C}$ ratio identical with thionine (uniformly labeled in the $^{3}\text{C}$ moiety) and $\text{L-}[G-^{3}\text{H}]$cystathionine, when subjected to the standard isolation procedure.

The over-all recovery of radioactivity in cystathionine sulfone was at least 50%.

The [${}^{14}\text{C}$]cystathionine synthesized was isolated as the sulfone after correction for any inhibition caused by the sample. For each series of tests concerned with a single ester, the following incubations were necessary to determine the uninhibited rate. The control incubation (1) lacked substrate, while the standard incubation (2) contained a known amount of homoserine ester. Each unknown sample was incubated alone (3) and with (4) the known amount of ester. The difference in incorporation of $^{14}\text{C}$ between 3 and 4, compared to 2, was a measure of inhibition which was applied to the incorporation of $^{14}\text{C}$ between 3 and 4, compared to 2,

The [${}^{14}\text{C}$]cystathionine synthesized was isolated as the sulfone in the standard way, except that the trichloroacetic acid mixture added to stop the reaction contained 0.42 mg of protein. Increasing amounts of $O$-phosphoryl- or $O$-oxalylhomoserine were incubated with [${}^{14}\text{C}$]homoserine in the presence of $B.\text{subtilis}$ extract containing 0.62 mg of protein. The amount of [${}^{14}\text{C}$]homoserine incorporated into cystathionine, isolated as the sulfone, was determined.

The [${}^{14}\text{C}$]cystathionine synthesized was isolated as the sulfone after correction for any inhibition caused by the sample. For each series of tests concerned with a single ester, the following incubations were necessary to determine the uninhibited rate. The control incubation (1) lacked substrate, while the standard incubation (2) contained a known amount of homoserine ester. Each unknown sample was incubated alone (3) and with (4) the known amount of ester. The difference in incorporation of $^{14}\text{C}$ between 3 and 4, compared to 2, was a measure of inhibition which was applied to the incorporation observed in 3. The corrected value for 3 was converted to nanomoles of ester by using the value for [${}^{14}\text{C}$] incorporation obtained in reaction 2 which contained the known amount of ester.

The [${}^{14}\text{C}$]cystathionine synthesized was isolated as the sulfone in the standard way, except that the trichloroacetic acid mixture added to stop the reactions contained 0.4 x $10^6$ dpm [${}^{14}\text{H}$]cystathionine. This addition permitted determination of recovery of cystathionine during the isolation procedure. Separate experiments showed that a mixture of authentic L-[${}^{14}\text{C}$]cystathionine (uniformly labeled in the $3\text{C}$ moiety) and L-[$G-^{14}\text{H}$]cystathionine, when subjected to the standard isolation procedure, yielded cystathionine sulfone with a $^{14}\text{H} : ^{14}\text{C}$ ratio identical with that of the original mixture. The over-all recovery of radioactivity in cystathionine sulfone was at least 50%.

The spinach enzyme used in both Methods A and B was prepared by concentration of the crude supernatant solution obtained after the initial centrifugation in the standard extraction procedure. The protein fraction that precipitated between 20 and 60% saturation with ammonium sulfate was dissolved in a minimal volume of 0.1 M potassium phosphate, pH 7.2, and subjected to the two gel filtration steps described for the standard extraction procedure. The enzyme extract of $B.\text{subtilis}$ used in Method B was prepared as described above, then concentrated 4- to 5-fold by "dialysis" against Aquacide II powder.

Isolation of $\alpha$-Aminobutyryl Donors—Briefly, $\alpha$-aminobutyryl donors were isolated and purified from a trichloroacetic acid extract of tissue by ion exchange chromatography followed by paper electrophoresis. Separation of $\alpha$-aminobutyryl donors by ion exchange chromatography (Fig. 3) was based on retention at neutral pH of acidic $\alpha$-aminobutyryl donors (e.g. O-succinyl-, O-malonyl- , O-oxalyl- , and O-phosphorylhomoserine) on Dowex 1-formate. Neutral $\alpha$-aminobutyryl donors (e.g. O-acetylhomoserine) were not retained. Acidic $\alpha$-aminobutyryl donors were eluted from the column of Dowex 1, and applied to a column of Dowex 50 $H^+$. Weakly acidic $\alpha$-aminobutyryl donors (e.g. O-succinyl- and O-malonylhomoserine) were retained while strongly acidic ones (e.g. O-phosphoryl- and O-oxalylhomoserine) were not. Addition of tracer amounts of radioactive homoserine esters to the trichloroacetic acid extract permitted monitoring of the fractionation procedure, and determination of recovery of the appropriate derivative at the end of the fractionation. Amounts of derivatives added were sufficiently small so that assays of homoserine esters present in tissues were not significantly affected by their inclusion.

The detailed procedure will be given for $Chlorella$; it was applied, with minor modifications, to all other tissues assayed for $\alpha$-aminobutyryl donors. Preparation of the trichloroacetic acid extract and all ion exchange chromatography was done at $4^\circ$.

To $Chlorella$ cells (8.6 ml packed volume) was added 10 ml of 5% trichloroacetic acid extract of tissue by ion exchange chromatography followed by paper electrophoresis. Separation of $\alpha$-aminobutyryl donors by ion exchange chromatography (Fig. 3) was based on retention at neutral pH of acidic $\alpha$-aminobutyryl donors (e.g. O-succinyl-, O-malonyl- , O-oxalyl- , and O-phosphorylhomoserine) on Dowex 1-formate. Neutral $\alpha$-aminobutyryl donors (e.g. O-acetylhomoserine) were not retained. Acidic $\alpha$-aminobutyryl donors were eluted from the column of Dowex 1, and applied to a column of Dowex 50 $H^+$.

The supernatant solution was retained, and the acetone-trichloroacetic acid extraction was repeated on the pellet twice more. All supernatants were combined. To the acetone-trichloroacetic acid extract was added O-phosphoryl-[${}^{14}\text{H}$]homoserine, 0.58 x $10^6$ dpm; O-oxalyl-[${}^{14}\text{H}$]-
homoserine, $1.54 \times 10^4$ dpm; and O-malonyl-[14C]homoserine, $0.4 \times 10^4$ dpm.

Trichloroacetic acid was removed by continuous extraction with ether for 6 hours. The aqueous phase was bubbled with $N_2$ and its pH adjusted to 6.8 by addition of 0.01N NaOH. The extract was clarified by centrifugation followed by filtration on paper, then applied to a Dowex 1-formate column (2.0 $\times$ 12.8 cm). The column was washed with 80 ml of water. Effluent and wash solutions were combined (Fraction I). The column was eluted with 260 ml of 2 N formic acid. The eluate, designated Fraction II, was extracted twice with an equal volume of ether to remove residual trichloroacetic acid. Fractions I and II were each lyophylized to dryness.

The residue from Fraction I was dissolved in 50 ml of water and applied to a Dowex 50-H$^+$ column (1.5 $\times$ 11.3 cm). The column was washed with 100 ml of water. The combined effluents were designated Fraction Ia. The column was eluted with 100 ml of 1.3 M pyridine acetate, pH 5.2. The eluate, designated Fraction Ib, was lyophylized to dryness, and dissolved in 1.0 ml of water. The pH was close to 7.

The residue from Fraction II was dissolved in 41 ml of water and applied to a Dowex 50-H$^+$ column (1.5 $\times$ 11.3 cm), which was washed with 100 ml of water. The combined effluent and wash was designated Fraction IIa. The column was eluted with 100 ml of 1.3 M pyridine acetate (pH 5.2) and the eluate designated Fraction IIb.

O-Phosphorylhomoserine and O-oxalylhomoserine would be contained in Fraction IIa. The fraction was lyophylized to dryness, dissolved in 2.4 ml of water, and the pH adjusted to 6.58 by addition of 0.01 M MOPS (60 mmoles, pH 7.25) followed by 7.5 ml of 0.01 N KOH. The fraction was again lyophylized to dryness and O-oxalylhomoserine separated from O-phosphorylhomoserine by electrophoresis. An amount of the fraction equivalent to 7.2 g wet weight of Chlorella was applied as a strip 4 cm wide to Whatman No. 3MM paper which had been exhaustively washed with glacial acetic acid and water. Electrophoresis was then performed in 7.9 M formic acid at 2000 volts. Special precautions were required to prevent overheating of the paper. An additional cast aluminum plate was substituted for the glass pressure plate normally used, and water at 4°C was circulated through both upper and lower plates. Pressure was applied evenly to the upper aluminum plate by means of several lead bricks. After electrophoresis, the paper was dried at room temperature, cut into 1-cm strips, and eluted with water. An aliquot of each eluate was used for determination of radioactivity and of the amount of α-aminobutyryl donor.

O-Malonylhomoserine and O-succinylhomoserine would be contained in Fraction IIb. This fraction was taken to dryness and dissolved in 4.2 ml of water. The pH was approximately 6. An amount equivalent to 8.56 g wet weight Chlorella was applied in a 10-cm wide streak to acid-washed paper, and subjected to electrophoresis at 3000 volts in 0.935 M pyridine acetate, pH 6.0, for 30 min. The paper was dried at room temperature, cut into 1-cm strips, and eluted for determination of radioactivity. In a separate experiment it was shown that under these conditions O-malonyl- and O-oxalylhomoserine migrated together 8 to 10 cm toward the anode. N-Malonylhomoserine was located at 18 to 20 cm toward the anode and homoserine was located 2 to 4 cm toward the cathode. From Fraction IIb, 91% of the total 14C radioactivity on the electrophoretogram was located in the area corresponding to O-malonylhomoserine and 9% in the area corresponding to homoserine. No N-malonylhomoserine was detected. Eluates from the area of the electrophoretogram corresponding to O-malonyl- and O-succinylhomoserine were combined for determination of α-aminobutyryl donors.

RESULTS

Characterization of O-Phosphorylhomoserine Preparations

Because of the major and novel role of O-phosphorylhomoserine to be elucidated below, it was of crucial importance to characterize fully the authentic samples of O-phosphorylhomoserine synthesized for this work. Two preparations of O-phosphorylhomoserine were synthesized, as described in detail under "Materials." O-Phosphorylhomoserine (I) contained negligible radioactivity and was detected in the characterization described below by its reaction with ninhydrin. O-Phosphoryl-[14C]homoserine (II) was detected and quantitated by its radioactivity. The two preparations, mixed as described in the legend to Fig. 4, were characterized simultaneously by their comigration as a single radioactive, ninhydrin-positive peak, and by their stoichiometric conversion to [14C]homoserine and P$_1$ in the presence of alkaline phosphatase. Both preparations are assumed to be the L-isomer, since yeast homoserine kinase is specific for L-homoserine (51).

Fig. 4 demonstrates the rapid release of P$_1$ and [14C]homoserine from the mixture of O-phosphorylhomoserine (I) and O-phosphoryl-[14C]homoserine (II) incubated with alkaline phosphatase. The close equivalence between these two variables is in agreement with the expected hydrolysis of O-phosphoryl-[14C]homoserine to [14C]homoserine. The time course shown in Fig. 4, together with the chromatographic and electrophoretic data to be presented below, indicates that hydrolysis of O-phosphorylhomoserine is essentially complete after 5.67 hours incubation with phosphatase. Approximately 95% of the radioactivity added as O-phosphoryl-[14C]homoserine (II) was converted to [14C]homoserine. No appreciable hydrolysis of O-phosphoryl-[14C]homoserine occurred in the absence of phosphatase.

Fig. 5 illustrates electrophoretic and paper chromatographic analysis of the O-phosphoryl-[14C]homoserine preparations after exhaustive incubation either in the absence or presence of phosphatase. In the absence of phosphatase (Fig. 5, A, C, and E) only one ninhydrin-positive compound was observed. Any ninhydrin-positive impurity representing 10%, or more, of the amount of O-phosphorylhomoserine could have been detected. Only one major peak of radioactivity was observed, which coincided with the ninhydrin-positive compound. After chromatography with Solvent A, an additional radioactive peak which contained less than 6% of the total radioactivity, was observed (Fig. 3B). This small peak probably represents "twinning" of O-phosphoryl-[14C]homoserine, since it yielded [14C]homoserine after treatment with phosphatase (Fig. 5F). Treatment with phosphatase resulted in complete disappearance of the original ninhydrin-positive and radioactive material, and quantitative recovery of radioactivity in a ninhydrin-positive compound of changed mobility which comigrated with authentic L-homoserine (Fig. 5, B, D, and F). The only significant radioactive impurity detected was that migrating at 2 to 5 cm in Fig. 5B. This minor peak contained approximately 4% of the total radioactivity.

Cystathionine γ-Synthase Activity

Properties of Cystathionine γ-Synthase in Green Plants—The results of a typical assay of cystathionine synthase activity in a crude extract of a green plant are presented in Fig. 6. This...
Enzymic hydrolysis of preparations of O-phosphoryl-[3H]homoserine. Hydrolysis was performed at 30°C in a reaction mixture containing O-phosphorylhomoserine (I) (10.0 μmoles based on the specific activity of 3H), O-phosphoryl-[G-3H]homoserine (II) (1.93 × 10⁷ dpm, 27.5 μmoles), Tris-HCl, pH 8.08 (55 μmoles), and alkaline phosphatase (2 units) in a final volume of 2.75 ml. A control reaction mixture, from which phosphatase was omitted, was run in parallel. At periods of 0.1, 0.33, 1, 2, and 3 hours after the incubation was begun, aliquots were inactivated with acid for determination of P₁ and [3H]homoserine. An additional 0.4 units of phosphatase was added after removal of samples at 3 hours, and further samples removed at 4, 5, and 5.67 hours. P₁ was determined by the method of Tassovsky and Shorr (50). Radioactivity in homoserine was determined by applying samples of approximately 20 μl, diluted in a final volume of 1 ml of water, to a column (0.9 X 2.9 cm) of Dowex 50-H⁺. O-Phosphoryl-[3H]homoserine was washed through the column with 4 ml of water, and [3H]homoserine eluted with 5 ml of 3 N NH₄OH. A chemical determination of [3H]homoserine was performed by automated amino acid analysis directly on the reaction mixture after 5.07 hours incubation with phosphatase. The specific radioactivity of [3H]homoserine was calculated from the radioactivity present in homoserine at these times, and the specific radioactivity of [3H]homoserine. [3H]Homoserine formed in the presence (△) or absence (○) of phosphatase; P₁, formed in the presence (●) or absence (〇) of phosphatase.

The figure illustrates electrophoretograms of the radioactive products obtained under our standard conditions after incubation of an extract of parsley leaves either in the absence of added homoserine ester or in the presence of O-acetyl- or O-phosphorylhomoserine. Incorporation of [14C]cysteine into [14C]cystathionine was completely dependent upon the addition of a homoserine ester. The presence of O-phosphorylhomoserine resulted in a large peak of radioactivity in [14C]cystathionine which had been converted during our standard purification procedure to the corresponding sulfone. Similar, well defined peaks of radioactivity were obtained with extracts of parsley and all other green plants after incubations in the presence of O-malonyl-, O-succinyl-, or O-osalylhomoserine (not shown in Fig. 6). In the presence of O-acetylhomoserine a much smaller peak of [14C]cystathionine sulfone was typically detected. The amount of O-Sulfurylhomoserine (assayed at a concentration of 0.02 M with spinach extract) showed only a trace of activity equal to that detected with O-acetylhomoserine; when present at 300-fold excess.

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the brei centrifuged at 48,000 g for 10 min to yield a supernatant solution (unfiltered extract). The Sephadex G-25 filtrate was prepared by subjecting the supernatant solution to gel filtration on a column of Sephadex G-25 equilibrated with the above buffer. The Dowex 1 filtrate was prepared by subjecting the Sephadex G-25 filtrate to chromatography on a column of Dowex 1 equilibrated with the above buffer. Assay conditions are those described under "Methods," except that 0.58 nmole of N⁵-methyltetrahydrofolate was present in the reaction mixtures as indicated, and incubation was for 30 min with all substrates.

Activity was stable during storage of the enzyme at -65°, and addition of potassium phosphate or Tris-HCl instead of MOPS resulted in up to 90% inhibition of activity with O-phosphorylhomoserine. The relationship between pH and activity was measured in the supernatant solution obtained after centrifugation at 100,000 × g for 2 hours. This was true whether the activity was assayed with O-acetyl-, O-oxalyl-, O-malonyl-, or O-succinylhomoserine.

The stoichiometry of cystathionine synthesis (Fig. 7) was determined by incubation of spinach extract with either O-malonyl-[⁵⁷C]homoserine and [H]cysteine (Fig. 7A), or O-phosphoryl-[³⁵C]homoserine and [³H]cysteine (Fig. 7B). At each time period, whether the α-aminobutyryl donor was O-malonyl- or O-phosphorylhomoserine, close to the same amount of radioactive cysteine and radioactive homoserine moiety was incorporated. The results are compatible with the synthesis of cystathionine according to Reaction 2.

Survey of Cystathionine γ-synthase Activities in Crude Extracts of Various Organisms—Table II summarizes the results of measurements of homoserine ester-dependent cystathionine γ-synthase activities in extracts from two bacteria, one blue-green alga, and representatives of the major divisions of green plants. The green plants studied ranged from the green algae Chlorella through the mosses, club-mosses, horsetails, ferns, conifers and their relatives, to the angiosperms. Cystathionine γ-synthase activity was determined in each of these extracts with O-acetyl-, O-oxalyl-, O-malonyl-, O-succinyl-, and O-phosphorylhomoserine. Each of the green plant tissues studied was active with each of the five homoserine esters. Considerable variation was observed in the specific activities for a given substrate assayed with the various tissue extracts. For example, the specific activity with O-phosphorylhomoserine varied from 1 in whole seedlings of Lathyrus sativus to 174 in leaves of Ginkgo biloba. However, the spectra of relative activities with the five substrates were rather similar in all green plant tissues. Representative data from Table II, shown in graphical form in Fig. 8, illustrate that extracts from green plants (Fig. 8, A and B) catalyzed the lowest rate of cystathionine synthesis in the presence of O-acetyl- and the highest rate in the presence of O-malonylhomoserine. Intermediate rates were observed with O-oxalyl-, O-succinyl- and O-phosphorylhomoserine. A single exception to this general pattern was observed with extracts of Ginkgo, in which O-phosphorylhomoserine was the most active substrate (Table II).

One general difference was noted between the activity patterns of the plants of early phylogenetic origin, and those that had evolved more recently. Extracts of the green alga, lower plants, and gymnosperms were more active with O-phosphorylhomoserine than with O-succinylhomoserine (e.g. Fig. 8A), while the reverse was true of the more recently evolved angiosperms (e.g. Fig. 8B). The last column of Table II shows that the ratio of activity with O-phosphorylhomoserine to that with O-succinylhomoserine for all of the plants of early phylogenetic origin

<table>
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<tr>
<th>Substrate</th>
<th>Cystathionine γ-synthase activity</th>
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<tr>
<td></td>
<td>Unfiltered extract</td>
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<tr>
<td></td>
<td>N⁵MeH₂ folate</td>
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<tr>
<td>0-Acetylhomoserine</td>
<td>1</td>
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<tr>
<td>0-Malonylhomoserine</td>
<td>38</td>
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<tr>
<td>O-Phosphorylhomoserine</td>
<td>33</td>
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Radioactivity in material less mobile than cystathionine sulfone was not correlated with time of incubation, presence or absence of homoserine ester, or amount of cystathionine synthesized. No attempt was made to identify this material.

Optimal assay conditions were defined with crude extracts of spinach. The concentrations of cysteine and homoserine ester used in the standard assay approximated those required for maximal rates. Because of the base lability of some of the homoserine esters (35), incubation times were chosen so that no more than 10% chemical decomposition of the homoserine ester would have occurred by the end of the incubation. These times were 10 min for O-acetyl-, 3 min for O-oxalyl-, 20 min for O-succinyl- and O-malonyl-, and 60 min for O-phosphorylhomoserine. Under standard assay conditions, cystathionine γ-synthase activity was linear with time and enzyme concentration. The relationship between pH and activity was measured in MOPS buffer between pH 6 and 8. A pH of 7.7 was selected for the assay, since, at this pH, activity with each substrate was near the optimum, while the rate of base-catalyzed rearrangement of each homoserine ester was not a serious problem. Use of potassium phosphate or Tris-HCl instead of MOPS resulted in up to 90% inhibition of activity with O-phosphorylhomoserine. Activity was stable during storage of the enzyme at -65°, and with repeated freezing and thawing.

The cystathionine γ-synthase of Neurospora, an enzyme which utilizes O-acetylhomoserine (12, 24), has been shown to have an absolute requirement for N⁵-methyltetrahydrofolate (52). The possibility that the low activity of plant extracts with O-acetylhomoserine reflected a limiting concentration of N⁵-methyltetrahydrofolate was ruled out by an experiment, the results of which are presented in Table I. With O-acetyl-, O-malonyl-, and O-phosphorylhomoserine it inhibited incorporation of O-phosphorylhomoserine by about 70%. O-Sulfurylhomoserine was not studied further in view of its relative inactivity, the lack of any evidence for its physiological occurrence, and the fact that, as mentioned under "Discussion," it was shown that the chief naturally occurring α-aminobutyryl donor for cystathionine synthesis in plants was not this compound.

and O-phosphorylhomoserine, addition of N⁵-methyltetrahydrofolate did not significantly stimulate spinach cystathionine γ-synthase activity even in enzyme extracts that had been subjected to treatments designed to remove traces of N⁵-methyltetrahydrofolate. These treatments included filtration through Sephadex G-25 (52), and combined filtration through Sephadex G-25 and Dowex 1 (53).

At least 80% of the recovered activity of spinach leaf cystathionine γ-synthase, assayed with either O-malonyl- or O-phosphorylhomoserine, appeared in the 48,000 × g supernatant solution obtained in the standard extraction procedure. Even when spinach leaves were ground in a medium (0.1 M potassium phosphate, pH 7.2, 0.4 M sucrose, 0.01 M NaCl) designed to maintain some degree of integrity of particulate components, over 80% of the recovered cystathionine γ-synthase appeared in the supernatant solution obtained after centrifugation at 100,000 × g for 2 hours. This was true whether the activity was assayed with O-acetyl-, O-oxalyl-, O-malonyl-, or O-succinylhomoserine.

The stoichiometry of cystathionine synthesis (Fig. 7) was determined by incubation of spinach extract with either O-malonyl-[⁵⁷C]homoserine and [³H]cysteine (Fig. 7A), or O-phosphoryl-[³⁵C]homoserine and [³H]cysteine (Fig. 7B). At each time period, whether the α-aminobutyryl donor was O-malonyl- or O-phosphorylhomoserine, close to the same amount of radioactive cysteine and radioactive homoserine moiety was incorporated. The results are compatible with the synthesis of cystathionine according to Reaction 2.

Survey of Cystathionine γ-Synthase Activities in Crude Extracts of Various Organisms—Table II summarizes the results of measurements of homoserine ester-dependent cystathionine γ-synthase activities in extracts from two bacteria, one blue-green alga, and representatives of the major divisions of green plants. The green plants studied ranged from the green algae Chlorella through the mosses, club-mosses, horsetails, ferns, conifers and their relatives, to the angiosperms. Cystathionine γ-synthase activity was determined in each of these extracts with O-acetyl-, O-oxalyl-, O-malonyl-, O-succinyl-, and O-phosphorylhomoserine. Each of the green plant tissues studied was active with each of the five homoserine esters. Considerable variation was observed in the specific activities for a given substrate assayed with the various tissue extracts. For example, the specific activity with O-phosphorylhomoserine varied from 1 in whole seedlings of Lathyrus sativus to 174 in leaves of Ginkgo biloba. However, the spectra of relative activities with the five substrates were rather similar in all green plant tissues. Representative data from Table II, shown in graphical form in Fig. 8, illustrate that extracts from green plants (Fig. 8, A and B) catalyzed the lowest rate of cystathionine synthesis in the presence of O-acetyl- and the highest rate in the presence of O-malonylhomoserine. Intermediate rates were observed with O-oxalyl-, O-succinyl-, and O-phosphorylhomoserine. A single exception to this general pattern was observed with extracts of Ginkgo, in which O-phosphorylhomoserine was the most active substrate (Table II).

One general difference was noted between the activity patterns of the plants of early phylogenetic origin, and those that had evolved more recently. Extracts of the green alga, lower plants, and gymnosperms were more active with O-phosphorylhomoserine than with O-succinylhomoserine (e.g. Fig. 8A), while the reverse was true of the more recently evolved angiosperms (e.g. Fig. 8B). The last column of Table II shows that the ratio of activity with O-phosphorylhomoserine to that with O-succinylhomoserine for all of the plants of early phylogenetic origin

TABLE I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cystathionine γ-synthase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfiltered extract</td>
</tr>
<tr>
<td></td>
<td>N⁵MeH₂ folate</td>
</tr>
<tr>
<td>O-Acetylhomoserine</td>
<td>1</td>
</tr>
<tr>
<td>O-Malonylhomoserine</td>
<td>38</td>
</tr>
<tr>
<td>O-Phosphorylhomoserine</td>
<td>33</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on September 22, 2017
Anabaena, and the two bacteria were quite different from those y-synthases in the two groups.

A true difference in the catalytic properties of the cystathionine 

in the relative rates with the two substrates appears to represent 

later group, the activities were additive. Thus, the difference 

significant (P less than 0.01). As is shown in Table III, when an 

activities less than 1.5). Statistical analysis by the t test demon-

strated that the difference between these two groups was highly 

extract of fern, a member of the early phylogenetic group, was 

assayed in a mixture with an extract of spinach, a member of the 

tested was 1.6 or greater, while in all of the angiosperms tested, 

activity with 0-phosphorylhomoserine was either approximately 

equal to, or less than, that with 0-succinylhomoserine (ratio of 

substrates. The ratio of 3H:14C in cystathionine did not change 

significantly (data not shown) when the product of each incuba-

tion and isolation of the sulfone.

The activity patterns observed with the blue-green alga, Anabaena, and the two bacteria were quite different from those

with green plants (Fig. 8, Table II). In extracts of Anabaena and E. coli, highest activities were observed with O-succinyl- 

homoserine; in extracts of B. subtilis, highest activity was ob-

served with O-acetylhomoserine. A striking aspect of the
the sensitivity for detection of O-acetylhomoserine, Fraction Ia was tested again with B. subtilis enzyme. No donor activity was detected (data not shown), and an amount of 0.2 nmole of O-acetylhomoserine per g of barley was set as the maximum concentration. Activity was initially detected in the crude extract, then in Fraction II (the eluate after chromatography on Dowex 1-formate) and finally in Fraction IIa (the flow-through from Dowex 50-H+ chromatography). All of the activity in these fractions was accounted for, within experimental error, by the O-phosphorylhomoserine content of the tissue. That the donor is O-phosphorylhomoserine was established by the following properties (Table IV): (a) it is stable during treatment with base, a property that excludes its being an O-acetylhomoserine ester (24, 35, 54); (b) its ability to sustain cystathionine synthesis is inactivated by preliminary incubation with highly purified E. coli phosphatase; and (c) it was recovered, along with the marker O-phosphoryl-[3H]homoserine added to the trichloroacetic acid extract, after electrophoresis under conditions which separate O-oxalyl- and O-phosphorylhomoserine (e.g. Fig. 10). Fraction I (which could contain, of the known donors, O-phosphorylhomoserine) and its derivatives were tested in this experiment (Table IV) with spinach enzyme. In order to increase the sensitivity for detection of O-acetylhomoserine, Fraction Ia was tested again with B. subtilis enzyme. No donor activity was detected (data not shown), and an amount of 0.2 nmole of O-acetylhomoserine per g of barley was set as the maximum amount of this ester which could have been present.

α-Aminobutyryl Donors from Other Tissues—Fractions obtained from several additional plants were assayed for the presence of α-aminobutyryl donors. Data in Table V show that

Endogenous α-Aminobutyryl Donor for Cystathionine Synthesis

The availability of enzyme preparations that catalyzed relatively rapid rates of cystathionine synthesis permitted the development of assays to detect α-aminobutyryl donors. It is emphasized that these assays would detect not only the homoserine esters described above but also any other compound active as an α-aminobutyryl donor in the presence of the enzymes used. Tissues from representative green plants were fractionated to separate possible α-aminobutyryl donors, and the assays for such donors were applied to each fraction. Results of the complete analysis of barley fractions will be presented, followed by corroborating evidence from other tissues.

Assay of α-Aminobutyryl Donor in Barley—Fractions obtained from axenic barley seedlings, as outlined in Fig. 3, were assayed for α-aminobutyryl donor activity. Each fraction was assayed at two concentrations, determined by the extent of inhibition of spinach cystathionine γ-synthase caused by each fraction. The resulting electrophoretograms are shown in Fig. 9. Both concentrations of Fraction IIa (Fig. 9, F and G) contained α-aminobutyryl donor activity capable of sustaining the synthesis of [14C]cystathionine from [14C]cysteine. Of known donors, O-oxalyl- and O-phosphorylhomoserine would be contained in this fraction. None of the other fractions (Ia, Ib, IIb) consistently and unequivocally showed α-aminobutyryl donor activity. To test the unlikely possibility that barley contained an α-aminobutyryl donor capable of reacting with barley, but not spinach, cystathionine γ-synthase, all fractions were assayed again using a crude preparation of barley enzyme. The results agreed with those obtained with spinach enzyme. No α-aminobutyryl donor activity was detected in Fractions Ia, Ib, or IIb, and the activity in Fraction IIa did not exceed that demonstrated by use of spinach cystathionine γ-synthase.

Values for all of the barley fractions are given in Table IV. The donor activity found in Fraction IIa was ultimately identified as O-phosphorylhomoserine, and the results with other fractions are expressed relative to the O-phosphorylhomoserine tissue concentration. Activity was initially detected in the crude extract, then in Fraction II (the eluate after chromatography on Dowex 1-formate) and finally in Fraction IIa (the flow-through from Dowex 30-H+ chromatography). All of the activity in these fractions was accounted for, within experimental error, by the O-phosphorylhomoserine content of the tissue. That the donor is O-phosphorylhomoserine was established by the following properties (Table IV): (a) it is stable during treatment with base, a property that excludes its being an O-acetylhomoserine ester (24, 35, 54); (b) its ability to sustain cystathionine synthesis is inactivated by preliminary incubation with highly purified E. coli phosphatase; and (c) it was recovered, along with the marker O-phosphoryl-[3H]homoserine added to the trichloroacetic acid extract, after electrophoresis under conditions which separate O-oxalyl- and O-phosphorylhomoserine (e.g. Fig. 10). Fraction I (which could contain, of the known donors, O-acetylhomoserine) and its derivatives were tested in this experiment (Table IV) with spinach enzyme. In order to increase the sensitivity for detection of O-acetylhomoserine, Fraction Ia was tested again with B. subtilis enzyme. No donor activity was detected (data not shown), and an amount of 0.2 nmole of O-acetylhomoserine per g of barley was set as the maximum amount of this ester which could have been present.

α-Aminobutyryl Donors from Other Tissues—Fractions obtained from several additional plants were assayed for the presence of α-aminobutyryl donors. Data in Table V show that

activity patterns with these three primitive organisms is that none showed any activity with O-phosphorylhomoserine. Green plants are therefore unique among the organisms surveyed in their ability to use O-phosphorylhomoserine as a substrate for cystathionine synthesis.

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activity patterns with these three primitive organisms is that none showed any activity with O-phosphorylhomoserine. Green plants are therefore unique among the organisms surveyed in their ability to use O-phosphorylhomoserine as a substrate for cystathionine synthesis.
homoserine was conclusively established after further purification. Perhaps the predominant proportion of this activity was recovered in Fraction IIa. Further, in every tissue examined, base treatment had no significant effect on the \( \alpha \)-aminobutyryl donor activity. These results are compatible with the proposal that \( O \)-phosphorylhomoserine is present in a range of tissues and is the major, perhaps the only, \( \alpha \)-aminobutyryl donor detected.

That the donor activity in Fraction IIa from the representative plant tissues, spinach and Chlorella, is indeed \( O \)-phosphorylhomoserine was conclusively established after further purification.

![Graph](http://www.jbc.org/)

**Fig. 9.** Assays of barley fractions for endogenous \( \alpha \)-aminobutyryl donor. Axenic barley seedlings were ground in a mortar with 1.5 times their volume of 5% trichloroacetic acid, and the brei was filtered through glass wool. \( O \)-Phosphoryl-\( [\text{H}] \)homoserine and \( O \)-acetyl-\( [\text{H}] \)homoserine (0.17 nmole and 1.2 \( \times \) 10^4 cpm of each, equivalent to 0.008 nmole ester per g of barley tissue) were added and the resulting extract was fractionated according to the procedure outlined in Fig. 3. Two amounts of each barley fraction were incubated with \( [\text{H}] \)cysteine and spinach enzyme as described for assay by Method B. \( [\text{H}], [\text{C}] \)Cystathionine was isolated by preliminary electrophoresis without oxidation, as described in the legend for Fig. 7. Areas of the electrophoreograms corresponding to \( [\text{H}] \)cystathionine were eluted, and the eluates were oxidized and subjected to electrophoresis in the standard way. \( [\text{H}] \) in cystathionine sulfone (-----) was used to correct for recovery during the purification procedure, while \( [\text{C}] \)cysteine incorporation (---) into cystathionine sulfone, corrected for recovery, was a measure of \( \alpha \)-aminobutyryl donor content. A \( [\text{C}] \) background of 11 cpm has not been subtracted. The numbers in parentheses shown below represent the equivalent amounts of fresh weight of barley assayed. A, no substrate; \( B \) (104 mg) and \( C \) (208 mg) of Fraction Ia; \( D \) (25.5 mg) and \( E \) (51 mg) of Fraction Ib; \( F \) (140 mg) and \( G \) (280 mg) of Fraction IIa; \( H \) (76 mg) and \( I \) (152 mg) of Fraction IIb.

\( \alpha \)-aminobutyryl donor activity was found in Fraction II from all tissues examined, and that, within experimental error, the predominant proportion of this activity was recovered in Fraction IIa. Further, in every tissue examined base treatment had no significant effect on the \( \alpha \)-aminobutyryl donor activity. These results are compatible with the proposal that \( O \)-phosphorylhomoserine is present in a range of tissues and is the major, perhaps the only, \( \alpha \)-aminobutyryl donor detected.

**Table IV**

\( \alpha \)-Aminobutyryl donor activity in barley

Preparation and assay of the extracts are described in Fig. 9. The results are expressed as a percentage of the concentration of \( O \)-phosphorylhomoserine (0.6 nmole per g of tissue) isolated by electrophoresis from Fraction IIa. The values in parentheses are the relative amounts of other donor activities which would have gone undetected. Treatment with base is described in Table V, while treatment with highly purified \( E \). coli phosphatase and electrophoretic separation of \( O \)-phosphorylhomoserine are essentially as described in Table VI.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Relative ( \alpha )-aminobutyryl donor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>210 %</td>
</tr>
<tr>
<td>Fraction I</td>
<td>0 (&lt;20)</td>
</tr>
<tr>
<td>Ia</td>
<td>0 (&lt;25)</td>
</tr>
<tr>
<td>Ib</td>
<td>0 (&lt;100)</td>
</tr>
<tr>
<td>Fraction II</td>
<td>109</td>
</tr>
<tr>
<td>IIa</td>
<td>100</td>
</tr>
<tr>
<td>IIb</td>
<td>0</td>
</tr>
<tr>
<td>IIa, base treated</td>
<td>82</td>
</tr>
<tr>
<td>IIa, phosphatase treated</td>
<td>0</td>
</tr>
<tr>
<td>IIa, isolated by electrophoresis</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table V**

\( \alpha \)-Aminobutyryl donor activity in other green plants

Preparation of the Chlorella trichloroacetic acid extract was as described under “Methods.” Paul’s Scarlet Rose cells were extracted in an equal volume of 5% trichloroacetic acid. The residue, after centrifugation, was extracted in 3 volumes of 88% acetone, followed by extraction in an equal volume of trichloroacetic acid. All extracts were combined. Corn and spinach tissues were ground in a mortar with an equal volume of 5% trichloroacetic acid. The brei was filtered through cheesecloth and centrifuged to yield a trichloroacetic acid extract. All extracts were subjected to the fractionation procedure outlined under “Methods” and in Fig. 3. Donor activity was measured by Method A. Base treatment consisted of incubation of the fraction in 6 mM NH4OH for 1 hour, followed by removal of NH4OH by evaporation. Units of activity are defined under “Methods.”

<table>
<thead>
<tr>
<th>Source</th>
<th>Fraction</th>
<th>( \alpha )-Aminobutyryl donor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella</td>
<td>II</td>
<td>2.5–3.4</td>
</tr>
<tr>
<td>Ia</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Corn shoot</td>
<td>II</td>
<td>1.1–1.3</td>
</tr>
<tr>
<td>Ia</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>IIa, base treated</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>IIa, base treated</td>
<td>0.2–0.4</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Corn root</td>
<td>II</td>
<td>1.3–1.6</td>
</tr>
<tr>
<td>Ia</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Ia, base treated</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Spinach leaf, Experiment 1</td>
<td>II</td>
<td>0.9</td>
</tr>
<tr>
<td>Ia</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Paul’s Scarlet Rose suspension culture cells</td>
<td>II</td>
<td>0.1</td>
</tr>
<tr>
<td>Ia</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>
marker O-phosphoryl-[aH]homoserine and O-oxalyl-[aH]homoserine (Peak 1) and O-oxalylhomoserine. The tritiated markers demonstrate a clean separation of O-phosphoryl-[aH]homoserine. No significant a-aminobutyryl donor activity was detected in the area corresponding to O-phosphorylhomoserine. 

The amount of O-phosphorylhomoserine determined after electrophoresis of spinach and Chlorella Fraction IIa agreed closely with that found before electrophoresis, demonstrating that, as in barley, a major proportion of the a-aminobutyryl donor can be recovered in an electrophoretic peak corresponding in location to O-phosphorylhomoserine. Finally, further characterization of O-phosphorylhomoserine and a-aminobutyryl donor originally added to the reaction mixtures. Appropriate corrections have been made for the molar amount of O-phosphorylhomoserine added as radioactive marker. The reaction mixture containing Chlorella extract was assayed after base treatment without prior electrophoresis.

**Table VI**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinach O-Phosphoryl- [H]homoserine</th>
<th>a-Aminobutyryl donor</th>
<th>Chlorella O-Phosphoryl-[H]homoserine</th>
<th>a-Aminobutyryl donor %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99</td>
<td>71</td>
<td>105</td>
<td>88</td>
</tr>
<tr>
<td>Base</td>
<td>101</td>
<td>100</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Discussion**

Properties of Plant Cystathionine γ-Synthase—Extracts from an array of green plants all have the capacity to synthesize cystathionine from cysteine and any of a number of homoserine esters. Under the optimal assay conditions developed during the present work the specific activities of plant extracts for this a-aminobutyryl transfer reaction are considerably higher than those...
The evolutionary acquisition of the ability to utilize O-phosphorylhomoserine for cystathionine biosynthesis may be localized to the green algae or to an ancestor of this group.

Physiological α-Aminobutyryl Donor—In many organisms the preferential activity of cystathionine γ-synthase with one homoserine ester is a helpful indicator of the physiologically important cystathionine precursor. Thus, the cystathionine γ-synthases of *S. typhimurium* (23) and *E. coli* (Table II) are most active in the presence of O-succinyllhomoserine, while independent evidence indicates that for each of these organisms this compound is the physiological substrate for cystathionine formation (21, 22, 24, 54). In like manner, the cystathionine γ-synthases of *N. crassa* (26) and *B. subtilis* (Table II) preferentially utilize O-acetylhomoserine, the ester which has been established by other studies as the actual cystathionine precursor in these forms (24, 26–28).

The cystathionine γ-synthase activity of crude extracts of green plants did not display sufficient specificity to permit an unequivocal decision as to the physiological α-aminobutyryl donor. To overcome this problem, we turned to an alternative approach. A typical plant preparation of cystathionine γ-synthase (from spinach) was used as the key component in assay systems which would permit the detection of substances active as donors of the α-aminobutyryl moiety of cystathionine. These assays, then, set no preconditions as to the chemical nature of an active compound other than that it be capable of transferring an α-aminobutyryl group to cysteine in the presence of plant enzyme. By use of this method it was possible to demonstrate the presence of an active α-aminobutyryl donor in all green plants studied, ranging from the green alga *Chlorella* to the higher flowering plants. This endogenous substrate for plant cystathionine γ-synthase could be distinguished from any of the O-acetylxylanolic esters studied by means of its base stability and its behavior during paper chromatography and paper electrophoresis, and from O-sulfurylhomoserine by its behavior during paper electrophoresis. Conversely, in all these respects, as well as in its rate of decomposition by highly purified alkaline phosphatase, this endogenous substrate has exactly the same properties as authentic O-phosphorylhomoserine. No other compound capable of sustaining significant cystathionine formation in the presence of plant enzyme was detected in plant extracts. The concentrations of O-phosphorylhomoserine reported here for green plants are some 10,000 times less than that in *Lactobacillus casei* (61), and some 100 times less than that in *B. subtilis* (62). It was therefore of crucial importance to exclude the possibility that the O-phosphorylhomoserine detected in plant extracts did not originate in contaminating bacteria or fungi. This was accomplished by the demonstration of O-phosphorylhomoserine in both *Chlorella* and barley (Hordeum vulgare) grown under axenic conditions (Table VII).

This evidence establishes O-phosphorylhomoserine as the chief, perhaps the only, compound both present in plants and capable of serving as an α-aminobutyryl donor with plant cystathionine γ-synthase. This fact, in conjunction with the demonstration that all green plants tested contain cystathionine γ-synthases

The difference between the activity patterns of the cystathionine γ-synthase of *B. subtilis* and those of *E. coli* and *S. typhimurium* (23 and Table II) provides further support for the proposal of Brush and Paulus (27) that there is an evolutionary divergence in the pathway for methionine biosynthesis between the Bacillaceae and the Enterobacteriaceae. Furthermore, the similarity of the patterns for *Anabaena flos-aquae* and the gram-negative bacteria *E. coli* and *S. typhimurium* (23 and Table II) is compatible with the similarity in another metabolic pathway between these two groups to which Schäfer and Vogel (60) have drawn attention.

### Table VII

Concentration of α-aminobutyryl donors in *Chlorella*, barley and spinach

The concentration of each homoserine ester was determined in the most purified fraction potentially containing that ester. O-Malonyl- and O-succinylhomoserine, which would be contained in the same fraction, were assayed together using authentic O-malonylhomoserine as standard. Concentrations of O-phosphorylhomoserine have been corrected for recovery. Values shown in parentheses represent estimates of the maximum concentration of other homoserine esters that could have been present and gone undetected. Values for recovery, sensitivity of the assay for each ester, and the amount of intuition caused by each fraction have been taken into account in setting these estimates. Values for barley were derived from those reported in Table IV. The concentrations of O-phosphorylhomoserine in *Chlorella* and spinach were determined by Method B from the electrophoreses shown in Fig. 10; these values are in agreement with those determined by Method A on separate batches of tissue (Table V).

For this comparison, units of α-aminobutyryl donor reported in Table V were converted to nanomoles of O-phosphorylhomoserine as described under "Methods."

<table>
<thead>
<tr>
<th>Homoserine ester</th>
<th><em>Chlorella</em></th>
<th>Barley</th>
<th>Spinach</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-Phosphoryl-</td>
<td>11</td>
<td>0.6</td>
<td>5</td>
</tr>
<tr>
<td>O-Malonyl-</td>
<td>0 (&lt;0.1)</td>
<td>0 (&lt;0.1)</td>
<td>0 (&lt;0.4)</td>
</tr>
<tr>
<td>O-Succinyl-</td>
<td>0 (&lt;7.7)</td>
<td>0 (&lt;0.2)</td>
<td>0 (&lt;0.2)</td>
</tr>
<tr>
<td>O-Oxalyl-</td>
<td>0 (&lt;0.7)</td>
<td>0 (&lt;0.2)</td>
<td>0 (&lt;0.5)</td>
</tr>
<tr>
<td>O-Acetyl-</td>
<td>0 (&lt;0.7)</td>
<td>0 (&lt;0.2)</td>
<td>0 (&lt;0.5)</td>
</tr>
</tbody>
</table>

Initially detected (2) and are reasonably comparable with the activities reported for *E. coli* (55) and measured by us for *E. coli* and *B. subtilis* (see Table I). With the homoserine esters at close to saturating concentrations, the crude green plant extracts generally catalyze the highest rates of cystathionine formation in the presence of O-malonylhomoserine, intermediate rates in the presence of O-oxalyl-, O-succinyl-, and O-phosphorylhomoserine, and very slow rates with O-acetylhomoserine. The activity patterns for the various homoserine esters were quite similar within the diverse phylogenetic groups surveyed, even in plant tissues which are unusual in that they accumulate large amounts of homoserine derivatives. For example, the activity pattern did not differ noticeably from the general one in extracts of pericarps of either garden-pea (*Pisum sativum*), a tissue which accumulates O-acetylhomoserine (56) or of grass-pea (*Lathyrus sativus*), a tissue which accumulates O-oxalylhomoserine (57). One significant difference was noted between extracts of plants which arose early phylogenetically and extracts of plants which arose more recently. At saturation, the former were more active with O-phosphorylhomoserine than with O-succinylhomoserine, whereas in the latter the reverse order generally prevailed. It should be emphasized that these properties were determined in crude extracts. Further work will be required to establish whether plants contain more than one cystathionine γ-synthase, and to clarify the molecular basis of the different activity patterns.

In their capacity to utilize O-phosphorylhomoserine for cystathionine formation, green plants are unique among the organisms tested. This capacity is not present in the bacteria *S. typhimurium* (23), *E. coli* or *B. subtilis*, nor the blue-green alga *Anabaena flos-aquae* (Table II), but does appear in the single-celled green alga *Chlorella sorokiniana*. Since the green algae are thought to be the ancestors of the multicellular green plants (58, 59), the evolutionary acquisition of the ability to utilize O-phosphorylhomoserine for cystathionine biosynthesis may be localized to the green algae or to an ancestor of this group.
capable of utilizing O-phosphorylhomoserine, leads us to propose that O-phosphorylhomoserine is indeed the dominant physiological cystathionine precursor in green plants.

This proposal is consistent with in vitro labeling experiments. Dougall and Fulton (18) observed that neither O-succinyl- nor O-acetylhomoserine were used in preference to homoserine for cystathionine synthesis by Paul's Scarlet Rose cells, and suggested that these homoserine esters were first converted to homoserine before being incorporated into cystathionine. In experiments in which radioactive homoserine was fed to pea seedlings, Grant and Voelkert (63) concluded that the relative specific radioactivities of O-acetylhomoserine and cystathionine were inconsistent with the former being a precursor of the latter. Finally, additional support for the proposed physiological role of O-phosphorylhomoserine is provided by a survey of the enzymes that esterify homoserine in green plants. Homoserine kinase was demonstrated in all tissues examined, whereas no significant synthesis of any of the O-acetylhomoserine esters studied here could be detected in plant tissues other than those of garden- or grass-peas. The latter species, respectively, contain readily measurable activities of acetyl-CoA homoserine acetyltransferase and the corresponding oxalyltransferase, in agreement with the exceptional accumulations of O-acetylhomoserine and O-oxalylhomoserine in the pericarps of these plants.

Thus, we have been able to demonstrate that green plants generally have the capacity to form O-phosphorylhomoserine, but we have not been able to demonstrate that they form or accumulate any O-acetylhomoserine ester. It is especially noteworthy that although most of the plant extracts showed highest cystathionine $\gamma$-synthase activity with O-malonylhomoserine, this ester could not be detected in plant tissues and its biosynthesis by plant extracts could not be demonstrated. These results provide another illustration that, as suggested by Nagai and Flavin (24), the specificity for enzymic homoserine esterification in an organism is a better indicator of the physiological cystathionine precursor in that organism than is the specificity of the corresponding cystathionine $\gamma$-synthase.

Concerning the contributions to homocysteine biosynthesis of the direct sulfhydration and the transsulfuration pathways, it has been reported that the acylhomoserine sulfhydrase of crude spinach extracts is relatively active with O-acetylhomoserine, but much less active with O-succinyl-, O-oxalyl-, or homoserine itself (3). Lack of O-acetylhomoserine, which could not be detected in the plants studied here, might then be expected to limit the contribution of the sulfhydration pathway. However, preliminary experiments indicate that O-phosphorylhomoserine as well as O-acetylhomoserine can serve as substrate for the sulfhydrate activities of crude spinach and Chlorella extracts. Clearly, a definitive answer as to the relative contributions of the two pathways must await further work.

The establishment of O-phosphorylhomoserine as the major cystathionine precursor in most green plant tissues suggests that the regulatory factors controlling the methionine and threonine pathways may be different in green plants than in other biological forms. For the moment, we may assume that O-phosphorylhomoserine is the immediate precursor of threonine in green plants, as it is in yeast (64), N. crassa (55, 60), E. coli (66), and B. subtilis (67). If so, and if O-phosphorylhomoserine is the physiological substrate for sulfhydrase, the last intermediate common to the formation of both methionine and threonine in green plants will be O-phosphorylhomoserine rather than homoserine, as it is in other organisms. Thus, it would be worthwhile to search for two separable homoserine kinases in plants, one involved in threonine, the other in methionine biosynthesis. Furthermore, the first enzymes beyond the branch-point may be threonine synthase and cystathionine $\gamma$-synthase or the sulfhydrase, or all three, and feedback inhibition and end product repression might be expected to impinge upon these enzymes.

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