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TRANSSULFURATION IN HIGHER PLANTS

PARTIAL PURIFICATION AND PROPERTIES OF β-CYSTATHIONASE OF SPINACH

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SUMMARY

Extracts of a range of higher plants metabolized cystathionine predominantly by β-cleavage to homocysteine, pyruvate and ammonia. γ-Cleavage of cystathionine (to cysteine, α-ketobutyrate and ammonia) could not be demonstrated in the same extracts, and, if present, was less than 0.5% as active as β-cleavage. It follows that transsulfuration in higher plants resembles that in bacteria in proceeding predominantly, perhaps exclusively, in the direction cysteine → (cystathionine) → homocysteine. The physiological significance of the transsulfuration pathway in the *de novo* synthesis of homocysteine and methionine in higher plants is discussed.

β-Cystathionase was purified over 400-fold from spinach leaves and some of its properties determined. The most active substrates are cystathionine, djenkolate and the mixed disulfide of cysteine and homocysteine. The effect of structure on the substrate activity is discussed, and a possible molecular conformation of active substrates is presented. The enzyme is competitively inhibited by β-cyanoalanine, and irreversibly inhibited by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide. The substrate specificity of the plant enzyme resembles that of the bacterial enzyme, while the inhibitor sensitivity resembles that of the fungal enzyme.

INTRODUCTION

Transsulfuration, the process whereby sulfur is transferred between homocysteine and cysteine, plays an important role in the metabolism of mammals[1], bacteria[2] and fungi[2]. Both the direction of sulfur flow and the enzymic mechanisms are different in the different taxa. In mammals the process proceeds only in the direction homocysteine → cysteine:

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

* Part of this study was made while the senior author was a Visiting Scientist from the Plant Physiology Unit, C.S.I.R.O., Division of Food Preservation and School of Biological Sciences, University of Sydney, Australia.

$$\text{Homocysteine} + \text{serine} \xrightarrow{\beta\text{-replacement}^{*}} \text{cystathionine} + \text{water} \tag{1}$$

$$\text{Cystathionine} + \text{water} \xrightarrow{\gamma\text{-elimination}} \text{cysteine} + \alpha\text{-ketobutyrate} + \text{ammonia} \tag{2}$$

Transsulfuration in bacteria proceeds only in the direction cysteine → homocysteine:

$$\text{Cysteine} + O\text{-succinylhomoserine} \xrightarrow{\gamma\text{-replacement}} \text{cystathionine} + \text{succinate} \tag{3}$$

$$\text{Cystathionine} \xrightarrow{\beta\text{-elimination}} \text{homocysteine} + \text{pyruvate} + \text{ammonia} \tag{4}$$

In bacteria, where cysteine is thought to be one of the first organic sulfur compounds formed, transsulfuration provides for the further assimilation of sulfur into homocysteine and methionine[4].

In fungi, pathways exist for both directions of sulfur transfer. Transsulfuration of homocysteine to cysteine proceeds *via* Reactions 1 and 2. Transsulfuration of cysteine to homocysteine in Neurospora proceeds *via* a γ-replacement reaction similar to Reaction 3, but specific for *O*-acetyl homoserine[5], followed by cleavage of cystathionine to homocysteine according to Reaction 4. While it is clear that transsulfuration of cysteine to homocysteine provides for the net synthesis of homocysteine, the physiological role of the transsulfuration of homocysteine → cysteine in fungi remains uncertain[6].

Very little is known about transsulfuration in higher plants, despite the potential significance of this process in the sulfur cycle of Nature. In this paper some insight into the pattern of transsulfuration was obtained by examination of the type of cleavage of cystathionine by extracts of higher plant tissues. The results suggest that transsulfuration in higher plants proceeds almost wholly in the direction cysteine → homocysteine. β-Cystathionase was partially purified from spinach leaves and some of its properties determined. A preliminary report has been presented[7].

MATERIALS AND METHODS

General methods

Protein was determined by the Lowry method[8]. Gel filtration was performed at 4°. All incubations were at 30°, unless stated otherwise.

Radioactive α-keto acids and amino acids were isolated on a column (0.9 cm × 2.9 cm) of Dowex 50-X4 (H^+ form), 200–400 mesh. α-Keto acids were determined in the column effluent[9], and total amino acids were recovered by elution of the column with 4 ml of 3 M NH_4OH. In the study of the metabolism of [^{14}C]serine, cystathionine was recovered in the ammonia eluate after elution of [^{14}C]serine with 0.4 M HCl[9]. DNP-hydrazone derivatives of α-keto acids were prepared and extracted by the method of ARONOFF[10], modified so that the initial organic extraction was performed 5 times.

The following solvents were commonly employed in paper chromatography: Solvent A: isopropanol–88% formic acid–water (7:1:2, by vol.); Solvent B: methan-

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[*] Since International Union of Biochemistry recommendations have not yet appeared on all the enzymes reported above, in general the terminology of FLAVIN[3] is employed. This terminology refers to the particular carbon atom on which the electronegative leaving group is situated.

ol–pyridine–1.25 M HCl (185:20:40, by vol.). Paper chromatograms and electrophoretograms were dipped in 0.5% ninhydrin in acetone to detect amino acids. Sulfur amino acids were oxidized with performic acid[11] in the presence of ammonium molybdate catalyst[12]. Methods for determination of radioactivity on paper and in liquid samples have been described[9].

Plant materials

Broccoli (*Brassica oleracea*, L. var. *botrytis*, L.) kale (*B. oleracea*, L.) lettuce (*Lactuca sativa*, L.) and carrots (*Daucus carota*, L. var. *sativa*, DC) were purchased from local markets. Seeds of barley (*Hordeum vulgare*, L.) and wheat (*Triticum aestivum*, L.) were grown for 8 days on cheese cloth suspended just above an aerated solution of 5 mM $CaCl_2$. Spinach (*Spinacia oleracea*, L.) and parsley (*Petroselinum crispum*, Nym.) were grown in a greenhouse maintained at 24°. Seeds of pea (*Pisum sativium*, L. var. *Telephone*) and bush bean (*Phaseolus vulgaris*, L. var. *humilis*, Alef.) were soaked in running tap water overnight, planted in vermiculite and allowed to grow at 22° for 18 days.

Preparation of plant extracts

Extracts of tissues used in Table I were prepared by grinding in a mortar and pestle 2.5 g of tissue with 1 g of sand and 6 ml of grinding medium. The resultant slurry was squeezed through two layers of cheese cloth and certrifuged for 1 min at 200 × *g*. The supernatant fluid was used as a source of enzyme. Grinding Medium A (pH 7.25) contained 0.1 M potassium phosphate, 0.1 mM EDTA and 0.14 mM 2-mercaptoethanol. The grinding medium used to determine the cellular distribution of β-cystathionase contained 0.4 M sucrose, 0.02 M Tris–HCl (pH 8.3), 0.01 M NaCl and 0.14 mM 2-mercaptoethanol.

Purification of β-cystathionase

Spinach acetone powder (25 g) was ground in a mortar and pestle with 25 g of aluminum oxide and 500 ml of Medium A. The slurry was squeezed through two layers of cheese cloth and centrifuged for 40 min. The supernatant solution was decanted and centrifuged for 1 h. All centrifugations were at 9000 × *g*. The final supernatant solution is designated Fraction 1.

$(NH_4)_2SO_4$ (Mann, Special Enzyme Grade) was added to Fraction 1 in the proportion of 13.45 g/100 ml (24% saturation). The precipitate was removed by centrifugation, and to each 100 ml of supernatant solution were added 5.5 g $(NH_4)_2SO_4$ to bring it to 34% saturation. The resulting precipitate was collected by centrifugation and dissolved in a minimal volume of Medium A. Fraction 2 was subjected to gel filtration with a buffer of pH 7.25 containing 5 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM pyridoxal phosphate and 0.14 mM 2-mercaptoethanol, then diluted with the same buffer to a protein concentration of 7.9 mg/ml. A slurry of bentonite was prepared by stirring for 15 min 2.4 g bentonite (Fisher, U.S.P.) with 40 ml of 0.01 M acetic acid. The dilute protein solution (100 ml) was added to the bentonite slurry, and stirred for a further 15 min. Bentonite was then removed by centrifugation for 10 min at 20 000 × *g*. The supernatant solution (Fraction 3) lost approx. 80% of its activity after 5 days storage at 4°, and was therefore immediately subjected to column chromatography on hydroxyapatite, as described below. The activity of Fraction 3 could be stabilized for at least 5 days by concentration (approx. 8-fold) to approx. 1.75 mg protein per ml by "dialysis" against Aquacide II (Calbiochem). The concentrated preparation was subjected before use to gel filtration with a buffer (Buffer I) of pH 7.25 containing 5 mM potassium phosphate, 0.1 mM EDTA and 0.14 mM 2-mercaptoethanol.

A column (0.9 cm × 2.2 cm) of hydroxyapatite, prepared by the method of TISELIUS *et al.*[13], was equilibrated with 5 mM K_2HPO_4 which had been adjusted to pH 5.6 with acetic acid. Fraction 3 (10 ml), which had been acidified with 2 ml of 25 mM acetic acid, was applied to the column. The column was washed with 2 ml of 0.1 M potassium acetate (pH 5.5), then eluted with 4 ml of 0.5 M potassium phosphate (pH 6.0). The eluate was concentrated approx. 10-fold as described above, and subjected to gel filtration with Buffer I. This fraction (Fraction 4) lost approx. 70% of its activity after storage at 4° for 5 days.

Assay of the rates of β- and γ-cleavage of cystathionine

The assay is based on the fact that β-cleavage produces pyruvate from the C_3 moiety of cystathionine; γ-cleavage produces α-ketobutyrate from the C_4 moiety of cystathionine. The rates of β- and γ-cleavage of cystathionine can therefore be assayed by determining the rates of formation of radioactive keto acid from $[3\text{-}^{14}C]$-cystathionine (radioactive in the β-carbon of the C_3 moiety) and $[^{14}C_4]$cystathionine (uniformly radioactive in the C_4 moiety), respectively.

The standard isotopic assay for β-cleavage includes the following components in μmoles in a final volume of 0.5 ml: $[3\text{-}^{14}C]$cystathionine, 9.8 nC, 0.0475; pyruvate, 5; Tris–HCl (pH 8.65), 100; and enzyme. After 30 min incubation in an atmosphere of N_2, the reaction was stopped by addition of 1.0 ml of 5% trichloroacetic acid, and pyruvate assayed by use of a column of Dowex 50 (H^+ form). The assay was corrected for a relatively small percentage of radioactivity in the α-keto acid fraction, which was formed in the presence of heat-inactivated plant extract. The β-cleavage assay was linear with enzyme concentrations that catalyzed up to 30% utilization of the substrate. The rate of β-cystathionase determined with the isotopic assay is approx. 45% of that determined with the spectrophotometric assay described below. This figure is in close agreement with a value of 42% expected on the basis of the K_m for cystathionine, and the relative concentrations of cystathionine in the two assays.

The standard assay for γ-cleavage contained the same reaction components as that for β-cleavage except that $[^{14}C_4]$cystathionine, 49.0 nC (0.0478 μmole) and α-ketobutyrate (1 μmole) were substituted for the first two components of the β-cleavage assay. α-Ketobutyrate was isolated on a column of Dowex 50 (H^+ form) then further purified by extraction of the DNP-hydrazone derivative. This extract was chromatographed in *n*-butanol saturated with 1.5 M NH_4OH, and γ-cystathionase activity determined from the amount of radioactivity cochromatographing with the DNP-hydrazone of authentic α-ketobutyrate.

Spectrophotometric assay of β-cystathionase

β-Cystathionase was assayed with a thermostated Cary Model 15 spectrophotometer, in a reaction mixture (pH 8.65) containing 10 mM cystathionine, 0.175 mM NADH, 30 μg lactate dehydrogenase (crystalline heart preparation from Calbiochem) and 0.2 M Tris–HCl. The reference cuvette contained identical components, except that enzyme was omitted. This assay was used chiefly with Fractions 3 and 4, where it was dependent upon the addition of all components of the assay. The assay could be used for Fraction 2 provided a correction (approx. 15%) was made for the substrate-independent rate of NADH oxidation.

Radioactive assay of the rate of cleavage of the mixed disulfide of cysteine and homocysteine

The rate of cleavage of the L- and L-*allo*-isomers of the mixed disulfide of cysteine and homocysteine was determined in a reaction mixture identical to that described for the standard isotopic assay for β-cleavage with the exception that the appropriate radioactive mixed disulfide was substituted for radioactive cystathionine. After 30 min incubation, the α-keto acid product of the reaction was isolated on a column of Dowex 50 (H^+ form), then further purified by extraction of the DNP-hydrazone derivative. The rate of cleavage of the mixed disulfide was calculated from the amount of radioactivity in the DNP-hydrazone extract.

Chemicals

[$^{14}C_4$]Cystathionine (radioactivity located uniformly in the C_4 moiety) and [3-^{14}C]cystathionine (radioactivity located in the β-carbon of the C_3 moiety) were prepared from either uniformly labeled L-[^{14}C]homocysteine or L-[3-^{14}C]serine, respectively, by the method of MUDD *et al.*[9]. Because of the method of synthesis, both preparations are assumed to be exclusively in the L-configuration. α-Keto [^{14}C]-butyrate was prepared by incubation of [$^{14}C_4$]cystathionine with an $(NH_4)_2SO_4$ fraction of rat liver γ-cystathionase[14]. The L- and L-*allo*-isomers of the mixed disulfide of cysteine and homocysteine were prepared by oxidation of a mixture of uniformly labeled L-[^{14}C]cysteine and either L- or D-homocysteine, respectively. The product was purified by multiple (3 times) paper chromatography in Solvent A. Multiple chromatography of each isomer in Solvent A showed that 85% of the radioactivity migrated with a ninhydrin-positive area whose R_F coincided exactly with that of an authentic sample of the mixed disulfide. The remaining 15% of the radioactivity co-chromatographed with cystine.

Miscellaneous materials

The preparation of solutions of pyridoxal phosphate is described in the accompanying report[15]. Solutions of L-homocysteine to be used in enzyme reaction mixtures were prepared from L-homocysteine thiolactone·HCl (Calbiochem) immediately before use[9]. (L + *meso*)-Lanthionine was obtained from Calbiochem, and contained 35% of the L-isomer, and 65% of the *meso*-isomer. L-Homolanthionine was a gift from Dr. H. T. Huang. The mixed disulfide of L-cysteine and L-homocysteine (a gift from Dr. J. E. Seegmiller) was homogeneous as judged by thin-layer chromatography on Eastman "chromagram" 6064 cellulose plates, with multiple (3 times) development in the solvent *n*-butanol–pyridine–glacial acetic acid–water (30:20:6:24, by vol.).

RESULTS

Type of cleavage of cystathionine by plant extracts

The results of Table I demonstrate a significant enzymic β-cleavage of cystathionine by extracts of each of the plant tissues studied. High rates of β-cleavage were observed in extracts from shoots of barley, and leaves of parsley, pea and spinach. β-Cleavage activity was not restricted to photosynthesizing tissues. Extracts of barley roots and cauliflower buds were also active. Furthermore, the specific activity of extracts prepared from etiolated pea leaves was only slightly (19%) less active than those prepared from illuminated leaves. Most (78%) of the activity originally present in the crude extract remained in the supernatant solution after centrifugation at 40 000 × *g* for 45 min.

TABLE I

RELATIVE RATE OF β-CLEAVAGE BY PLANT EXTRACTS

Unless otherwise stated, all plants were grown in the light and Grinding Medium A was used for the preparation of extracts.

Plant extract	*Specific activity (pmoles/min per mg protein)*
Spinach leaves	300
Parsley leaves	320
Parsley leaves (Grinding Medium B)	280
Pea leaves	390
Barley shoots	400
Barley roots	90
Bean leaves	160
Cauliflower buds	90
Kale leaves	70
Wheat leaves	55

No γ-cleavage activity could be detected in any of the plant extracts. Although radioactivity from [$^{14}C_4$]cystathionine appeared at a slow rate in the acidic fraction, further purification (see MATERIALS AND METHODS) showed that this radioactivity was not present in α-ketobutyrate. The identity of this radioactive material was not further studied. The following separate experiments suggest that failure to detect γ-cleavage activity cannot be ascribed to an inefficient recovery of α-keto [^{14}C]-butyrate, or to a masking of γ-cleavage activity by inhibitors in the plant extracts: (a) Authentic α-keto [^{14}C]butyrate when incubated with each of the plant extracts described in Table I, could be recovered as the DNP-hydrazone derivative in a minimum yield of 59%. (b) Extracts of spinach leaves, parsley leaves and barley shoots caused less than 30% inhibition of the activity of a partially purified preparation of rat liver γ-cystathionase.

The γ-cleavage assay could detect a minimum rate of approx. 1.5 pmoles α-keto [^{14}C]butyrate per min per mg protein. Therefore, in the more active plant tissues of Table I (leaves of spinach, parsley and peas, and shoots of barley) γ-cleavage, if present, was less than 0.5% as active as β-cleavage. It is concluded that cystathionine is cleaved in plant tissues predominantly, perhaps exclusively, by β-cleavage.

Purification of β-cystathionase

Typical results of the partial purification of β-cystathionase from an acetone

TABLE II

PURIFICATION OF SPINACH β-CYSTATHIONASE

Details of the fractionation are described in MATERIALS AND METHODS. Fractions 2–4 were assayed spectrophotometrically. The activity of Fraction 1 was determined isotopically, and converted as described in MATERIALS AND METHODS, to a value equivalent to that obtained with the spectrophotometric assay.

Fraction	*Specific activity (nmoles/min per mg protein)*	*Yield (%)*
1. Extract of acetone powder	0.26	100
2. 24–34% $(NH_4)_2SO_4$	1.9	91
3. Supernatant after bentonite treatment	18.4	40
4. Eluate from hydroxyapatite column	111	17

powder of spinach leaves are presented in Table II. The most purified preparation (Fraction 4) represented an overall purification of over 400-fold and a yield of 17%. Further purification was impractical due to the instability of this fraction.

Stoichiometry of the β-cleavage of cystathionine

The stoichiometric conversion of cystathionine to pyruvate was demonstrated by incubation of Fraction 4 with 4.9 nC (47.6 nmoles) of [3-^{14}C]cystathionine under the conditions of the standard isotopic assay. The reaction was stopped with 5% trichloroacetic acid containing 0.15 μmole of carrier cystathionine and applied to a column of Dowex 50 (H^+ form). A total of 3.1 nC (30.1 nmoles) was not retained by Dowex 50 (H^+ form), and was characterized (see below) as [^{14}C]pyruvate. Elution of the column with 3 M NH_4OH yielded a radioactive compound (1.9 nC) which co-

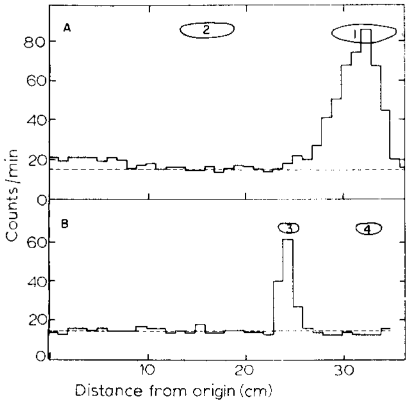

Fig. 1. Characterization of [^{14}C]pyruvate formed from metabolism of [3-^{14}C]cystathionine. Distribution of radioactivity along paper chromatogram of DNP-hydrazone extract (A), and of this extract after catalytic reduction with hydrogen (B). 1, DNP-hydrazone of pyruvate originally added to reaction mixture; 2, unidentified DNP-hydrazone. Carrier alanine and α-aminobutyrate were added to the chromatogram of B, and appear as the ninhydrin-positive spots 3 and 4, respectively. The broken lines represent background radioactivity.

chromatographed under a variety of conditions (see below) with authentic cystathionine. It follows that 3.0 nC (4.9–1.9) or 29.1 nmoles of cystathionine were consumed during the formation of 30.1 nmoles of pyruvate, providing strong evidence for the quantitative conversion of the C_3 moiety of cystathionine to pyruvate.

The radioactive α-keto acid produced from the metabolism of [3-^{14}C]cystathionine was characterized as [^{14}C]pyruvate by the following criteria: (a) Co-chromatography of the DNP-hydrazone derivative with that of authentic pyruvate in the solvent toluene–acetic acid–water (3:3:1, by vol.)[16] (Fig. 1A). (b) Co-chromatography of the DNP-hydrazone derivative after catalytic reduction with H_2 (ref. 17), with authentic alanine (the expected product) in the solvent *n*-butanol–acetic acid–water (120:30:50, by vol.) (Fig. 1B).

The radioactive compound eluted from the column with 3 M NH_4OH was characterized as [^{14}C]cystathionine by its co-migration with the authentic compound when subjected to paper chromatography (in Solvents A and B) either directly, or after oxidation with performic acid.

Evidence for the stoichiometric conversion of cystathionine to homocysteine was obtained by incubation of [$^{14}C_4$]cystathionine with β-cystathionase, followed by paper chromatography of the amino acids, after performic oxidation, in Solvent B. Radioactivity appearing in homocysteic acid accounted for 75% of the radioactivity disappearing from the oxidized product of cystathionine. This relationship is in reasonable agreement with a stoichiometric conversion of the C_4 moiety of cystathionine to homocysteine.

The radioactive product of the metabolism of [$^{14}C_4$]cystathionine was further characterized as [^{14}C]homocysteic acid by its co-migration with authentic homocysteic acid during paper electrophoresis for 3 h at 900 V in formic acid (pH 1.6). Because of its characterization as [^{14}C]homocysteic acid, no direct evidence was obtained for the oxidation state of the C_4 sulfur amino acid. By analogy with other systems[3], it is reasonable to assume that the enzymic product is homocysteine.

The combined results of the metabolism of [3-^{14}C]cystathionine and [$^{14}C_4$]-cystathionine therefore provide support for the enzymic cleavage of cystathionine to pyruvate and homocysteine, as indicated in Reaction 4.

Evidence for pyridoxal phosphate as a prosthetic group

The following observations suggest that spinach β-cystathionase is probably a pyridoxal phosphate enzyme, which is isolated as the holoenzyme: (a) Addition of free pyridoxal phosphate did not result in a significant increase in activity of any preparation of spinach β-cystathionase. (b) As shown in Table III, hydroxylamine-treated enzyme was inactive when assayed either without preincubation, or when preincubated in the absence of pyridoxal phosphate. Preincubation of hydroxylamine-treated enzyme with pyridoxal phosphate resulted in recovery of 73% of the specific activity originally observed with the holoenzyme. Holoenzyme showed little change in activity by preincubation either in the absence or presence of pyridoxal phosphate. These results suggest that treatment of the holoenzyme with hydroxylamine yields an apoenzyme that requires preincubation with pyridoxal phosphate to restore the active holoenzyme. An alternative interpretation that restoration of activity is an artifact resulting from the chemical reaction of the aldehyde group of pyridoxal phosphate with trace inhibitory amounts of hydroxylamine is considered unlikely

TABLE III

PYRIDOXAL PHOSPHATE REQUIREMENT OF HYDROXYLAMINE-TREATED ENZYME

The hydroxylamine-treated enzyme was prepared from a concentrated preparation of Fraction 3 by dialysis for 15 h against Buffer I containing 0.2 mM hydroxylamine, followed by gel filtration with Buffer I. The relative specific activity is expressed as a percentage of that determined with the holoenzyme in the absence of preincubation. Preincubation was performed where shown for 30 min in 0.2 M Tris–HCl (pH 8.65) (final volume 0.3 ml), either in the absence or presence of 1.67 mM pyridoxal phosphate. Enzyme activity was then determined by the standard spectrophotometric assay. The presence of 0.5 mM pyridoxal phosphate in the assay after preincubation with pyridoxal phosphate had no significant effect on the initial rate.

Conditions	*Relative specific activity (%)*	
	Hydroxylamine-treated enzyme	*Holoenzyme*
No preincubation	0	100
Preincubation	0	87
Preincubation + pyridoxal phosphate	73	94

for the following reasons: (i) The hydroxylamine-treated enzyme was subjected to gel filtration before use. This treatment was designed to remove free hydroxylamine. (ii) The presence of hydroxylamine-treated enzyme had no effect on the activity of the untreated enzyme. (iii) Reactivation of the apoenzyme was relatively specific for pyridoxal phosphate. Pyridoxal and a number of other pyridine derivatives related to pyridoxal phosphate were tested for their ability to reactivate the apoenzyme under conditions similar to those described in Table III. The concentration of each compound was 0.9 mM, which is approx. 7 times that required to attain a maximum rate of holoenzyme formation with pyridoxal phosphate. The concentration was selected in order to amplify any small rate of reactivation with the pyridine derivatives. Pyridoxal was found to be only 23% as effective as pyridoxal phosphate. The other pyridine derivatives which included pyridoxine, pyridoxamine phosphate, pyridoxine phosphate, 4-pyridoxic acid, pyridoximine and 4-deoxypyridoxine showed no significant activity.

Further evidence for the participation of pyridoxal phosphate in spinach β-cystathionase is provided in the accompanying report[15].

pH Optimum

Fractions 3 and 4 showed pH optima of 8.8 and 8.55 in Tris–HCl and potassium borate buffers, respectively.

Substrate specificity

The relative activity of the enzyme at various stages of purification in cleaving a number of amino acids is shown in Table IV. A summary of the structures of the most pertinent compounds is presented in Table VI. The highest v_{max} values were obtained with djenkolate, cystathionine and the mixed disulfide of cysteine and homocysteine; the v_{max} value for djenkolate was consistently higher than that for cystathionine (see also Fig. 2). At a concentration of 0.13 mM, activity with the

TABLE IV

SUBSTRATE SPECIFICITY OF β-CYSTATHIONASE

Rates of cleavage of the mixed disulfides at a concentration of 0.13 mM were determined with the radioactive assay. All other rates of cleavage were determined by substitution of the appropriate compound for cystathionine in the standard spectrophotometric assay. In order to prevent disulfide exchange with the small amount of ^{14}C-labeled mixed disulfide, enzyme preparations used in this assay were subjected to gel filtration against Buffer I in which 2-mercaptoethanol had been omitted. Enzyme preparations used in all other assays were subjected to gel filtration against Buffer I. Rates with each compound are expressed as a percentage relative to the rate determined with cystathionine at the same concentration. For D-cystathionine and the mixed disulfide, relative rates were determined only at the concentration shown. Separate experiments with each of the remaining amino acids showed that the rates reported are equivalent to at least 90% of v_{max}. Activities less than 5% of that with cystathionine could not be accurately determined. ND = not determined.

Compound	*Concn. (mM)*	*Relative rate (%)*		
		Fraction 2	*Fraction 3*	*Fraction 4*
L-Cystathionine	5.0	100	100	100
L-Djenkolate	5.0	107	105	120
L-*allo*-Mixed disulfide	0.13	49	32	35
L-Mixed disulfide	0.13	23	15	16
L-Mixed disulfide	2.5	24	ND	60
L-Homocystine	5.0	<5	6	20
L-Cystine	5.0	13	<5	14
meso-Cystine	5.0	ND	6	ND
L-Homocysteine	5.0	5	<5	5
L + *meso*-Lanthionine	5.0	13	<5	<5
L-Homolanthionine	2.5	15	<5	<5
L-Cysteine	5.0	<5	ND	<5
S-Methyl-L-cysteine	5.0	<5	<5	<5
L-Homoserine	5.0	5	<5	<5
L-Serine	5.0	<5	<5	<5
D-Cystathionine	0.15	ND	ND	<5
D-Cystathionine	5.0	ND	ND	11

L-*allo*-isomer of the mixed disulfide was significantly greater than that with the L-isomer. The only other compounds showing significant activity in Fraction 4 were L-homocystine and L-cystine. The low activities observed with L + *meso*-lanthionine and L-homolanthionine in Fraction 2 were removed during subsequent purification. No activity was detected with D-cystathionine at a concentration at which L-cystathionine is approximately one-half saturating; at a concentration of 5 mM, where L-cystathionine is at least 95% saturating, a relative activity of only 11% was observed with D-cystathionine.

Kinetics of cystathionine and djenkolate cleavage, in the presence and absence of β-cyanoalanine

The relationship between the reciprocal of the rate of substrate utilization and the reciprocal of substrate concentration (Fig. 2) was used to determine K_m values of 0.13 and 0.25 mM for cystathionine (Curve 1) and djenkolate (Curve 2), respectively. The relative v_{max} of djenkolate was 109% of that of cystathionine, in general agreement with the value of 120% reported in Table IV. PFEFFER AND RESSLER[18] have previously reported that β-cyanoalanine is an inhibitor of rat liver γ-cystathionase, and suggested that the inhibition was competitive. The results presented

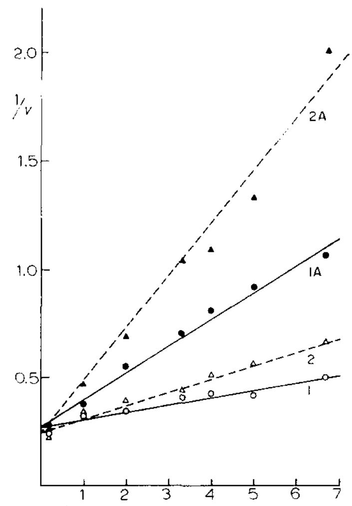

Fig. 2. Kinetics of cystathionine and djenkolate cleavage, in the presence and absence of β-cyanoalanine. Enzyme activity of Fraction 4 was determined in the standard spectrophotometric assay, except for the change in the type and concentration of substrate, and for the addition of 0.1 mM β-cyanoalanine as shown. Curves 1 and 2 were determined with cystathionine and djenkolate, respectively. Curves 1A and 2A were determined with cystathionine and djenkolate, respectively, in the presence of 0.1 mM β-cyanoalanine. Velocity is expressed as nmoles/min. [S] = substrate concentration (mM).

in Fig. 2 demonstrate that β-cyanoalanine* is an effective inhibitor also of spinach β-cystathionase, and further establish the competitive kinetics of the inhibition. K_i values for β-cyanoalanine of 0.040 and 0.032 mM were calculated in the presence of cystathionine and djenkolate, respectively.

Metabolism of serine

In addition to catalyzing the γ-elimination of cystathionine (Reaction 2), γ-cystathionase of rat liver[19,20] catalyzes a slow γ-replacement reaction:

$$\text{Homoserine} + \text{cysteine} \rightarrow \text{cystathionine} + H_2O \tag{5}$$

It was of interest to determine whether β-cystathionase of spinach catalyzes an analogous β-replacement reaction:

$$\text{Serine} + \text{homocysteine} \rightarrow \text{cystathionine} + H_2O \tag{6}$$

An attempt was made to demonstrate Reaction 6 in a reaction mixture containing the following components in μmoles in a final volume of 0.8 ml: L-[3-^{14}C]serine, 2; sodium pyruvate, 8; L-cystathionine, 0.12; Tris–HCl (pH 8.65), 160; Fraction 3, and varying amounts (0, 0.05, 0.5 and 5) of L-homocysteine. Since any [^{14}C]cystathionine formed could be enzymically cleaved to [^{14}C]pyruvate, the rate of cysta-

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* Inhibition by β-cyanoalanine was not the result of its cleavage by β-cystathionase to cyanide (*plus* pyruvate and ammonia). Thus, the cleavage of β-cyanoalanine could not be detected experimentally, and ,if present, proceeded at a rate less than 5% of that with cystathionine. The maximum concentration of cyanide formed from β-cyanoalanine cleavage would be less than 0.1% of that required to produce the observed inhibition.

thionine synthesis was determined from the total radioactivity incorporated into the cystathionine and α-keto acid fractions. A small amount of radioactivity, which was at the limit of detection of the assay, was incorporated into the cystathionine fraction. This small incorporation was equivalent to a rate of cystathionine synthesis of approx. 0.1% of the rate of cystathionine cleavage. Radioactivity was incorporated into the α-keto acid fraction at a rate of approx. 25% of that of cystathionine cleavage. However, neither the rapid incorporation into the α-keto acid fraction nor the equivocal incorporation into the cystathionine fraction was dependent upon the presence of homocysteine. Furthermore, as explained below, the radioactive product in the α-keto acid fraction was hydroxypyruvate, and not pyruvate. These combined observations therefore suggest that spinach β-cystathionase does not catalyze the synthesis of cystathionine *via* Reaction 6 at a significant rate.

TABLE V

EFFECT OF DTNB AND *N*-ETHYLMALEIMIDE ON β-CYSTATHIONASE

Fraction 4 was subjected to gel filtration with Buffer I from which 2-mercaptoethanol had been omitted. The enzyme was then preincubated at 30° in 0.1 M potassium phosphate (pH 7.3) for the time, and with the inhibitor concentration shown. The reaction mixture was subjected to gel filtration as described above, and assayed with the standard spectrophotometric assay.

Inhibitor	*Concn. (mM)*	*Time (min)*	*Inhibition (%)*
DTNB	0.01	1	93
	0.01	10	100
N-Ethylmaleimide	5	10	26
	10	10	88
	20	10	92

The formation of radioactive α-keto acid noted above had an absolute requirement for pyruvate. The radioactive α-keto acid was characterized as hydroxypyruvate by its reduction to glycerate in the presence of NADH and lactate dehydrogenase[21]. The reaction taking place thus appears to be the one catalyzed by L-alanine: hydroxypyruvate aminotransferase, which has previously been reported in leaves of spinach and other plants[22]:

$$[^{14}\text{C}]\text{Serine} + \text{pyruvate} \rightarrow [^{14}\text{C}]\text{hydroxypyruvate} + \text{alanine}$$

Effect of DTNB and N-ethylmaleimide

The results of Table V show that spinach β-cystathionase (Fraction 4) is strongly inhibited by DTNB. *N*-Ethylmaleimide inhibited at relatively high concentrations. Both inhibitors acted irreversibly, since the reported activities were determined after gel filtration of the inhibited enzyme. Progressive recovery of activity of a DTNB-treated enzyme was observed after addition of certain thiols to the assay. Thus addition of 20 mM 2-mercaptoethanol or dithiothreitol to the assay mixture containing enzyme that had been preincubated for 10 min with 0.01 mM DTNB resulted in 90% recovery of activity after approx. 6 min.

DISCUSSION

The natural occurrence of cystathionine was first reported almost 30 years ago by HORN AND JONES[23], who isolated an isomorphous mixture of cystathionine and its selenium analogue from the vetch *Astragalus pectinatus*. To our knowledge, no subsequent reports have appeared that unequivocally demonstrate the presence of cystathionine in higher plants. When the studies described in this paper were initiated, the limited data on the natural distribution of cystathionine and selenocystathionine* provided essentially the only indication of a possible physiological role of cystathionine in higher plants.

The wide distribution of β-cleavage activity in plant extracts, coupled with the failure to detect γ-cleavage activity, suggests that transsulfuration in higher plants proceeds predominantly, perhaps exclusively, in the direction cysteine → (cystathionine) → homocysteine. The operation of this pathway in higher plants is supported by the demonstration[27] of the enzymic synthesis by spinach extracts of cystathionine *via* reactions similar to those reported in bacteria and Neurospora. Further evidence for the role of cystathionine in the biosynthesis of homocysteine and methionine is provided by the *in vivo* studies of DOUGALL AND FULTON[28].

The enzymic synthesis of homocysteine and methionine can proceed also by a pathway independent of transsulfuration, involving the direct sulfhydration of *O*-acetyl homoserine by either sulfide or methylmercaptan[29,30]. The relative physiological significance of the transsulfuration and direct sulfhydration pathway in *de novo* synthesis of homocysteine and methionine in higher plants is presently being examined.

The 400-fold purified preparation of spinach β-cystathionase was most active with djenkolate, cystathionine and the mixed disulfide of cysteine and homocysteine. The fact that the relative activity with all three substrates remained essentially unchanged throughout extensive purification (Table IV) suggests that one enzyme catalyzes the cleavage of all these substrates. Further evidence that cystathionine and djenkolate are cleaved by the same enzyme is provided by the following observations: (a) The rate determined with saturating concentration of one substrate is not significantly stimulated by addition of the other. (b) Both substrates show similar competitive kinetics with β-cyanoalanine (Fig. 2), and similar competitive protection of β-cystathionase from inactivation by rhizobitoxine[15].

Kinetic values were determined for cystathionine and djenkolate. $v_{\max}$ for cystathionine is slightly less than that for djenkolate (Table IV and Fig. 2); K_m for cystathionine (0.13 mM) is appreciately less than that for djenkolate (0.25 mM). Insufficient amounts of the mixed disulfides were available for determination of kinetic values. To our knowledge, this is the first report of a plant enzyme that is relatively specific for cystathionine. Previously reported plant enzymes that cleave cystathionine exhibit only marginal activity with this substrate. Thus an enzyme from turnip[31] is much more active with cystine than with cystathionine (N. W.

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* The selenium analogue of cystathionine has been demonstrated in a number of plants whose growth is stimulated by selenium, and which accumulate the element in high concentration[24,25]. Selenocystathionine has been detected also in the fruits of *Lecythis ollaria*[26]. It is not clear what relationship exists between the metabolism of cystathionine and its selenium analogue.

ANDERSON, personal communication). Similarly, the rate of cleavage of cystathionine by a C–S lyase from *Albizzia lophanta* is only 4% of that with djenkolate[32].

Spinach β-cystathionase appears to catalyze a β-elimination reaction with each of its active substrates. Evidence has been presented for the β-cleavage of cystathionine to pyruvate and homocysteine *via* Reaction 4. The products of djenkolate cleavage were not characterized other than by demonstration of the formation of an α-keto acid that was a substrate for lactate dehydrogenase. The products of djenkolate cleavage by spinach β-cystathionase are presumed to be the same as those formed by rat liver γ-cystathionase[33]:

$$\begin{array}{l}
\text{Djenkolate} \rightarrow \text{pyruvate} + NH_3 + \text{thiomethylcysteine} \\
\text{Thiomethylcysteine} \xrightarrow{\text{Non-enzymic}} \text{cysteine} + \text{formaldehyde} + H_2S \\
\hline
\text{Sum: Djenkolate} \rightarrow \text{pyruvate} + NH_3 + \text{cysteine} + \text{formaldehyde} + H_2S
\end{array}$$

The radioisotopic data demonstrated that the C_3 moiety of the mixed disulfide is cleaved to an α-keto acid. By analogy with the cleavage of cystine[34], the mixed disulfide would be expected to yield the following:

$$\begin{array}{l}
\text{Mixed disulfide} \rightarrow \text{pyruvate} + NH_3 + \text{thiohomocysteine} \\
\text{Thiohomocysteine} \xrightarrow{\text{Non-enzymic}} \text{homocysteine} + S \\
\hline
\text{Sum: Mixed disulfide} \rightarrow \text{pyruvate} + NH_3 + \text{homocysteine} + S
\end{array}$$

Failure to demonstrate a significant synthesis of cystathionine from serine *via* Reaction 6 is probably not surprising, since serine is a very poor substrate for spinach β-cystathionase (Table IV). A correlation between the effectiveness of substrates in γ-elimination and γ-replacement reactions has previously been noted[35].

The substrate specificity of the enzyme was studied in some detail (Table IV). Compounds (*e.g.* serine, cysteine, *S*-methylcysteine) that consist of only one α-amino acid moiety show negligible activity. All active substrates consist of two α-amino acid moieties that are joined by a sulfur-containing bridge*. The manner in which a variation in these structures affects substrate activity is shown in Table VI, and may be summarized as follows: (1) All active substrates contain a C_3 L-α-amino acid. Changing the configuration of the C_3 moiety of an active substrate (*e.g.* cystathionine) from L to D results in a striking decrease in activity (Table IV). With cystathionine, which contains both a C_3 and a C_4 moiety, elimination proceeds almost completely from the C_3 moiety. (2) The sulfur-containing bridge may be a thioether, thioacetal, or disulfide. (3) The remaining moiety may be in either the L- or D-configuration. All active substrates listed in Table VI have the remaining moiety in the L-configuration. In Table IV, it was shown that the L-*allo*-isomer of the mixed disulfide is approx. twice as active as the L-isomer, from which it differs only by having the 4C moiety in the D-configuration. Other structural changes in the "remaining moiety" of the molecule may lead to major changes in activity. For example a severe loss in activity occurs upon reduction of the carbon atoms from 4 (cystathionine and mixed disulfide) to 3 (lanthionine and cystine). The mechanism by which substrate specificity is conferred by this portion of the molecule is not immediately evident. Speci-

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* The data did not show whether moieties other than α-amino acids, and bridges other than those containing sulfur, would be active.

TABLE VI

SUBSTRATE SPECIFICITY, SUMMARIZED IN RELATION TO NATURE OF MOIETIES AND BRIDGE

The relative rates are shown in parentheses after each compound, and are those listed in Table IV for Fraction 4.

Type of moieties	*Type of bridge linking each moiety*		
	Thioether *(-S-)*	*Thioacetal* *(-S-C-S-)*	*Disulfide* *(-S-S-)*
$C_3 + C_3$	L + *meso*-Lanthionine (<5)	L-Djenkolate (120)	L-Cystine (14)
$C_3 + C_4$	L-Cystathionine (100)	—	L-Mixed disulfide (60)
$C_4 + C_4$	L-Homolanthionine (<5)	—	L-Homocystine (20)

ficity is not determined simply by the length of the chain attached to the C_3 moiety. If this were so, cystathionine and cystine, which each have the C_3 moiety linearly extended by 5 atoms, should be equally active. Examination of molecular models indicated that all highly active substrates could achieve at least one spatial arrangement which could not be attained by compounds which were inactive as substrates. This arrangement is illustrated in Fig. 3. In this figure, all compounds are shown with the amino groups *cis* to one another. Under these circumstances, only compounds which are highly active as substrates permit a spatial separation of carboxyl groups such that the carboxyl group of the "remaining moiety" extends below the lower broken line of Fig. 3. In the absence of data on the crystal structure of many of these compounds, it is not possible to assess the thermodynamic stability of the spatial arrangement shown in Fig. 3, or of other possible arrangements. The figure is

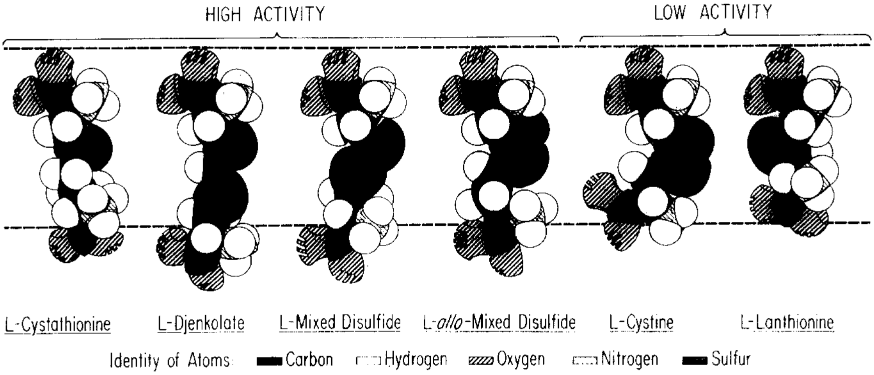

Fig. 3. Comparison of compounds which are active or inactive as substrates for β-cystathionase. Details of the comparison are discussed in the text. As a result of the C–S–S–C dihedral angle of approx. 90° (ref. 36), each disulfide can exist in two rotational-isomeric forms in which an S–C bond can project either above $\left(\begin{smallmatrix}C & C\\ | & | \\ S & {-}S\end{smallmatrix}\right)$ or below $\left(\begin{smallmatrix}C & C\\ | & \vdots \\ S & {-}S\end{smallmatrix}\right)$ the plane of the remaining (C–S–S) bonds. Only the former isomer of each pair is shown. However, the comparison discussed in the text applies also to the latter isomers. In the stereoformulae shown above, the full lines of normal thickness (—) denote bonds lying in the plane of the paper; the thickened line (**—**) denotes a bond projecting from the plane of the paper toward an observer; the broken line (- - -) denotes a bond projection away from an observer. CPK space-filling models (The Ealing Corp., Cambridge, Mass.) were used throughout.

presented chiefly to illustrate that it is possible to rationalize the contribution of the "remaining moiety" to the experimentally observed pattern of substrate specificity of spinach β-cystathionase.

Spinach β-cystathionase catalyzes the same type of cystathionine cleavage (Reaction 4), and probably has the same pyridoxal phosphate prosthetic group, as the corresponding enzymes isolated from bacteria and fungi. The substrate specificity of the spinach enzyme resembles that of the bacterial enzyme[37]. Thus both plant and bacterial enzymes are most active with cystathionine and djenkolate, and are far less active with cystine, *S*-methylcysteine, and serine; significant differences in relative rates of the two enzymes were observed only with the mixed disulfide and lanthionine. The substrate specificity of the spinach enzyme contrasts with that of the fungal enzyme[38] whose activity with cystine, lanthionine and serine approximates that with cystathionine. Conversely, with respect to sensitivity to the inhibitors DTNB and *N*-ethylmaleimide, the plant enzyme resembles the fungal enzyme, and is clearly different from the bacterial enzyme[2,38]. Thus spinach β-cystathionase differs from the analogous enzymes from either bacteria or fungi.

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