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Homoserine Esterification in Green Plants

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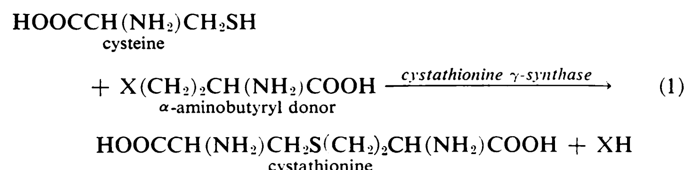
ABSTRACT

Extracts of phylogenetically diverse plans were surveyed for their ability to synthesize the following homoserine esters which are potential precursors for methionine and threonine synthesis in green plants: *O*-acetyl-, *O*-oxalyl-, *O*-succinyl-, *O*-malonyl-, and *O*-phosphohomoserine. Synthesis of *O*-acylhomoserine esters was detected only in *Pisum sativum* L. and *Lathyrus sativus* L. Extracts of *P. sativum*, a plant known to accumulate *O*-acetylhomoserine, catalyzed the specific synthesis of this ester from homoserine and acetyl-CoA. Extracts of *L. sativus*, a plant known to accumulate *O*-oxalylhomoserine, catalyzed the specific synthesis of this ester from homoserine and oxalyl-CoA. None of the other plants surveyed, including representatives of the green algae, horsetails, gymnosperms, and angiosperms, catalyzed the synthesis of any of the *O*-acylhomoserine esters studied. In contrast, synthesis of *O*-phosphohomoserine by the reaction catalyzed by homoserine kinase was demonstrated in extracts of all plants examined, including the two exceptional legumes.

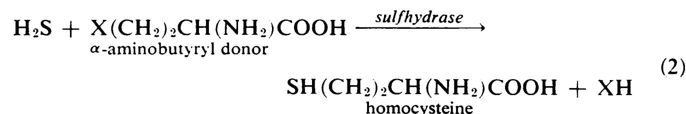
These results suggest that, among the five homoserine esters studied, *O*-phosphohomoserine is the major activated homoserine derivative in plants. Direct confirmation of the dominant physiological role of *O*-phosphohomoserine in the synthesis of cystathionine in the transsulfuration pathway of methionine biosynthesis in plants has recently been provided (Datko, A. H., Giovanelli, J., and Mudd, S. H. 1974. J. Biol. Chem. 249: 1139–1155).

Homoserine has been established as the precursor of the four-carbon skeletons of methionine and threonine (2, 4, 9, 10, 29, 30, 34, 37, 46, 47). In microorganisms, activation of homoserine by esterification of its hydroxyl group is required prior to conversion to either methionine or threonine. For threonine formation, *O*-phosphohomoserine is the physiologically important precursor in both bacteria (41, 48, 51) and fungi (16, 35, 50). For methionine biosynthesis, *O*-acetylhomoserine is used by all fungi studied (18, 19, 27, 36, 42) and by some bacteria[1] (1, 3, 25, 28), whereas *O*-succinylhomoserine is used by other bacteria (5, 17, 19, 22, 27, 38–40).

Green plants can potentially synthesize methionine by two alternative pathways—the transsulfuration pathway and the direct sulfhydration pathway. While the relative physiological contributions of these two pathways have not yet been clarified (8), the following observations demonstrate that activation of homoserine is essential for both. Synthesis of cystathionine (reaction 1) has been demonstrated in extracts of a wide range of plants (3).



High rates were catalyzed with *O*-malonylhomoserine as the α-aminobutyryl donor, intermediate with *O*-oxalyl-, *O*-succinyl-, and *O*-phosphohomoserine, and low rates with *O*-acetylhomoserine. Preliminary data (6) on the synthesis of homocysteine by the direct sulfhydration pathway (reaction 2) in extracts of spinach suggested that *O*-acetylhomoserine is more active than *O*-oxalyl- or *O*-succinylhomoserine.



Neither reactions 1 nor 2 proceeded with homoserine itself.

The details of the conversion of homoserine to threonine have not been studied in plants. However, by analogy with all other biological systems studied, it is to be expected that this conversion will also require activation of homoserine.

Previous studies on homoserine esterification in green plants have been limited to the legumes *Pisum* and *Lathyrus*. *P. sativum* L. (garden pea) accumulates *O*-acetylhomoserine (11), and the *in vivo* conversion in this plant of homoserine to *O*-acetylhomoserine has been established (9, 10, 30). *L. sativus* L. (grass pea) and five other *Lathyrus* species have been demonstrated to accumulate *O*-oxalylhomoserine, although the ester could not be detected in 10 other species of *Lathyrus* (31, 32). Evidence has been presented (14, 24) for the synthesis of *O*-oxalylhomoserine from homoserine and oxalyl-CoA in extracts of *L. sativus*. At present it is not known whether these genera are unique in accumulating the homoserine esters in question, since the papers which reported the presence of these esters did not mention studies of other genera. Nor have other investigations on the natural distribution and synthesis of these or other homoserine esters in the plant kingdom been published.

At the time this study was initiated, the homoserine derivatives actually used for biosynthesis of methionine and threonine in green plants had not been defined. One approach to this problem, which has been successfully employed for studies with microorganisms (1, 27) is to determine the nature of the homoserine esters that are synthesized in crude tissue extracts. In this work we examined the ability of extracts of a

[1] *Corynebacterium acetophilum* may possibly be an exception to the generalization that activation of homoserine is accomplished by esterification of the hydroxyl group, since available evidence does not definitely settle whether the precursor of methionine in this organism is *O*-acetylhomoserine or the ether, *O*-ethylhomoserine (12, 26).

wide phylogenetic range of plants to catalyze the synthesis of the following homoserine esters: *O*-acetyl-, *O*-oxalyl-, *O*-succinyl-, *O*-malonyl-, and *O*-phosphohomoserine. A preliminary report has appeared (7).

MATERIALS

Acylhomoserine Derivatives. *N*-Acylhomoserine derivatives were prepared by base treatment of the corresponding *O*-acylhomoserine compounds (13).

O-Oxalylhomoserine-G-^{3}H was prepared by incubation of an extract of *L. sativus* seedlings with homoserine-^{3}H and oxalyl-CoA, as previously described (3). The combined effluent and wash solutions obtained after Dowex-50(H^+) chromatography were chilled in ice, and titrated with cold 0.1 M $KHCO_3$ to a pH of 5. Two separate batches of *O*-oxalylhomoserine-^{3}H were prepared by this method. In one batch, whose characterization is described in "Results," radiopurity was approximately 85%, with *N*-oxalylhomoserine-^{3}H comprising the only detectable impurity. In the second batch, no impurity could be detected, and radiopurity was estimated to be greater than 90%. In both batches, homoserine-^{3}H was converted to *O*-oxalylhomoserine-^{3}H in a yield of at least 50%.

O-Succinylhomoserine-^{3}H was prepared essentially by the method previously described (3) for synthesis of *O*-malonylhomoserine-^{3}H. L-Homoserine-^{3}H (1.1 μmoles, 3.66 × 10^8 dpm) was lyophilized to dryness in a 300-μl Reactivial (Pierce Chemical Co.), then incubated at 25 C for 10 min with 10 μl of a mixture prepared by adding 0.15 ml of succinyl dichloride and 25 μl of 60% perchloric acid to 0.6 ml of acetone. The reaction mixture, after processing as previously described (3), was subjected to electrophoresis for 30 min in 0.02 M K phosphate, pH 6.6. Two peaks of radioactivity in addition to that of unreacted homoserine were obtained, and were eluted from the electrophoretogram. Peak A, migrating at 9 cm toward the anode, contained approximately 27% of the recovered radioactivity. Peak B, migrating at 15 cm toward the anode, contained approximately 16% of the recovered radioactivity. Peak B was tentatively characterized as *N*-succinylhomoserine-^{3}H. It was inseparable from authentic *N*-succinylhomoserine during chromatography in 1-butanol-acetic acid-water, 12:3:5, v/v (solvent A), or electrophoresis in 0.02 M K phosphate, pH 6.6, for 30 min, and the rate of migration in these systems was unchanged after incubation for 30 min at 25 C with 3 N NH_4OH. Peak A was resolved into two radioactive peaks (A1 and A2) after chromatography for 18 hr in solvent A. Peaks A1 and A2 migrated at 14 cm and 29 cm, respectively. Peak A2 was not further characterized. Approximately 37% of the radioactivity of peak A was recovered in peak A1, which was characterized as *O*-succinylhomoserine-^{3}H on the basis of the following properties. It co-chromatographed with authentic *O*-succinylhomoserine in solvent A. Only one radioactive derivative, which co-chromatographed with authentic *N*-succinylhomoserine, was obtained after incubation of peak A1 for 30 min at 25 C in 3 N NH_4OH. The over-all yield of *O*-succinylhomoserine-^{3}H was 5% of the added homoserine-^{3}H.

Other Chemicals. Acetyl-, succinyl-, and malonyl-CoA, and acetylphosphate were obtained from P-L Biochemicals. Oxalyl-CoA was prepared by the method of Quayle (33), and assayed by the delayed nitroprusside reaction (20). The sources and preparation of other materials have been previously described (3).

Tissues. Seeds of *L. sativus* L. and *P. sativum* L. var. Alaska were soaked for 15 min in 20% Clorox containing approximately 0.03% (v/v), So-Kleen (Babbitt Products, Lakeville, Conn.) detergent. The seeds were drained, washed in distilled water, and germinated at 25 C on moist filter paper covered with cheesecloth. Seedlings were harvested after 3 days (*L. sativus*) or 5 days (*P. sativum*). Mature tissues (flowers, leaves, etc.) of these species were obtained from plants grown in a greeenhouse. Seeds of *Hordeum vulgare* L. var. Betzes were soaked overnight in running tap water, then transferred to cheesecloth suspended over aerated 5 mM $CaCl_2$. Germination was continued for 5 days at 20 C and irradiance of 3 microeinsteins $m^{-2}sec^{-1}$. Irradiance was measured only in the 400 to 700 nm waveband, as previously described (3). The sources of other tissues have been previously described (3).

METHODS

General Methods. Methods for paper chromatography and paper electrophoresis have been described (3). Solvent A, consisting of a 1-butanol-acetic acid-water, 12:3:5, v/v, was commonly used for paper chromatography. Amino acids and *O*-esters of homoserine were visualized on paper with ninhydrin. *N*-Acylhomoserine derivatives were visualized by steaming the paper chromatogram or electrophoretogram for 5 min, followed by dipping in a solution of bromocresol green (44).

Unless stated otherwise, evaporations were performed *in vacuo* at 23 C with a rotary Evapo-Mix (Buchler Instruments, Inc.). Protein was determined by the method of Lowry *et al.* (23). When extracts contained thiol compounds, or had not been subjected to gel filtration, protein was precipated with trichloroacetic acid prior to assay by the Lowry method. Unless stated otherwise, values for radioactivity have been determined with an error of 5% or less, and have been corrected for spillover and background. Details of the determination of radioactivity have been described (8). Dowex-50 chromatography was performed with AG 50W-X4, 200 to 400 mesh (Bio-Rad Laboratories) on a 0.9 × 2.9 cm column. The material to be chromatographed was applied to the column, which was washed with water to yield 5.0 ml final volume of combined effluent and wash solutions. Where appropriate, the column was then eluted with 3 N NH_4OH to yield a final volume of 5.0 ml of eluate solution. Trichloroacetic acid was removed from reaction mixtures by extraction three times with 0.5 ml of fresh ethyl ether.

Preparation of Enzyme Extracts. All operations involved in preparation of enzyme extracts were performed at 4 C. All extracts were assayed shortly after their preparation.

Extracts of multicellular organisms were prepared by grinding tissue (1 g fresh wt/ml grinding medium) with either phosphate buffer (0.1 M, pH 7.3) or medium A. Medium A was a modification on that described by Steinhart *et al.* (45) and contained 18% (v/v) polyethylene glycol 400, 2.6 mM dithiothreitol, 48 mM K phosphate (pH 7.3), 0.26 M sucrose, and 8 mM EDTA. A small amount of sand was added to facilitate thorough grinding. The brei was passed through cheesecloth, and the filtrate was centrifuged either for 2 min at 120*g* (120*g* supernatant), or for 15 min at 10,000*g* to yield a 10,000*g* supernatant and pellet fractions. The 10,000*g* supernatant fraction, unless stated otherwise, was subjected to gel filtration with Sephadex G-25 equilibrated with either 5 mM tris-HCl, pH 7.5 (for tissues ground in phosphate buffer), or medium A from which EDTA had been omitted (for tissues ground in medium A). The pellet was suspended in the same buffer as used for gel filtration.

Chlorella sorokiniana Shihira and Krauss, strain 7-11-05, was grown photoautotrophically as previously described (3) and disrupted in 0.1 M K phosphate, pH 7.3, in a French pressure cell operated at 24,000 p.s.i. The homogenate was treated as described above to yield gel filtered 10,000*g* supernatant and pellet fractions. Cells of *Escherichia coli* and an equal weight of aluminum oxide were ground in a mortar with

5 mM K phosphate, pH 7.5, –0.1 mM EDTA (5 ml/g fresh weight cells). The slurry was centrifuged 15 min at 10,000*g*, and the resultant supernatant fraction was centrifuged at 48,000*g*. The 48,000*g* supernatant fraction was used without further treatment as a source of homoserine succinyltransferase.

Standard Assay of *O*-Acetyl-, *O*-Succinyl-, and *O*-Malonylhomoserine Synthesis. Enzyme extract was incubated at 30 C for 30 min with the following components in a final volume of 100 μl: 0.22 mM L-homoserine-^{14}C or -^{3}H, 0.1 M tris-HCl or HEPES, pH 7.6, and 4 mM acyl-CoA. The reaction was stopped with 20 μl of 1.5 M trichloroacetic acid, and the precipitate was removed by centrifugation. Assay of the synthesis of *O*-acetyl-, *O*-succinyl-, and *O*-malonylhomoserine is based on a method, originally developed by Nagai and Flavin (27), that utilizes the fact that these esters are retained on a column of Dowex-50(H$^+$) before, but not after, base treatment. In the presence of mild base, *O*-acylhomoserine esters undergo an *O* to *N* acyl migration (13) with consequent loss of their cationic property. One aliquot of 50 μl of supernatant solution was diluted and applied to the column without prior alkaline treatment. The increment in radioactivity of the combined effluent and wash solutions resulting from base treatment is a measure of *O*-acetyl-, *O*-succinyl-, or *O*-malonylhomoserine synthesis. Rates reported in this assay have been corrected for a relatively small activity observed in the absence of acyl-CoA. The rate of *O*-acetylhomoserine synthesis was linear with time for 30 min, and approximately linear with enzyme concentrations that catalyzed up to 10% esterification of homoserine.

Sensitive Assay of *O*-Acetyl-, *O*-Succinyl-, and *O*-Malonylhomoserine Synthesis. Sensitivity of the standard assay of *O*-acylhomoserine synthesis was increased approximately 10-fold by more extensive purification of the enzyme reaction products, as described below. Reaction mixtures contained 0.22 mM L-homoserine-^{14}C, 1.93 $\times$ 10^6 dpm, 0.1 M HEPES, pH 7.6, 4 mM acetyl-, succinyl-, or malonyl-CoA and enzyme extract in a final volume of 0.2 ml. After incubation at 30 C for 30 min, the reaction was stopped with 0.5 ml of 5% trichloroacetic acid and precipitated protein was removed by centrifugation. The supernatant solution (0.5 ml) was extracted with ether, then subjected to the procedures summarized in Figure 1. The enzymic product (*O*-acylhomoserine-^{14}C) and unesterified homoserine-^{14}C are retained during the first chromatography on Dowex-50 (H$^+$), whereas *N*-acylhomoserine-^{14}C passes through the column and is discarded. Under the basic conditions used for elution of the column (3 N NH_4OH), *O*-acylhomoserine-^{14}C is quantitatively converted to the *N*-acylhomoserine-^{14}C derivative. The 3 N NH_4OH eluate therefore contains *N*-acylhomoserine-^{14}C equal to the amount of *O*-acylhomoserine-^{14}C originally formed, and homoserine-^{14}C. The eluate was evaporated to dryness, dissolved in 1 ml of water, and reapplied to a column of Dowex-50 (H$^+$). *N*-Acylhomoserine-^{14}C is recovered in the effluent and wash solutions, and is separated from homoserine-^{14}C which is retained on the column. The combined effluent and wash solutions were evaporated to dryness, then incubated at 100 C for 1 min with 0.5 ml of 85 mM KOH to hydrolyze any *N*-acylhomoserine-^{14}C lactone formed in the preceding steps. The solution was then desalted on a column of Dowex-50 (NH_4^+), and the combined effluent and wash solutions chromatographed in solvent A for 24 hr in the presence of a known amount of radioactivity from an appropriate authentic sample of *N*-acylhomoserine-^{3}H. Addition of ^{3}H markers greatly facilitated the precise localization of any acylhomoserine-^{14}C, and furthermore allowed the amount of acylhomoserine-^{14}C synthesized to be calculated from a determination of the ^{14}C/^{3}H ratio of acylhomoserine and a knowledge of the amount of ^{3}H added as the authentic compound.

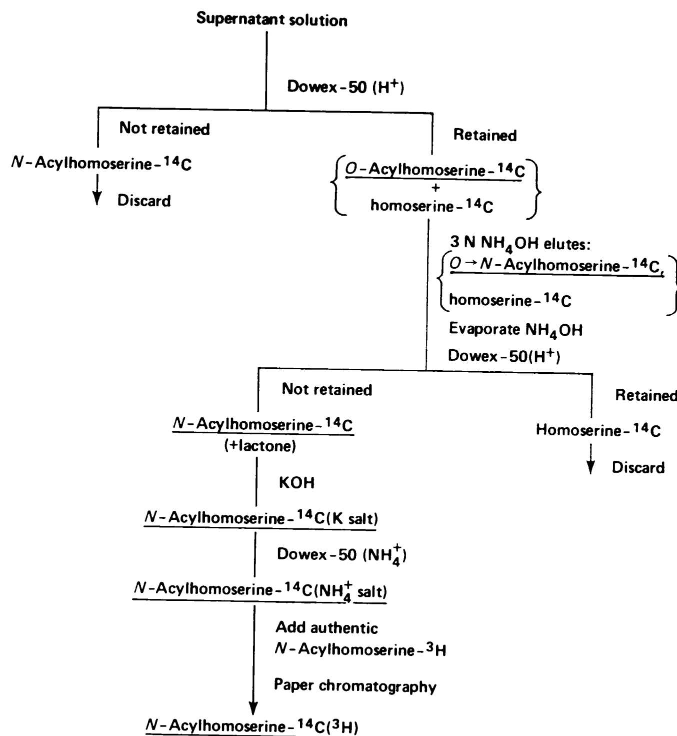


FIG. 1. Sensitive assay of *O*-acetyl-, *O*-succinyl-, and *O*-malonylhomoserine synthesis. The enzymic product, *O*-acylhomoserine-^{14}C, and *N*-acylhomoserine-^{14}C derived therefrom, are underlined to assist in tracing their isolation and purification.

Assay of *O*-Oxalylhomoserine Synthesis. The reaction mixture and conditions were identical to those of the sensitive assay described above, except that the acyl-CoA derivative was oxalyl-CoA. The reaction mixture was then subjected, as described above in the sensitive assay, to the first chromatography on a column of Dowex-50 (H$^+$). Unesterified homoserine-^{14}C is retained on the column, and *N*-oxalylhomoserine-^{14}C and *O*-oxalylhomoserine-^{14}C recovered in the combined effluent and wash solutions. *O*-Oxalylhomoserine is not retained on Dowex-50 (H$^+$), due to its strongly acidic oxalyl group. A known amount of radioactive authentic *O*-oxalylhomoserine-^{3}H was added to this solution, which was then cooled in ice and titrated to approximately pH 5 with cold 0.1 M $KHCO_3$. *O*-Oxalylhomoserine-^{14}C was separated from *N*-oxalylhomoserine-^{14}C and other ^{14}C impurities by electrophoresis, followed by paper chromatography. Under the conditions of electrophoresis (1.5 hr in 83 mM pyridine-46 mM acetic acid, pH 5.3) *O*-oxalylhomoserine migrates at 24 cm toward the anode, and is efficiently separated from *N*-oxalylhomoserine at 37 cm toward the anode. Those paper strips corresponding to authentic *O*-oxalylhomoserine-^{3}H were eluted, and the eluate was chromatographed in solvent A for 24 to 48 hr. The amount of *O*-oxalylhomoserine-^{14}C synthesized was calculated from the ^{14}C/^{3}H ratio of *O*-oxalylhomoserine on the chromatogram.

The assays described above are specific for acylation of the hydroxyl group of homoserine. Any modification of these assays which results in a loss of this specificity should be approached with caution, since significant nonenzymic synthesis of *N*-malonyl-, *N*-succinyl-, and *N*-oxalylhomoserine was observed under the assay conditions in the presence of the appropriate acyl-CoA compound.

Assay of *O*-Phosphohomoserine Synthesis. *O*-Phosphohomoserine synthesis was assayed by incubation of enzyme extract with the following components in a final volume of 100 μl:

0.41 mM L-homoserine-^{14}C or -^{3}H, 5 mM ATP, 5 mM $MgCl_2$, 10 mM dithiothreitol and 0.1 M K phosphate, pH 7.8. After incubation at 30 C for 30 min, the reaction was stopped with 0.5 ml of 5% trichloroacetic acid, and precipitated protein was removed by centrifugation. An aliquot of the supernatant solution was applied to a column of Dowex-50 (H^+), and the strongly acidic *O*-phosphohomoserine was recovered in the combined effluent and wash solutions. The rate of *O*-phosphohomoserine synthesis was calculated from a determination of the radioactivity in this solution. The rates reported for *O*-phosphohomoserine synthesis have been corrected for a relatively low activity observed in the absence of ATP. *O*-Phosphohomoserine synthesis was linear over 30 min, with enzyme concentrations that catalyzed up to 35% esterification of homoserine.

Determination of Homoserine Esterification in Mixed Extracts. It was frequently necessary to demonstrate that failure to detect synthesis of a particular homoserine ester was not due to a masking of that activity by inhibitors in the plant extract. An inhibition due to the plant extract was determined by comparing the activity of an enzyme known to synthesize a particular homoserine ester with the activity of the same enzyme assayed in the presence of plant extract. Enzymes (with their source in parentheses) that synthesize the following homoserine esters were used: *O*-succinylhomoserine (*E. coli*), *O*-oxalylhomoserine (*L. sativus*), *O*-acetylhomoserine (*P. sativum*) and *O*-phosphohomoserine (*S. oleracea*).

RESULTS

Our initial studies of homoserine esterification concentrated on *P. sativum* (garden pea) and *L. sativus* (grass pea) which are known to accumulate *O*-acetylhomoserine (11) and *O*-oxalylhomoserine (31), respectively.

Synthesis of *O*-Acetylhomoserine by Extracts of *P. sativum*. The results of Table I demonstrate that extracts of young fruits of *P. sativum* catalyze esterification of homoserine specifically in the presence of acetyl-CoA. No detectable enzymic esterification occurred when succinyl-, malonyl-, or oxalyl-CoA were substituted for acetyl-CoA.

As explained in "Methods," assays of homoserine acetyltransferase involved a determination of the amount of *N*-

Table I. *Esterification of Homoserine by P. sativum Extract*

Activity with acetyl- and oxalyl-CoA were determined in standard assays, and that with succinyl- and malonyl-CoA with the sensitive assay. Each reaction mixture contained 10,000*g* supernatant fraction (equivalent to 0.6 mg of protein) prepared by extracting young *P. sativum* fruit in phosphate buffer. The heated extract was prepared by incubation at 85 C for 5 min. Values in parentheses are estimated maximal rates that could have escaped detection. The maximal rate reported in the presence of oxalyl-CoA has been corrected for an inhibition of 60% of the activity of homoserine oxalyltransferase from *L. sativus* caused by the presence of *P. sativum* extract. No inhibition of *E. coli* homoserine succinyltransferase was observed in the presence of *P. sativum* extract.

Changes in Standard Reaction Mixture	Homoserine Esterified
	nmoles/mg protein·hr
None	5.2
Extract heated	0.2
Succinyl-CoA substituted for acetyl-CoA	0 (<0.2)
Malonyl-CoA substituted for acetyl-CoA	0 (<0.1)
Oxalyl-CoA substituted for acetyl-CoA	0 (<2)

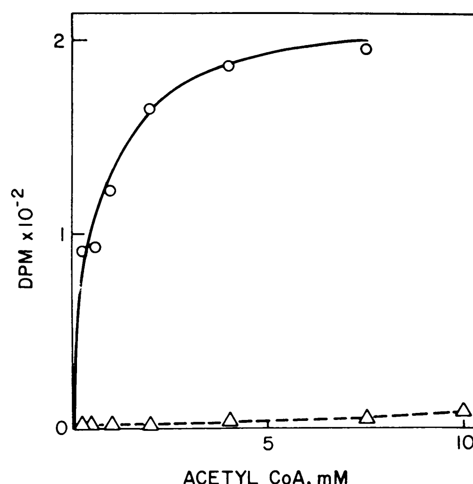


FIG. 2. Evidence for *O*-acetylation of homoserine by an extract of *P. sativum*, and effect of acetyl-CoA concentration of the rate of reaction. The reaction mixtures were identical to that described under the assay of *O*-acetylhomoserine synthesis, except for variation of the concentration of acetyl-CoA as shown. The extract of *P. sativum*, equivalent to 0.2 mg of protein, was prepared as described in Table I. After incubation for 30 min at 30 C, duplicate aliquots of each reaction mixture were applied to columns of Dowex-50 (H^+), either without further treatment (– – –) or after incubation with KOH (——) as described in the standard assay of *O*-acetylhomoserine. The ordinate represents radioactivity from each reaction mixture that appeared in the combined effluent and wash solutions after Dowex-50 (H^+) chromatography.

acetylhomoserine formed as a result of incubation of the reaction product with mild base. Evidence that enzymic acetylation of homoserine proceeds predominantly at the hydroxyl, rather than the α-amino, group of homoserine is provided in Figure 2. Reaction mixtures containing homoserine-^{14}C and an extract of *P. sativum* were incubated with increasing concentrations of acetyl-CoA. Only after base treatment of the reaction mixture did appreciable radioactivity appear in the effluent of a column of Dowex-50 (H^+). These observations are consistent with *O*-acetylhomoserine-^{14}C, which is retained by Dowex-50 (H^+), being the major product. After incubation with base, *O*-acetylhomoserine-^{14}C is converted to *N*-acetylhomoserine-^{14}C, which appears in the effluent after chromatography on Dowex-50 (H^+). Figure 2 further demonstrates that the rate of *O*-acetylhomoserine synthesis approaches a maximum at the concentration of 4 mM acetyl-CoA used in the assays of homoserine acetyltransferase.

Characterization of the radioactive product formed after base treatment as *N*-acetylhomoserine is presented in Figure 3. The radioactive compound co-migrated with authentic *N*-acetylhomoserine during paper electrophoresis (Fig. 3A) or paper chromatography in two solvents (Fig. 3, B and C).

The results of Table II show that the most active extracts of *P. sativum* are obtained from young fruits, and that activity decreases during maturation of the fruit. Low activities were detected in the stems of the mature plant, and in the roots and shoots of seedlings. No activity was detected in leaves of the mature plant, or in cotyledons of seedlings.

Activity in tris-HCl buffer was maximal at pH 7.6, the pH of the standard assay. At least 90% of the recovered homoserine acetyltransferase activity of young fruits of *P. sativum* extracted in phosphate buffer appeared in the 10,000*g* supernatant solution. Activity of extracts that had not been subjected to gel filtration was stable for many months at −65 C.

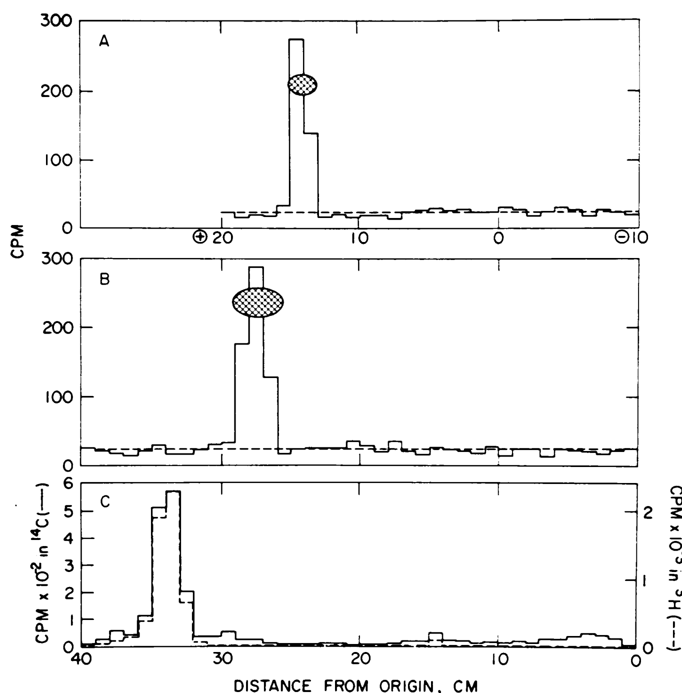


FIG. 3. Characterization of radioactive N-acetylhomoserine. Reaction mixtures containing either L-homoserine-G-^{3}H (A and B) or L-homoserine-U-^{14}C (C) were incubated with acetyl-CoA and extract of *P. sativum*, as described in Table I. The reaction was stopped with 20 μl of 0.6 M trichloroacetic acid and precipitated protein was removed by centrifugation. The supernatant solution was extracted with ether, then incubated for 1 min at 100 C with 0.75 M KOH. Water (1 ml) was added, and the solution was applied to a column of Dowex-50 (H$^+$). The combined effluent and wash solutions were evaporated to dryness. The residue was incubated with 0.5 ml of 85 mM KOH at 100 C for 1 min to hydrolyze any N-acetylhomoserine lactone to N-acetylhomoserine, diluted with 1 ml of water and applied to a column of Dowex-50 (NH$_4^+$). The combined effluent and wash solutions were either subjected to electrophoresis in 83 mM pyridine-46 mM acetic acid, pH 5.3, for 75 min (A), or chromatography in ethanol-water, 11:4, v/v (B) or solvent A (C). The hatched areas in A and B represent the locations of authentic carrier N-acetylhomoserine. Radioactivity in A and B has not been corrected for background, which is indicated by the broken lines. In C, the broken lines represent radioactivity of authentic N-acetylhomoserine-^{3}H, added as a marker to the origin of the paper chromatogram.

Gel filtration resulted in decreased stability to storage at −65 C. Young fruit stored at −65 C lost approximately 75% of its original activity over a period of 2.5 years.

Homoserine succinyl transferase from *Salmonella* and homoserine acetyltransferase from *Neurospora* and *Saccharomyces* catalyze rapid exchanges of homoserine with O-succinylhomoserine and O-acetylhomoserine, respectively (27, 36). Extracts of *P. sativum*, by contrast, did not catalyze any detectable exchange of homoserine with O-acetylhomoserine, and if this exchange reaction were present it proceeded at a rate of less than 30% of the rate of O-acetylhomoserine synthesis.

Synthesis of O-Oxalylhomoserine by Extracts of *L. sativus*. Figure 4 illustrates the radioactive products recovered in the effluent of a column of Dowex-50 (H$^+$) when a high concentration of *L. sativus* extract is incubated with radioactive homoserine and oxalyl-CoA under the conditions described in "Materials" for synthesis of O-oxalylhomoserine-G-^{3}H. O-Oxalylhomoserine is the major product, comprising over 85% of the total radioactive material in the column effluent. The remaining radioactivity resided in N-oxalylhomoserine. In separate experiments, it was demonstrated that N-oxalylhomoserine was synthesized nonenzymically from homoserine and oxalyl-CoA at a rate sufficient to account for the relatively small amounts illustrated in Figure 4. The major radioactive product was characterized as O-oxalylhomoserine by demonstration of its co-migration with the authentic compound during chromatography in two solvents (Fig. 4, A and B) and electrophoresis at pH 5.3 (Fig. 4C) and pH 1.9 (data not shown). Furthermore, incubation of the radioactive product with base quantitatively converted it to a compound which coelectrophoresed with authentic N-oxalylhomoserine (Fig. 4D).

When the concentration of *L. sativus* extract was reduced approximately 5-fold to a range in which synthesis of O-oxalylhomoserine is approximately linear with time and protein concentration, it was observed that the total radioactivity recovered in the effluent of a column of Dowex-50 (H$^+$) was only an approximation of the amount of O-oxalylhomoserine formed. This is due chiefly to the fact that as the concentration of enzyme is decreased, the relative rate of nonenzymic synthesis of N-oxalylhomoserine becomes more significant. In the assay of O-oxalylhomoserine synthesis described in detail in "Methods," plant extract was incubated with homoserine-^{14}C and oxalyl-CoA, and the product of the enzymic reaction, O-oxalylhomoserine-^{14}C, was purified by electrophoresis of the column effluent in pyridine-acetic acid, pH 5.3, followed by chromatography in solvent A. These purification steps were necessary to avoid false positive results in assays of O-oxalylhomoserine synthesis with extracts of plants other than *L. sativus*, and were used routinely for the assay of extracts of all plants examined. The results of Figure 5 illustrate a typical assay of homoserine oxalyltransferase from *L. sativus*. Electrophoresis of the column effluent, to which authentic O-oxalylhomoserine-^{3}H had been added (Fig. 5, A, B, C, and D), demonstrates the synthesis of O-oxalylhomoserine as a peak of ^{14}C that migrated with the authentic ^{3}H compound. As previously shown (Fig. 4), O-oxalylhomoserine is effectively resolved from N-oxalylhomoserine during this electrophoresis. Significant synthesis of O-oxalylhomoserine-^{14}C was observed only in the presence of oxalyl-CoA and *L. sativus* extract (Fig. 5, A and B). No synthesis was observed in the presence of

Table II. *Distribution of Homoserine Acetyltransferase in Organs of P. sativum*

Fruits were harvested at three stages of development, corresponding to the following mean lengths: early (29 mm), intermediate (47 mm), and late (52 mm). Extracts were prepared as described in Table I, and assayed by the standard procedure. Values have been corrected for any inhibition in the activity of extracts of young fruits when mixed separately with an equal volume of extract from each of the other organs. Values in parentheses are estimated maximal rates that could have escaped detection.

Organ	Activity	
	nmoles/g fresh wt·hr	*nmoles/mg protein·hr*
Fruits, early stage	68	10
Fruits, intermediate stage	20	6
Fruits, late stage	0 (<4)	0 (<1)
Stems	7	3
Leaves	0 (<3)	0 (<3)
Seedling, shoots	9	1
Seedling, roots	3	0.6
Seedling, cotyledons	0 (<1)	0 (<0.1)

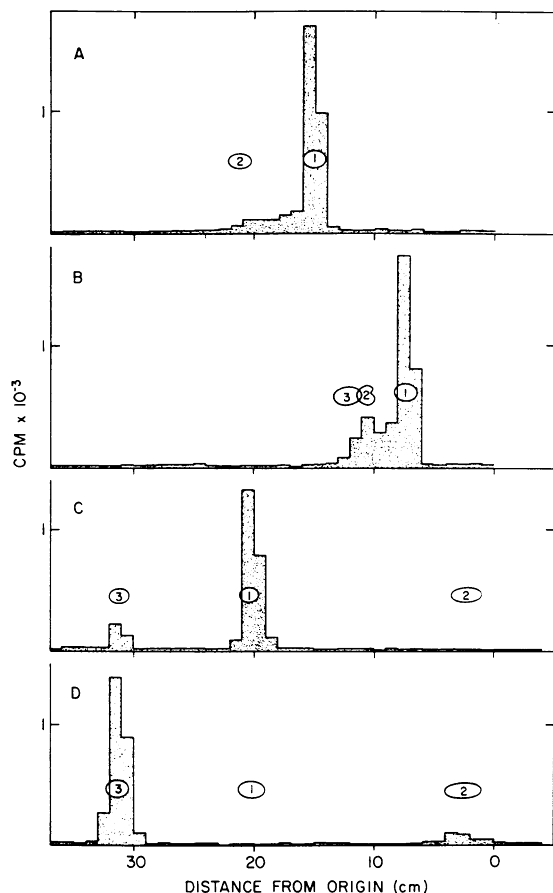


FIG. 4. *O*-Oxalylhomoserine as the product of homoserine esterification by an extract of *L. sativus*. A 10,000*g* supernatant fraction (containing 4 mg of protein) of *L. sativus* seedlings extracted in phosphate buffer was incubated with homoserine-^{3}H and oxalyl-CoA as previously described (3). Recovery of the radioactive product in the effluent of a column of Dowex-50 (H^+) is described in "Materials" under the preparation of *O*-oxalylhomoserine-G-^{3}H. A and B: Chromatography of the radioactive product in ethanol-water, 11:4, v/v, and solvent A, respectively; C: electrophoresis of the radioactive produce in 83 mM pyridine-46 mM acetic acid, pH 5.3, for 80 min. Migration was toward the anode; D: the radioactive product was incubated for 1 min at 100 C with 0.75 M KOH, desalted by passage through a column of Dowex-50 (NH_4^+), and electrophoresed as in C. Internal markers of *O*-oxalylhomoserine (1) and homoserine (2) were visualized with ninhydrin. An internal marker of *N*-oxalylhomoserine (3) was visualized with bromocresol green. Radioactivity has not been corrected for a background of 20 cpm.

heated extract (Fig. 5C) or in the absence of oxalyl-CoA (Fig. 5D). While no separation of the peaks of ^{14}C and ^{3}H obtained after electrophoresis could be observed, the ratios of ^{14}C/^{3}H across these peaks were not constant, indicating the presence of ^{14}C material other than *O*-oxalylhomoserine at this stage of purification.

The enzymic ^{14}C product was further purified, after elution, by chromatography in solvent A (Fig. 5, E, F, G, and H). In this procedure, some conversion of *O*-oxalylhomoserine to homoserine occurs, which was reflected on the chromatogram in the formation of an anterior peak (homoserine) and a posterior shoulder (*O*-oxalylhomoserine). Chromatography of the product of the reaction containing 66 μg of *L. sativus* protein (Fig. 5E) exhibited a constant ratio of ^{14}C/^{3}H across the homoserine peak, which was used to calculate the amount of *O*-oxalylhomoserine-^{14}C synthesized. The higher ratio of ^{14}C/^{3}H in the shoulder corresponding to *O*-oxalylhomoserine indicated the persistence of ^{14}C impurity in this region. In the presence of 132 μg of *L. sativus* extract (Fig. 5B) a relatively constant ratio of ^{14}C/^{3}H was observed across both the homoserine peak and *O*-oxalylhomoserine shoulder.

Synthesis of *O*-oxalylhomoserine was proportional to the concentration of *L. sativus* extract and was approximately linear over 30 min. The rate of *O*-oxalylhomoserine synthesis at 0.4 mM oxalyl-CoA was as rapid as that at 4 mM oxalyl-CoA. Esterification of homoserine is specific for oxalyl-CoA. Thus no esterification was detected when oxalyl-CoA was replaced by acetyl-, succinyl-, or malonyl-CoA, and any undetected esterification would have been less than 15% of that obtained with oxalyl-CoA. No inhibitions of the appropriate homoserine acyltransferases of *P. sativum* and *E. coli* were observed in the presence of *L. sativus* extract, indicating that failure to detect synthesis of *O*-acylhomoserine esters other than *O*-oxalylhomoserine with extracts of *L. sativus* is probably not due to the

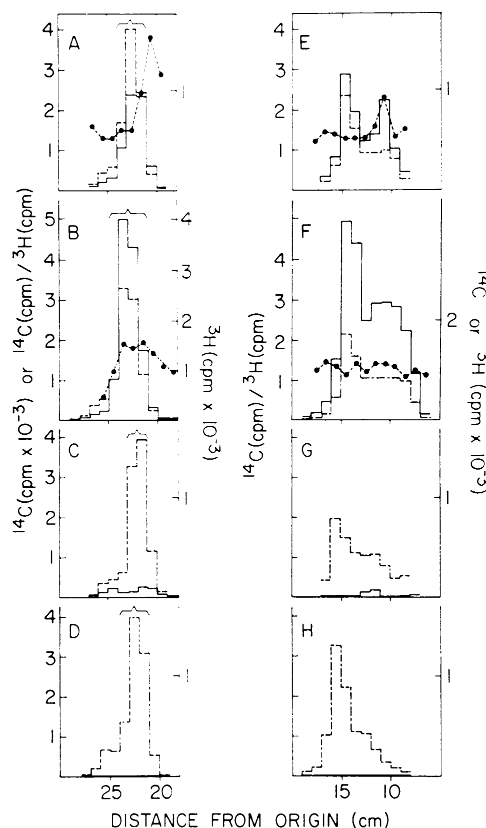


FIG. 5. Assay of *O*-oxalylhomoserine synthesis in *L. sativus* extracts. The standard reaction mixture (A) contained 0.29 mM L-homoserine-U-^{14}C, 0.1 M HEPES pH 7.7, 4 mM oxalyl-CoA, and a 10,000*g* supernatant fraction containing 66 μg of protein of *L. sativus* extracted in phosphate buffer. Incubation was at 30 C for 30 min; B: *L. sativus* extract increased to 132 μg protein; C: *L. sativus* extract (132 μg) preincubated 100 C for 5 min; D: oxalyl-CoA omitted. Equal aliquots of the effluent from a column of Dowex-50 (H^+) were mixed with 2×10^5 dpm (A, C, and D) or 4×10^5 dpm (B) of authentic *O*-oxalylhomoserine-^{3}H and subjected to electrophoresis in pyridine-acetic acid, pH 5.3 (A, B, C, and D), as described in Figure 4. Appropriate areas (represented by brackets) were eluted from each of the electrophoretograms, and the eluates chromatographed in solvent A (E, F, G, and H). Further details are described under the assay of homoserine oxalyltransferase in "Methods". ——: ^{14}C; - - -: ^{3}H; ·-·-·-: ^{14}C/^{3}H.

presence of inhibitors. Enzyme activity was stable for many months at −65 C.

Enzymic synthesis of *O*-oxalylhomoserine was restricted mainly to the seedlings of *L. sativus*. No activity was detected in flowers or in fruits harvested at an early, intermediate or late stage of development, or in the stems or leaves of mature plants. Activity, if present in these organs, was less than 5% of that in seedlings on a fresh wt basis.

No exchange of homoserine with *O*-oxalylhomoserine could be detected in the presence of *L. sativus* extracts, and, if present, proceeded at a rate of less than 1% of the rate of *O*-oxalylhomoserine synthesis.

Failure to Detect General Synthesis of *O*-Acylhomoserine Esters in Green Plants. The experiments described above demonstrate the specific synthesis of *O*-oxalylhomoserine or *O*-acetylhomoserine in two plants in which the esters corresponding to those synthesized are known to accumulate. It was of considerable interest to determine whether the capacities to synthesize *O*-acylhomoserine esters are restricted to such plants or whether these capacities are general properties of plants.

A survey of *O*-acylhomoserine synthesis was performed on extracts of seven plants that were selected to cover a wide range of phylogenetic diversity. These plants included the green alga *C. sorokiniana*, the horsetail *Equisetum arvense*, the gymnosperm *Ginkgo biloba*, and four angiosperms, *Liriodendron tulipifera, Fagus grandifolia, Zea mays*, and *Spinacia oleracea*. No synthesis of any *O*-acylhomoserine derivative could be detected with extracts of any of these plants. The results obtained with *C. sorokiniana* are typical, and will be described in detail. Figure 6 shows the results of sensitive assays of *O*-malonyl-, *O*-succinyl-, and *O*-acetylhomoserine synthesis with a supernatant fraction of *C. sorokiniana*. Addition of malonyl-CoA (Fig. 6B), succinyl-CoA (Fig. 6C), or acetyl-CoA (Fig. 6D) resulted in very small increments of ^{14}C incorporated above that observed in the absence of any added acyl-CoA derivative (Fig. 6A). Results obtained by supplementation of reaction mixtures with acetyl phosphate were identical to those supplemented with acetyl-CoA. None of these small increments of ^{14}C co-migrated exactly with ^{3}H of the corresponding authentic *N*-acylhomoserine derivative. *O*-Oxalylhomoserine synthesis was assayed in the supernatant and pellet fractions of *C. sorokiniana*. Examination of chromatographs obtained in the final step of the assay showed that reaction mixtures lacking oxalyl-CoA synthesized negligible ^{14}C material that co-migrated with authentic *O*-oxalylhomoserine-^{3}H. Two well defined peaks of ^{14}C were detected upon addition of oxalyl-CoA to the reaction mixtures. However, neither of these peaks of ^{14}C ($R_{homoserine}$ of 0.85 and 1.1) co-migrated with authentic *O*-oxalylhomoserine-^{3}H, and no attempt was made to characterize them. The small increments of ^{14}C incorporation resulting from addition of acyl-CoA derivatives to the reaction mixtures were used as a basis for estimating the upper limits of the rates of homoserine acyltransferase that would have escaped detection. The limits for each of the plants in the survey will be specified subsequently (Table V).

Synthesis of *O*-Phosphohomoserine in All Green Plants Studied. Enzymic esterification of homoserine to *O*-phosphohomoserine was demonstrated in extracts of all green plants examined. Synthesis of *O*-phosphohomoserine was dependent upon ATP and markedly stimulated by $MgCl_2$, as illustrated by the results obtained with a crude extract of spinach (Fig. 7). A maximal rate was obtained at 5 mM ATP and 5 mM $MgCl_2$, the concentrations used in the assay of homoserine kinase. The requirement for ATP appeared specific since

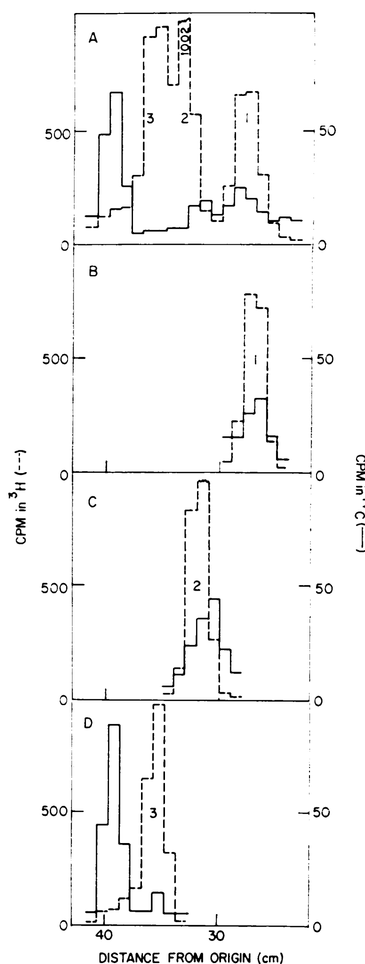


FIG. 6. Lack of synthesis of *O*-acetyl-, *O*-succinyl-, and *O*-malonylhomoserine with supernatant fraction of *C. sorokiniana*. Synthesis of each ester was determined with the sensitive assay. Distribution of ^{14}C in equal aliquots of column effluent chromatographed in solvent A (——); Distribution of radioactivity of authentic *N*-malonylhomoserine-^{3}H (1), *N*-succinylhomoserine-^{3}H (2) and *N*-acetylhomoserine-^{3}H (3) (- - -). A, B, C, and D were derived from reaction mixtures that were either unsupplemented (A), or supplemented with malonyl-CoA (B), succinyl-CoA (C), or acetyl-CoA (D).

uridine-, guanosine-, cytidine-, inosine-, and xanthosine-5′-triphosphates were all less than 15% as active as ATP at concentrations of 5 mM. Dithiothreitol at 10 mM stimulated the rate approximately 20%. The most active buffer tested was K phosphate, pH 7.8. The presence of K phosphate appears also to stimulate the reaction, since activity determined in the presence of 0.1 M bicine, pH 7.8, was increased approximately 75% by the simultaneous presence of K phosphate at the same concentration and pH. The enzyme was stable to storage at −60 C.

The homoserine ester formed by an extract of parsley was characterized as *O*-phosphohomoserine by demonstration of its co-migration with the authentic compound during paper chromatography (Fig. 8A), and paper electrophoresis at pH 1.9 (Fig. 8B) and pH 8.0 (Fig. 8C). Incubation of the radioactive ester with highly purified phosphatase converted it to a radioactive derivative which was retained on Dowex-50 (H^{+}), and exhibited the chromatographic and electrophoretic properties of authentic homoserine (Fig. 9). The homoserine ester

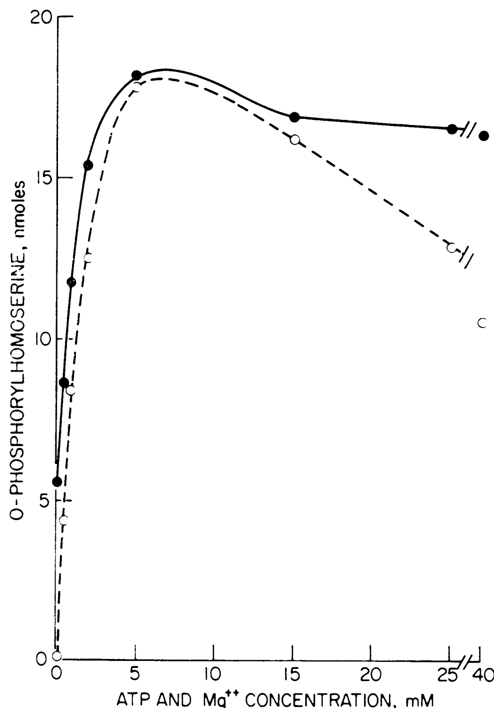


FIG. 7. Effect of ATP and Mg^{2+} concentration on rate of synthesis of *O*-phosphohomoserine by an extract of spinach. Each reaction mixture contained a 10,000*g* supernatant fraction (equivalent to 0.5 mg of protein) prepared by extraction of spinach leaves in phosphate buffer. Effect of increasing concentrations of ATP at a fixed concentration (5 mM) of $MgCl_2$ (——); effect of increasing concentrations of $MgCl_2$ at a fixed concentration (5 mM) of ATP (– – –). The rates of synthesis have been corrected for the relatively small values determined in the absence of enzyme.

formed by an extract of spinach was similarly characterized as *O*-phosphohomoserine.

The composition of the extraction medium was important for isolation of homoserine kinase from many, but not all, plants examined. The results of Table III demonstrated that little or no homoserine kinase activity could be detected in seedlings of *Hordeum vulgare*, shoots of *Zea mays*, or leaves of *Petroselinum crispum* extracted with phosphate buffer. Increases in activities ranging from at least 5-fold (*P. crispum* pellet) to at least 14-fold (*Zea mays* supernatant fraction) were obtained when these tissues were extracted with medium A. Leaves of *S. oleracea*, by contrast, yielded approximately equal amounts of homoserine kinase activity when extracted with either medium. Extracts of *C. sorokiniana* prepared in phosphate buffer also yielded high homoserine kinase activity (Table IV). The depressed homoserine kinase activities of extracts prepared in phosphate buffer do not appear to be caused by either the presence of inhibitors or by inactivation of the enzyme during isolation. Thus, it was demonstrated that extracts of spinach retained full activity when assayed in the presence of inactive extracts of other plants prepared in phosphate buffer. Furthermore, equal amounts of spinach and parsley leaves combined before extraction in phosphate buffer yielded a supernatant fraction in which the homoserine kinase activity equaled that obtained with spinach alone.

Table IV demonstrates that homoserine kinase activity was detected in each of a wide range of plants, including representatives of the green algae, club-mosses, horsetails, gymnosperms, and angiosperms. The distribution of homoserine kinase activity between supernatant and pellet fractions was found to vary from tissue to tissue under the standard conditions of enzyme extraction using medium A. For example, approximately 75% of the recovered activity of spinach was consistently observed in the supernatant fraction, and essentially all the activity remaining in the pellet fraction was removed by a single wash with the medium A. By contrast, homoserine kinase of parsley was recovered in the pellet fraction only, and could not be solubilized by repeated washing, passage through a French pressure cell, or by sonication for 10 min at 4 C. Approximately 30% of the activity was solubilized by suspension of the pellet in water, followed by freezing and thawing.

DISCUSSION

The relative rates of homoserine ester synthesis in extracts of a wide phylogenetic range of plants are summarized in Table V. Two important observations emerge from these results:

1. *O*-Acylhomoserine synthesis was detected only in *P. sativum* and *L. sativus*. Our results demonstrate the enzymic

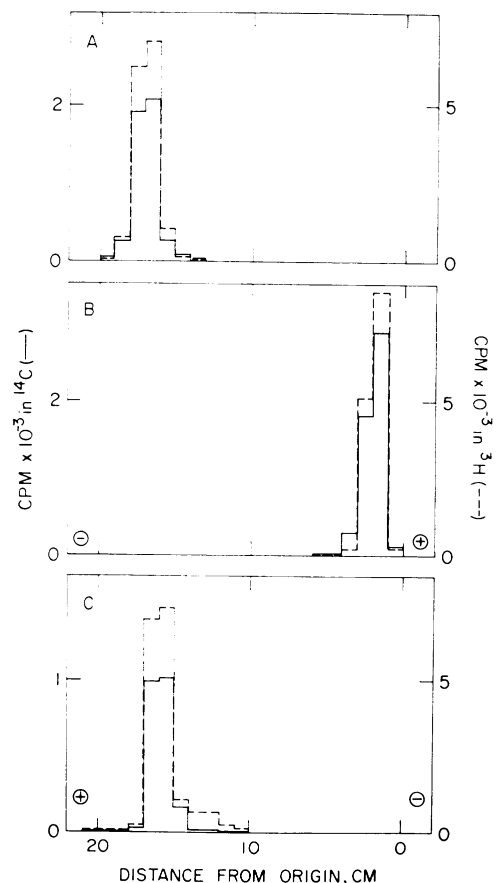


FIG. 8. *O*-Phosphohomoserine as the product of homoserine esterification by an extract of parsley. The reaction mixture, which contained homoserine-^{14}C and a pellet fraction of parsley leaves extracted in medium A, and method of isolation of the radioactive product are described in the assay of *O*-phosphohomoserine synthesis. The supernatant solution obtained after addition of trichloroacetic acid was extracted with ether prior to its chromatography on a column of Dowex-50 (H^+). Aliquots of the combined effluent and wash solutions from the column were mixed with authentic *O*-phosphohomoserine-^{3}H and subjected to the following procedures. A: Chromatography in 2-propanol-formic acid-water, 7:1:2, v/v; B: electrophoresis in formic acid, pH 1.9, for 45 min; C: electrophoresis in 125 mM triethanolamine-HCl, pH 8.0, for 45 min.

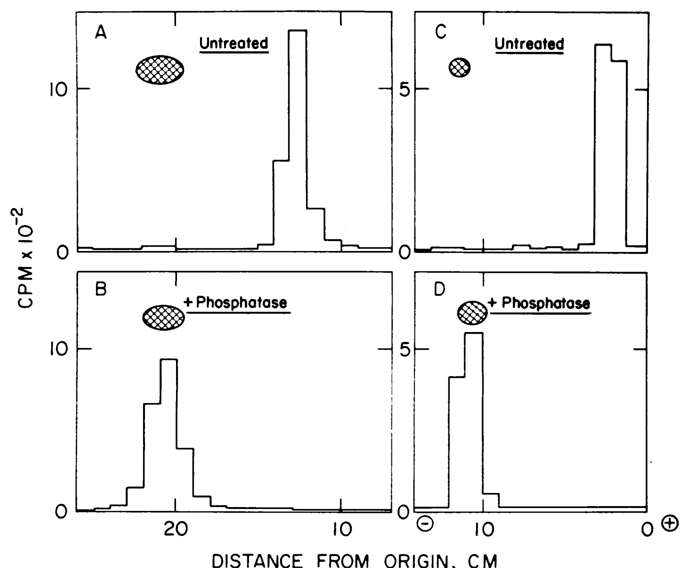


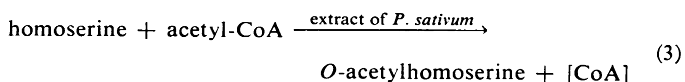
FIG. 9. Conversion of the homoserine ester formed by an extract of parsley to homoserine after incubation with phosphatase. The homoserine ester, prepared as described in the legend to Figure 8, was purified by elution of the radioactive peak obtained after chromatography in 2-propanol-formic acid-water, 7:1:2, v/v. The purified preparation was incubated for 10 min at 30 C in 33 mM tris-HCl, pH 8.05, either in the absence or presence of 0.3 units of highly purified alkaline phosphatase. Aliquots of these reaction mixtures were then chromatographed in the solvent described above, or electrophoresed for 45 min in formic acid, pH 1.9. A and B: Chromatography after incubation in the absence or presence, respectively, of phosphatase; C and D: electrophoresis after incubation in the absence or presence, respectively, of phosphatase. Internal markers of homoserine (hatched areas) were added to the origins of the paper chromatograms or electrophoretograms.

Table III. *Effect of Extraction Medium on Homoserine Kinase Activity*

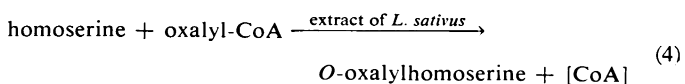
Tissue fractions were prepared as described in "Methods" in either phosphate buffer or medium A. Values in parentheses are estimated maximal values that could have escaped detection.

Species	Fraction	Extraction Medium	
		Phosphate	Medium A
		nmoles/hr·g fresh wt	
Hordeum vulgare, var. Betzes, seedling	Supernatant	16	190
Zea mays, shoots	Supernatant	0 (<10)	140
Petroselinum crispum, leaves	Supernatant	0 (<4)	0 (<4)
	Pellet	0 (<4)	20
Spinacia oleracea, leaves	Supernatant	540	590

synthesis of the two *O*-acylhomoserine esters known to accumulate in plants, according to the following reactions:



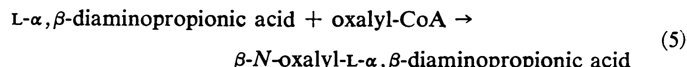
$$\text{homoserine} + \text{acetyl-CoA} \xrightarrow{\text{extract of } P.\ sativum} O\text{-acetylhomoserine} + [\text{CoA}] \quad (3)$$



$$\text{homoserine} + \text{oxalyl-CoA} \xrightarrow{\text{extract of } L.\ sativus} O\text{-oxalylhomoserine} + [\text{CoA}] \quad (4)$$

No attempt was made to demonstrate directly either the production of CoA or the strict stoichiometry of these reactions. Synthesis of *O*-acylhomoserine ester by each of the extracts was quite specific for the particular ester that accumulates in the tissue. Thus, extracts of *P. sativum* catalyzed specifically the synthesis of *O*-acetylhomoserine (Table I), and those of *L. sativus* specifically the synthesis of *O*-oxalylhomoserine. Neither plant extract catalyzed a detectable exchange of homoserine with the appropriate homoserine ester. By contrast, homoserine succinyltransferase from *Salmonella* and homoserine acetyltransferase from *Neurospora* and *Saccharomyces* (27, 36) each catalyze a rapid exchange of homoserine with the appropriate homoserine ester.

Enzymic synthesis of *O*-oxalylhomoserine has been previously reported in extracts of *L. sativus* (14, 24), although the products were not definitively characterized. Malathi *et al.* (24) partially purified oxalyl-CoA-α,β-diaminopropionic acid oxalytransferase:



$$\text{L-}\alpha,\beta\text{-diaminopropionic acid} + \text{oxalyl-CoA} \rightarrow \beta\text{-}N\text{-oxalyl-L-}\alpha,\beta\text{-diaminopropionic acid} \quad (5)$$

from germinating seeds of *L. sativus*, and noted that the preparation also catalyzed the synthesis of *O*-oxalylhomoserine (according to reaction 4) and *N*-oxalylhomoserine at rates of 10% and 15%, respectively, of that of reaction 5. In our experiments, enzymic oxalylation proceeded predominantly, per-

Table IV. *Syntheses of O-Phosphohomoserine with Extracts of Various Plants*

Except where noted in the table, leaves were the source of enzyme extracts from the multicellular organisms. Extracts of *C. sorokiniana* were prepared in phosphate buffer, and those of all other tissues in medium A. Values in parentheses are estimated maximal values that could have escaped detection.

Species	10,000*g* Supernatant		Pellet	
	nmoles/g fresh wt·hr	*nmoles/mg protein·hr*	*nmoles/g fresh wt·hr*	*nmoles/mg protein·hr*
Green algae				
Chlorella sorokiniana	4080	230	2020	100
Club-Mosses				
Lycopodium lucidulum	5	24	87	6
Horsetails				
Equisetum arvense, shoots	106	13	7	1
Gymnosperms				
Ginkogo biloba, microsporangiate strobilus	135	31	63	9
Angiosperms				
Liriodendron tulipifera	185	8	75	6
Pisum sativum, young fruit	7	1	43	9
Lathyrus sativus, seedling	5	1	9	1
Fagus grandifolia	0 (<2)	0 (<2)	9	2
Zea mays, shoots	140	37	16	5
Hordeum vulgare, var. Betzes, seedling	190	82	7	3
Spinacia oleracea	330	54	101	11
Lysimachia nummularia	0 (<2)	0 (<2)	32	3
Petroselinum crispum	0 (<4)	0 (<8)	21	2
Senecio aureus, flower bud	16	3	4	1

Table V. *Relative Rates of Synthesis of Homoserine Esters*

The organs used for preparation of extracts were those described in Table IV. Assay of *O*-acetylhomoserine synthesis was determined in the presence of acetyl-CoA or acetylphosphate, as shown below. Extracts of *C. sorokiniana*, *P. savitum*, and *L. sativus* required for assay of *O*-acylhomoserine synthesis were extracted in phosphate buffer. Assay of *O*-acylhomoserine synthesis in all other species was performed in extracts prepared in medium A. Values marked with an asterisk were determined with the standard assay of *O*-acylhomoserine synthesis. The sensitive assay was used for all other assays of *O*-acylhomoserine synthesis. Assays of *O*-phosphohomoserine synthesis in *C. sorokiniana* and *S. oleracea* were determined on the supernatant and pellet fractions used for assay of *O*-acylhomoserine synthesis. *O*-Phosphohomoserine synthesis in all other species represents the sum of activities in the 10,000*g* supernatant and pellet fractions obtained after extraction in medium A. Values in parentheses are the maximal rates that could have escaped detection, and for *O*-acetyl-, *O*-succinyl-, and *O*-oxalylhomoserine synthesis, have been corrected for any inhibition determined by the mixing experiments described in "Methods".

Species	Fraction Assayed for *O*-Acylhomoserine Synthesis	Homoserine Ester Synthesized					
		Acetyl[1]	Acetyl[2]	Succinyl	Malonyl	Oxalyl	Phospho
		nmoles/g fresh wt·hr					
Green algae							
Chlorella sorokiniana	10,000*g* Supernatant	0 (<1)	0 (<1)	0 (<2)	0 (<2)	0 (<4)	4080
	Pellet	0 (<1)	0 (<1)	0 (<2)	0 (<2)	0 (<5)	2020
Horsetails							
Equisetum arvense	120*g* Supernatant	0 (<2)	—	0 (<15)*	0 (<15)*	0 (<20)	113
Gymnosperms							
Ginkgo biloba	120*g* Supernatant	0 (<1)	—	0 (<10)*	0 (<10)*	0 (<33)	198
Angiosperms							
Liriodendrom tulipifera	120*g* Supernatant	0 (<1)	—	0 (<15)*	0 (<15)*	0 (<10)	260
Fagus grandifolia	120*g* Supernatant	0 (<2)	—	0 (<2)	0 (<2)	0 (<8)	9
Zea mays	120*g* Supernatant	0 (<1)	—	0 (<1)	0 (<2)	0 (<20)	156
Spinacia oleracea	10,000*g* Supernatant	0 (<1)	0 (<1)	0 (<1)	0 (<1)	0 (<20)	330
	Pellet	0 (<1)	0 (<1)	0 (<1)	0 (<1)	0 (<20)	101
Pisum sativum	120*g* Supernatant	14	—	0 (<1)	0 (<1)	0 (<20)	50
Lathyrus sativus	120*g* Supernatant	0 (<100)	—	0 (<150)	0 (<125)	1390	14

[1] Assayed in the presence of acetyl-CoA.
[2] Assayed in the presence of acetylphosphate.

haps exclusively, at the hydroxyl group of homoserine (Fig. 4), since the relatively minor production of *N*-oxalylhomoserine could be accounted for by a nonenzymic reaction. Further work will be required to determine whether oxalyl-CoA-α,β-diaminopropionic acid oxalyltransferase and homoserine oxalyltransferase activities are catalyzed by the same or different enzymes.

Pathways for synthesis of acetyl-CoA are widely distributed in plants, and oxalyl-CoA synthetase has been demonstrated in extracts of *L. sativus* (14, 24). Coupling of the synthesis of acetyl- or oxalyl-CoA with the appropriate acyltransferase described here thus provides routes for accumulation of acetylhomoserine and oxalylhomoserine in *P. sativum* and *L. sativus*, respectively.

No *O*-acylhomoserine synthesis could be detected in any of the plants examined other than *P. sativum* and *L. sativus*. Table V illustrates that any undetected synthesis of *O*-acetylhomoserine in plants other than the two legumes studied would have been less than 15% of the activity of *P. sativum;* any undetected synthesis of *O*-oxalylhomoserine would have been less than 3% of the activity of *L. sativus*.[2]

Two trivial reasons that could have explained our failure to detect a general synthesis of *O*-acylhomoserine esters in plants were examined. (*a*) Could the extraction methods have been unsatisfactory for isolation of homoserine acyltransferases? This possibility is considered unlikely, since it was demonstrated in separate experiments not reported here that active 10,000*g* supernatant fractions of homoserine acetyltransferase of *P. sativum*, homoserine oxalyltransferase of *L. sativus*, and homoserine succinyltransferase of *E. coli* were readily obtained after extraction in either phosphate buffer or medium A. The possibility that *O*-acylhomoserine synthesis occurred in the pellet fraction obtained after centrifugation for 15 min at 10,000*g* was specifically excluded for *C. sorokiniana* and *S. oleracea* (Table V). (*b*) Was *O*-acylhomoserine synthesis not detected because of inhibition by plant extracts? This possibility is considered unlikely since activities of homoserine acetyltransferase of *P. sativum*, homoserine oxalyltransferase of *L. sativus*, and homoserine succinyltransferase of *E. coli* were readily detected when added to each of the plant extracts. The maximum undetected rates of synthesis of *O*-acetyl-, *O*-oxalyl-, and *O*-succinylhomoserine shown in Table V have been corrected for any inhibition observed.

Our results strongly suggest that the capacity to synthesize *O*-acylhomoserine esters is not a general property of plants, but is restricted to plants such as *P. sativum* and *L. sativus* that are highly unusual in accumulating these esters. *P. sativum* and *L. sativus* are also unusual in belonging to a small group of plants, all of which are legumes, in which homoserine can constitute a major component of the soluble amino acid pool (15, 21, 32, 43, 49). These combined observations indicate that the metabolism of homoserine in the two legumes studied here is not representative of that in green plants in general.

2. *O*-Phosphohomoserine synthesis was demonstrated in all plants examined. Synthesis of *O*-phosphohomoserine was dem-

[2] In Table V, a valid comparison may be made of values of the same enzyme activity extracted from different tissues. However, in the absence of a more comprehensive knowledge of the substrate kinetics and other properties of the enzymes studied, considerable caution should be exercised in comparing the relative values of different enzyme activities.

onstrated in a wide range of plants, including the two unusual legumes described above. Although the nucleotide product was not characterized and the stoichiometry not determined, it appears that synthesis of *O*-phosphohomoserine in plants proceeds by the reaction previously reported to be catalyzed by homoserine kinase in bacteria (41, 48, 51) and fungi (16, 50):

$$\text{homoserine} + \text{ATP} \xrightarrow{Mg^{2+}} O\text{-phosphohomoserine} + [\text{ADP}] \quad (6)$$

Two unusual properties of the plant enzyme require comment. First, in some plants (barley, corn, parsley) active extracts were obtained only in medium A, whereas in spinach active extracts were obtained in either phosphate buffer or medium A (Table III). Second, the distribution of homoserine kinase between supernatant and pellet fractions prepared in medium A varied from tissue to tissue (Table IV). In general, activities of the most active tissues were found predominantly in the supernatant fractions, whereas those of the less active tissues were found predominantly in the pellets. Appropriate mixing experiments with tissue extracts and with whole tissues suggested that these two results could be explained neither by the presence of inhibitors in the extracts, nor by precipitation and inactivation of a soluble homoserine kinase during its isolation. These findings raise the possibility that plant homoserine kinase may exist in at least two forms with different sensitivities to extraction in phosphate buffer, and with different cellular locations.

The two major findings discussed above suggest to us that, of the five homoserine esters studied, *O*-phosphohomoserine might be the most important homoserine ester in plants. Direct confirmation that *O*-phosphohomoserine is the dominant physiological substrate for synthesis of cystathionine in the transsulfuration pathway of methionine biosynthesis in plants has recently been presented by Datko *et al.* (3). This work demonstrated that *O*-phosphohomoserine is a substrate for plant cystathionine γ-synthase, and the only naturally occurring α-aminobutyryl donor active with plant cystathionine γ-synthase that was detected in plants. The method used to search for naturally occurring α-aminobutyryl donors set no preconditions as to the chemical nature of a donor other than that it be capable of transferring an α-aminobutyryl group to cysteine to form cystathionine in the presence of plant cystathionine γ-synthase.

The nature of the physiologically important homoserine ester(s) in the direct sulfhydration pathway of methionine synthesis, and for threonine synthesis, remains to be established. Subject to the possible exceptions discussed below for *P. sativum* and *L. sativus*, the present work excludes a dominant role for any of the *O*-acylhomoserine esters studied. *O*-Phosphohomoserine could be the physiological substrate for these pathways as well, but a possible role for homoserine esters not included in this study cannot be excluded at this time. Further experiments of the type described by Datko *et al.* (3) will be required to clarify these questions.

P. sativum and *L. sativus* are highly atypical in their capacity to synthesize *O*-acylhomoserine esters. These legumes are potentially capable of activating homoserine by two pathways. One pathway, common to all plants examined here, is *via O*-phosphohomoserine. The other pathway in *P. sativum* is *via O*-acetylhomoserine, and in *L. sativus* is *via O*-oxalylhomoserine. *O*-Acetylhomoserine and *O*-oxalylhomoserine are each substrates for plant cystathionine γ-synthase (3), and for synthesis of homocysteine in the direct sulfhydration pathway (6). The observation that synthesis of *O*-acetylhomoserine and *O*-oxalylhomoserine was not widely distributed throughout the various organs of *P. sativum* and *L. sativus* suggests that these esters may not play major sustained physiological roles in methionine and threonine synthesis in these plants. This suggestion is consistent with the results of experiments in which Grant and Voelkert (10) fed radioactive homoserine to *P. sativum* seedlings. Relative specific radioactivities were inconsistent with the possibility that *O*-acetylhomoserine was a precursor of cystathionine. The primary aim of the present work was to determine the major general pathways of homoserine esterification in plants, and further experiments will be required for a final evaluation of the relative physiological significance in legumes of these two pathways of homoserine esterification.

Note Added in Proof. We have recently demonstrated that under certain experimental conditions the sulfhydration reaction catalyzed by *Chlorella* or spinach extracts is more active with *O*-phosphohomoserine than with *O*-acetylhomoserine.

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