Phytochemistry, Vol 27, No 11, pp 3453–3460, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00
Pergamon Press plc

INCORPORATION OF CYSTEINE AND SELENOCYSTEINE INTO CYSTATHIONINE AND SELENOCYSTATHIONINE BY CRUDE EXTRACTS OF SPINACH

J.C DAWSON and J.W. ANDERSON

Botany Department, La Trobe University, Bundoora, Victoria, 3083, Australia

(*Received* 16 *November* 1987)

Key Word Index—*Spinacia oleracea*, Chenopodiaceae, cystathionine; selenocystathionine, cysteine; selenocysteine; *O*-phosphorylhomoserine, cystathionine γ-synthase; selenium

Abstract—Crude extracts of spinach catalysed the incorporation of [^{3}H]cysteine into cystathionine in the presence of *O*-phosphorylhomoserine (PHS) or *O*-succinylhomoserine (SHS). They also supported the incorporation of [U-^{14}C]PHS into cystathionine in the presence of cysteine. These reactions were completely inhibited by 0.1 mM propargylglycine and 1 mM amino-oxyacetate and less strongly by vinylglycine, β-cyanoalanine and isonicotinic acid hydrazide These properties are consistent with the synthesis of cystathionine by cystathionine γ-synthase (CγS) (EC 4 2 99 9) activity Spinach extracts also incorporated [^{75}Se]selenocysteine into selenocystathionine in the presence of SHS in a reaction which was sensitive to propargylglycine. In the presence of selenocysteine, spinach extracts incorporated [^{14}C]PHS into a compound with chromatographic characteristics indistinguishable from selenocystathionine at a rate similar to that for cysteine When cysteine and selenocysteine were supplied together the amount of [^{14}C]PHS incorporated was slightly less than for either substrate alone. Conversely, selenocysteine strongly inhibited the incorporation of [^{3}H]cysteine into cystathionine. It was concluded that selenocysteine acts as an alternative substrate of CγS to cysteine resulting in the formation of the selenium isologue of cystathionine In crude spinach extracts, CγS exhibits a greater affinity for selenocysteine (K_m *ca* 70 μM) than cysteine (K_m *ca* 240 μM).

INTRODUCTION

The reaction catalysed by cystathionine γ-synthase (CγS) is a component of the transsulphuration pathway in plants [1]. The enzyme catalyses a γ-addition of an α-aminobutyryl donor to cysteine according to the reaction:

Cysteine + X-*O*-homoserine ⟶ cystathionine + XOH.

Although homoserine itself is inactive, crude extracts from a wide phylogenetic range of plants catalyse the synthesis of cystathionine with *O*-malonyl-, *O*-oxalyl-, *O*-succinyl- (SHS), *O*-phosphoryl- (PHS) and *O*-acetylhomoserine (AHS) as α-aminobutyryl donors [2] Various lines of evidence discussed in ref. [1] strongly suggest, however, that in plants PHS is the physiologically important donor for the synthesis of cystathionine. Some properties of CγS have been reported in crude extracts of several plant species [2–8] and the enzyme has been purified *ca* 12-fold from leaf tissue of barley seedlings [9]. Studies of the subcellular localization of CγS in pea and barley leaf protoplast lysates suggest that it is confined to chloroplasts [10]

The reaction catalysed by CγS is of interest with respect to selenium metabolism particularly in those plants known as selenium accumulators which, under appropriate conditions, produce large amounts of various selenoamino acids—in particular selenium isologues of intermediates (and their derivatives) of the sulphate assimilation pathway [11, 12] In this paper, we report that crude cell-free, extracts of spinach (a non-accumulator species) catalyse γ-addition to both cysteine and selenocysteine resulting in the synthesis of cystathionine and selenocystathionine respectively. Several features of these two reactions are consistent with CγS activity. Furthermore, it is likely that both substrates compete for the same catalytic site on the enzyme.

RESULTS

Synthesis of cystathionine

When crude spinach extracts were incubated with [^{3}H]cysteine and unlabelled PHS a ^{3}H-labelled product was formed which could be separated from the substrate by treatment with Dowex 50-H$^+$, oxidation with performic acid and a second Dowex treatment (method A). The isolated ^{3}H-product was free from other ^{3}H-compounds as determined by paper electrophoresis in buffer A and was indistinguishable from cystathionine sulphone synthesized by performic acid oxidation of authentic cystathionine. The ^{3}H-labelled product was not formed in the absence of extract or PHS. It was concluded that the ^{3}H-labelled material isolated as described for method A provides a suitable means for the quantitative estimation of cystathionine synthesis by CγS activity.

Crude extracts in the presence of L-cysteine also supported the incorporation of [^{14}C]PHS into a compound which was chromatographically indistinguishable from authentic cystathionine in solvent A (R_f 0.25). The ^{14}C-labelled product was well resolved from [^{14}C]PHS (R_f 0.57) and homoserine (R_f 0.49). The product was not formed in the absence of L-cysteine or enzyme. These

characteristics are consistent with the production of [^{14}C]cystathionine When material from terminated reaction mixtures was subjected to treatment on Dowex 50-H^+ as described for method B assays, analysis by paper chromatography in solvent A of the material eluted by aq NH_3 showed that a cysteine-dependent peak of ^{14}C-label was associated exclusively with cystathionine Accordingly, ^{14}C-labelled material eluted from Dowex was used to quantify method B assays.

Various properties of cystathionine synthesis by CγS activity were studied Using method B, the rate of cystathionine synthesis increased with cysteine concentration up to *ca* 0 5 mM (Fig 1) Higher concentrations were inhibitory, the rate at 2 mM for example was 57% of the rate at 0 5 mM The K_m for L-cysteine was *ca* 0 25 mM As studied by method A, the rate increased with the concentration of PHS up to *ca* 5 mM, higher concentrations were inhibitory The K_m for PHS was 0.75 mM Method B was used to study the specificity of the reaction with respect to cysteine, spinach extracts did not support the synthesis of cystathionine when L-cysteine was replaced with D-cysteine (0 5 mM), L-cystine (0 3 mM), seleno-DL-cystine (0 6 mM), L-cysteic acid, L-methionine, *S*-methyl-L-cysteine (each 10 mM) and DL-homocysteine and seleno-DL-methionine (each 20 mM) DL-Homocysteine (20 mM) and L-cystine (0 3 mM) inhibited enzyme activity with L-cysteine (0 5 mM) as substrate by 85 and 36% respectively but none of the other compounds listed above significantly affected CγS activity as determined by method B The substrate specificity of CγS was examined with respect to the α-aminobutyryl donor by method A. SHS (5 mM) and AHS (5 mM) supported cystathionine synthesis at 41 and 4% respectively of the rate obtained with 5 mM PHS When both PHS (5 mM) and SHS (5 mM) were supplied together the rate of [^{3}H]cysteine incorporation did not exceed by more than 10% that for PHS alone, suggesting that the two compounds serve as alternative substrates L-Homoserine, L-serine, *O*-phospho-L-serine, *O*-acetyl-L-serine, L-serine-*O*-sulphate and L-asparate (each 5 mM) did not support the incorporation of [^{3}H]cysteine into cystathionine nor did they affect the synthesis of cystathionine by more than 10% when PHS was supplied as the aminobutyryl donor.

Pre-incubation of spinach crude extract with low concentrations of DL-propargylglycine, an irreversible inhibitor of the CγS of *Lemna paucicostata* [6, 7, 13], strongly inhibited the spinach enzyme (Fig 2) Aminooxyacetate, a carbonyl group reagent and a well known inhibitor of pyridoxal phosphate-dependent enzymes [14, 15], abolished CγS activity The spinach enzyme also exhibited varying degrees of sensitivity to DL-vinylglycine, β-cyanoalanine and isonicotinic acid hydrazide (Table 1). The effect of cystathionine and related compounds on the rate of cystathionine synthesis was determined by methods A and B (Table 2) L-Cystathionine (7.5 mM) inhibited activity by *ca* 15% when assayed by both methods A and B L-Selenocystathionine, inhibited activity by 22% by method B but 64% by method A Cystine (0.32 mM) and selenocystine (1 5 mM) both decreased enzyme activity by similar amounts when assayed by method A but these effects were not as pronounced when measured by method B

Synthesis of [^{75}Se]*selenocystathionine by spinach extracts*

The catalytic activity of CγS towards selenocysteine *in lieu* of cysteine was examined by supplying [^{75}Se]selenocysteine and SHS (method C) In principle,

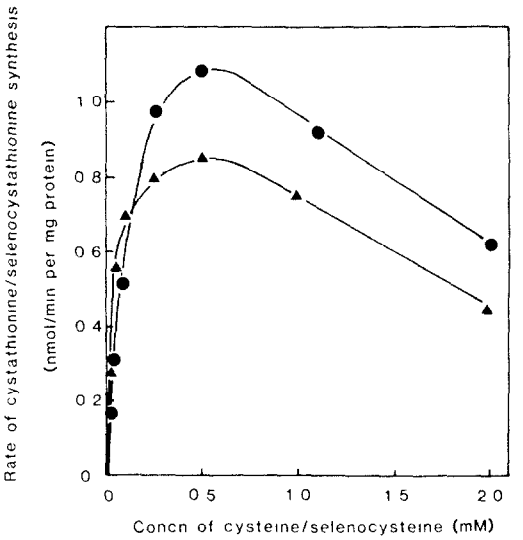

Fig 1 Effect of concentration of cysteine (●) and selenocysteine (▲) on the rate of incorporation of [^{14}C]PHS (5 mM) into cystathionine and selenocystathionine catalysed by crude spinach extracts All other conditions were as described for method B (cysteine as substrate) and method D (selenocysteine as substrate). Separate spinach extracts were used to investigate the effects of cysteine and selenocysteine concentration The extract used to study selenocysteine exhibited an activity of 0 96 nmol/min/mg protein when selenocysteine was replaced with 0 5 mM cysteine

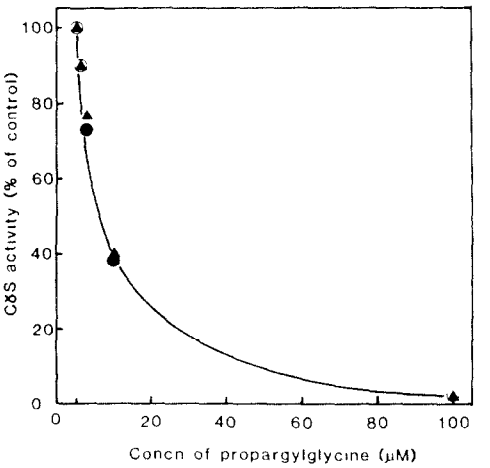

Fig 2 Effect of DL-propargylglycine concentration on the rate of [^{14}C]PHS incorporation into cystathionine/selenocystathionine in the presence of cysteine (▲) and selenocysteine (●) Crude spinach extract was preincubated for 10 min at 30° in 20 mM HEPES buffer, pH 7 8, at the concentrations of propargylglycine specified All other details were as described for method B (with cysteine as substrate) and method D (selenocysteine as substrate) Activity is expressed relative to appropriate controls lacking propargylglycine (1 00 and 1 15 nmol/min/mg protein for methods B and D respectively)

this method has much in common with method A but as discussed below the post-incubation analytical procedures employed in method A could not be used. Examination of ^{75}Se-labelled material in terminated reaction mixtures by electrophoresis after the first Dowex treatment shows that crude spinach extracts do indeed catalyse the synthesis of a ^{75}Se-labelled product which is electrophoretically indistinguishable from authentic selenocystathionine (Fig. 3A) This product is not formed in the absence of SHS (Fig. 3B) or in the absence of enzyme. Moreover, the formation of the product is completely abolished by 0 1 mM DL-propargylglycine (results not shown). However, a substantial amount of the ^{75}Se-label eluted from Dowex is not associated with the compound which co-migrates with selenocystathionine (Fig 3A). This label is associated with several slower-migrating compounds which also move towards the cathode upon electophoresis in buffer A These compounds are also detected in incubations lacking SHS (Fig. 3B) and enzyme thus suggesting that they are oxidation products of selenocysteine which are formed non-enzymically. The level of these compounds, but not the level of the ^{75}Se-labelled product which co-migrates with selenocystathionine, is readily decreased by successive Dowex treatments. If a performic acid oxidation treatment, analogous to that employed in method A, is conducted between the two Dowex treatments then very little ^{75}Se-label is recovered; no label co-migrated with selenocystathionine

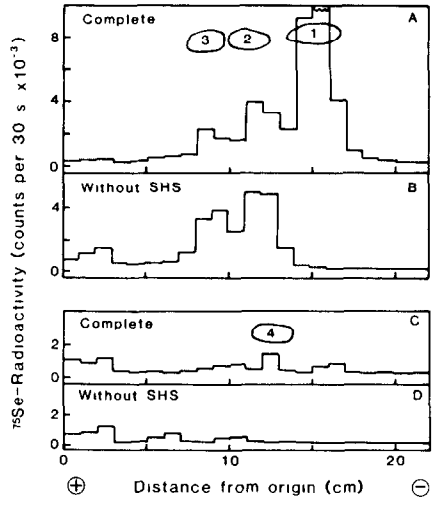

Fig 3 Radioelectrophoretogram traces of the ^{75}Se-labelled products formed from [^{75}Se]selenocysteine in the presence and absence of SHS by crude extracts of spinach (A and B). The deleterious effect of performic acid oxidation on the recovery of the ^{75}Se-labelled products are also shown (C and D) Conditions for the enzyme incubation (with and without SHS) and the first Dowex treatment were as described for method C A sample of the ^{75}Se-labelled material eluted from the first Dowex treatment with aq NH_3 was dried, dissolved in 0.17% TCA (20 5 ml) and reapplied to Dowex After washing, re-eluting with aq NH_3 and drying a second time, the residue was dissolved in 88% formic acid (0 2 ml) and subjected to PE in buffer A for 1 75 hr at 2 5 kV (traces A and B). Alternatively, the ^{75}Se-labelled material eluted from the first Dowex treatment was dissolved and oxidized with performic acid as described in ref [7] before reapplying to Dowex After washing and eluting with aq NH_3 the dried material was dissolved in 88% formic acid (0 2 ml) and subjected to PE as described above (traces C and D) Authentic samples of selenocystathionine (1), selenocystine (2), selenocysteine (3) and cystathionine sulphone (4) migrated as shown The value of the truncated peak in trace A is 16 2

Table1 Effect of some inhibitors on the CγS activity of crude spinach extracts

Inhibitor*	Concentration (mM)	CγS activity† (% of control)
DL-Vinylglycine	0.1	93
	1	71
β-Cyanoalanine	1	22
	10	5
Amino-oxyacetate	1	0
Isonicotinic acid hydrazide	1	100
	10	25

*Crude extracts were preincubated with the appropriate inhibitor for 10 min at 30° in 20 mM HEPES buffer (pH 7 8) at the concentrations specified.

†Activity was determined by method A The rate of [^{3}H]cysteine incorporation in a control incubation lacking inhibitor was 0 58 nmol/min/mg protein

Table 2 Effect of cystathionine and cystine and their selenium isologues on CγS activity of crude spinach extracts as determined by [^{3}H]cysteine incorporation (method A) and [^{14}C]PHS incorporation (method B)

Addition	Concentration (mM)	CγS activity* (% of control) Method A	Method B
L-Cystathionine	7 5	84 0	86.3
L-Selenocystathionine	7 5	35 8	78 1
L-Cystine	0 32	26 3	55 3
Seleno-DL-cystine	1 5	15 5	89 8

*CγS activity was assayed by the standard procedures for method A and B, the standard concentrations for PHS and cysteine for both methods were 5 and 0 5 mM respectively The rates of cystathionine synthesis in control incubations lacking any additions for methods A and B were 0.56 and 0 84 nmol/min/mg protein respectively.

and only a trace amount co-migrated with cystathionine sulphone (Fig 3C) Incubation mixtures lacking SHS treated in a similar way contained negligible label in this region (Fig 3D) The deleterious effect of performic acid oxidation of selenocystathionine was examined by eluting enzymically synthesized [^{75}Se]selenocystathionine from electrophoretograms (e g Fig. 3A), mixing it with 100 nmol of authentic unlabelled selenocystathionine, and subjecting it to various oxidative procedures prior to paper electrophoresis Treatment with performic acid for as little as 5 min (cf 60 min for method A assays) resulted in only 40% recovery of the ^{75}Se-label in the region comigrating with cystathionine sulphone after electrophoresis in buffer A Oxidation with H_2O_2 [16, 17] was equally unsatisfactory Collectively, these studies show that oxidative procedures employed to separate labelled cysteine from cystathionine on Dowex (method A) are unsuitable for the separation of selenocysteine from selenocystathionine In theory, the incorporation of [^{75}Se]selenocysteine into selenocystathionine can be quantified by subjecting terminated reaction mixtures to Dowex treatment followed by electrophoresis to remove labelled contaminants which are formed non-ezymically

Alternative approaches for separating [^{75}Se]selenocysteine and its oxidation products from [^{75}Se]selenocystathionine were investigated They involved terminating incubations with hot ethanol containing one of the alkylating reagents *N*-ethylmaleimide and iodoacetate to promote a reaction with residual [^{75}Se]selenocysteine [18, 19] Analysis of the resulting mixture by electrophoresis revealed the presence of a ^{75}Se-labelled product which co-migrated with selenocystathionine This is shown in Fig 4A using iodoacetate as the alkylating agent This product did not form in the absence of enzyme or SHS (Fig 4B) The data in Fig. 4C show that although several ^{75}Se-labelled compounds are formed non-enzymically, treatment with iodoacetate permits clear resolution of enzymically synthesised selenocystathionine from the carboxymethyl adduct of unreacted [^{75}Se]selenocysteine The discontinuation of commercial supplies of [^{75}Se]selenocystine (from which [^{75}Se]selenocysteine was synthesized) forced the termination of this work

Synthesis of [^{14}C]*selenocystathionine from* [^{14}C]*PHS and unlabelled selenocysteine*

Direct examination of terminated reaction mixtures (as for method D) by paper chromatography in solvent A showed the presence of a single ^{14}C-labelled product with chromatographic characteristics indistinguishable from selenocystathionine. The formation of the product was completely dependent on the presence of selenocysteine and enzyme, characteristics which are consistent with the synthesis of selenocystathionine by C$_\gamma$S activity Treatment of the reaction mixtures on Dowex effected complete removal of [^{14}C]PHS from [^{14}C]selenocystathionine as judged by paper chromatography and electrophoresis thereby affording a method for the quantitative estimation of selenocystathionine synthesis This procedure, which is incorporated in the standard procedure for method D, eliminates the problems associated with the non-enzymic formation of ^{75}Se-labelled oxidation products of [^{75}Se]selenocysteine (method C) though this method does not directly demonstrate incorporation of selenium *per se*

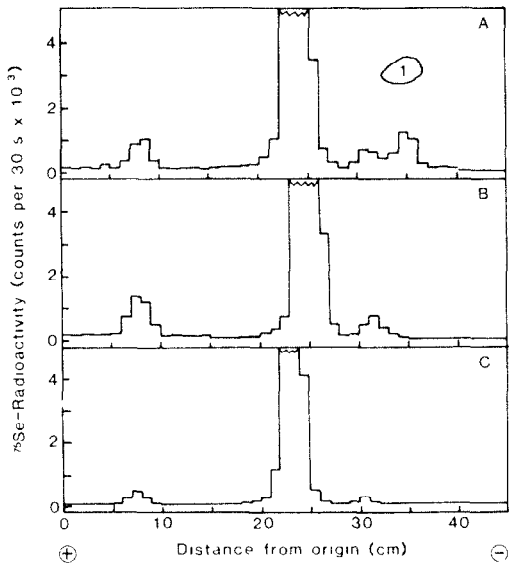

Fig 4 Radioelectrophoretogram traces of the ^{75}Se-labelled products formed from [^{75}Se]selenocysteine by crude spinach extracts in the presence of SHS when reactions were terminated with ethanol containing iodoacetic acid Incubation mixtures (50 μl) were as described for method C Reactions were terminated by addition of 1 ml of hot (50°) 13% ethanol containing a 20% molar excess of iodoacetic acid (0 3 M stock in 0 2I M NaOH) over the total selenol and thiol concentration After alkylation for 15 min, insoluble material was removed by centrifugation Samples were subjected to PE in buffer A for 3 hr at 2 5 kV The distribution of ^{75}Se-label is shown for a complete incubation (A) and an incubation lacking SHS (B), authentic selenocystathionine (1) migrated as depicted in (A) The electrophoretic characteristics of [^{75}Se]carboxymethylselenocysteine are shown in (C) The values of the truncated peaks in A, B and C were 19 3, 21 1 and 9 9 respectively

Several properties of selenocystathionine synthesis by spinach extracts were examined by method D. Crude extracts from acetone powders supported selenocystathionine synthesis at rates of *ca* 0 85–0 95 nmol/min/mg protein The rate of the reaction increased with the concentration of selenocysteine up to *ca* 0 5 mM with respect to the L-isomer (Fig 1) Concentrations greater than 0 5 mM were inhibitory; for example the activity at 2 mM was 53% of the activity at 0 5 mM The K_m for seleno-L-cysteine, determined from non-inhibitory levels of substrate, was 70 μM The synthesis of selenocystathionine, like cystathionine was extremely sensitive to DL-propargylglycine, concentrations as low as 10 μM inhibited activity by *ca* 60% (Fig 2)

Evidence on whether crude spinach extracts contained a single or separate enzyme to support the incorporation of cysteine/selenocysteine into cystathionine/selenocystathionine was sought by examining the effect of selenocysteine on the incorporation of [^{3}H]cysteine into cystathionine while concomitantly measuring the total incorporation of [^{14}C]PHS into cystathionine and selenocystathionine in the presence of unlabelled cysteine and/or selenocysteine (Table 3) The incorporation of 0 5 mM [^{3}H]cysteine into cystathionine was inhibited (85%) by selenocysteine (0 5 mM) The incorporation of

Table 3 Effect of cysteine and selenocysteine on the CγS activity of crude spinach extracts as determined by [^{3}H]cysteine incorporation and [^{14}C]PHS incorporation

Labelled substrate	Unlabelled substrates and additions	CγS activity* (% of control)
[^{3}H]Cysteine (0.5 mM)	PHS (5 mM)	100†
	PHS (5 mM) plus selenocysteine (0.5 mM)	15 3
[^{14}C]PHS (5 mM)	Cysteine (0 5 mM)	100‡
	Selenocysteine (0 5 mM)	100
	Cysteine (0 5 mM) plus selenocysteine (0 5 mM)	87 5

*CγS activity with [^{3}H]cysteine as the labelled substrate was determined as for method A except for the modifications shown [^{14}C]PHS incorporation was measured as for method B except that cysteine was replaced or supplemented with selenocysteine as indicated Under these conditions [^{14}C]PHS would be incorporated into selenocystathionine as well as cystathionine

†0 65 nmol/min/mg protein

‡0 96 nmol/min/mg protein

[^{14}C]PHS into cystathionine/selenocystathionine proceeded at similar rates when either cysteine or selenocysteine was supplied as the aminopropyl donor. When both cysteine and selenocysteine were supplied the total incorporation of [^{14}C]PHS was only slightly less (12%) than the incorporation for either substrate when measured alone The results imply that cysteine and selenocysteine compete with each other. The large inhibition of [^{3}H]cysteine incorporation by selenocysteine probably reflects a combination of direct competition between the two substrates, slight inhibition caused by a combined substrate concentration of 1 mM and higher affinity for selenocysteine than cysteine The lower rate of [^{14}C]PHS incorporation when both substrates were supplied together relative to the rates obtained with each substrate alone is also attributed to the inhibitory affect of a combined substrate concentration of 1 mM

The nature of the competition between cysteine and selenocysteine was examined further by studying the kinetics of the selenocysteine inhibition of [^{3}H]cysteine incorporation into cystathionine (Fig 5). A Hanes–Woolf plot of this data is shown in Fig. 6 It is apparent that even if all the experimental points are weighted equally the data are not consistent with non-competitive competition between [^{3}H]cysteine and selenocysteine. Both K_m and V_{max} vary with increasing selenocysteine concentration typical of a mixed competitive/non-competitive relationship. However, both cysteine and selenocysteine are inhibitory at concentrations greater than *ca* 0 3–0.5 mM (Fig 1). When only those points are considered where the combined concentrations of cysteine and slenocysteine does not exceed 0.3 mM the kinetics of selenocysteine inhibition of cysteine incorporation more closely resemble competitive inhibition (Fig 6). Assuming this is correct, then the K_i value for selenocysteine, calculated by the method of ref [20] at a selenocysteine concentration of 50 μM, is *ca* 40 μM

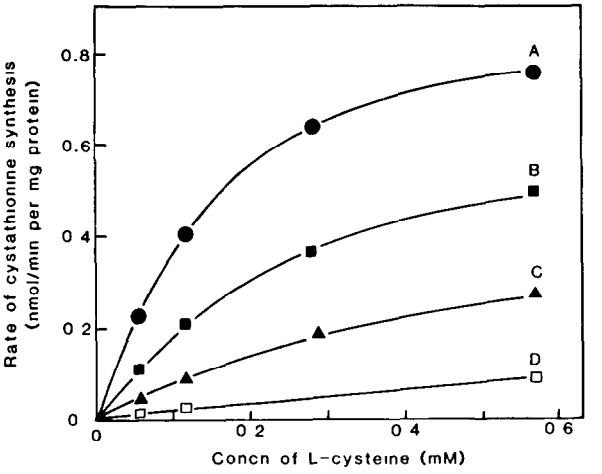

Fig. 5. Effect of selenocysteine on the incorporation of [^{3}H]cysteine into cystathionine catalysed by crude spinach extracts in the presence of PHS. [^{3}H]Cysteine incorporation was measured by method A without any additions (A) and in the presence of 0 05 mM (B), 0 125 mM (C) and 0 25 mM (D) selenocysteine respectively

DISCUSSION

Crude extracts of spinach supported the synthesis of cystathionine as determined by methods A and B using cysteine and PHS as substrates. The properties of the reaction are consistent with CγS activity from other sources [6, 7]. Cysteine could not be replaced as the donor of the sulphur and aminopropyl moieties of cystathionine while various *O*-esters of homoserine (but not homoserine itself) acted as aminobutyryl donors. Maximum activity of the spinach enzyme was detected in the presence of PHS, the physiologically active substrate [2] The K_m value for cysteine (0.25 mM) is similar to that reported from other plants [5, 7, 9] and *Salmonella* [21]. The K_m value for PHS is one of the lowest reported for a crude plant extract [5, 7] but is considerably greater than the value reported for the purified barley leaf enzyme [9] Inhibition of the spinach enzyme by high concentrations of cysteine and PHS has been reported for enzymes from other sources [6, 22]. Inhibition of cystathionine synthesis by propargylglycine, vinylglycine and β-cyanoalanine (Fig 2, Table 1) is also consistent with the involvement of CγS activity [6, 9, 22]. The inhibition of activity

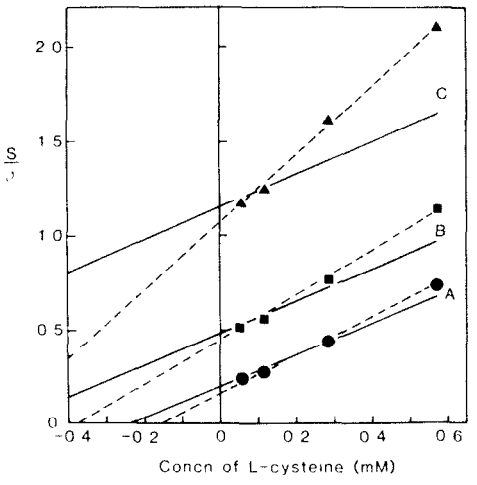

Fig 6 Hanes–Woolf plot of the kinetics of selenocysteine inhibition of [^{3}H]cysteine incorporation into cystathionine The primary data are shown in Fig 5 The dotted lines depict the lines of best fit for all available data The continuous lines show the lines of best fit for those estimations where the combined (cysteine plus selenocysteine) concentrations do not exceed the presumed inhibitory level of 0 3 mM Without selenocysteine (A), 0 05 mM selenocysteine (B) and 0 125 mM selenocysteine (C)

by amino-oxyacetate and isonicotinic acid hydrazide is in agreement with the reported role of these reagents as inhibitors of pyridoxal phosphate-requiring enzymes [14, 15]

It is not clear whether the inhibition of cystathionine synthesis by 0 32 mM cystine (Table 2) results from variations in the concentrations of cysteine or interaction with the enzyme The presence of a 25-fold molar excess of DTT over the initial cysteine concentration could result in reduction of cystine, augmenting the cysteine concentration from 0 5 mM to an inhibitory level of 1 1 mM (Fig. 1). Presumably the greater inhibition by cystine of method A assays compared to method B results from radiochemical dilution of the [^{3}H]cysteine substrate in addition to the inhibitory effects of elevated cysteine concentrations; method B assays involving [^{14}C]PHS would not be affected in this way Qualitatively similar results were obtained with selenocystine This is consistent with the possibility that selenocystine was reduced to selenocysteine which competed with cysteine thereby inhibiting [^{3}H]cysteine incorporation but not the incorporation of [^{14}C]PHS The inhibitory effect of selenocystathionine on method B assays (Table 2) is consistent with that for cystathionine but the relatively greater inhibition in method A assays implies the presence of a contaminant or a compound formed from selenocystathionine by spinach extracts which inhibits [^{3}H]cysteine incorporation but has little effect on [^{14}C]PHS incorporation.

The studies with [^{75}Se]selenocysteine incorporation (Figs 3 and 4) provide evidence that the selenium atom of selenocysteine is incorporated into selenocystathionine The electrophoretic characteristics of the product, the requirement for the aminobutyryl donor SHS and the sensitivity of [^{75}Se]selenocysteine incorporation to propargylglycine establish the identity of selenocystathionine as the product and the role of the enzyme CγS in its synthesis However, techniques were not perfected for a satisfactory quantitative assay for the incorporation of [^{75}Se]selenocysteine into selenocystathionine; procedures analogous to those employed for the quantitative incorporation of [^{3}H]cysteine into cystathionine (method A) involving the oxidation of cystathionine to its sulphone could not be used due to uncontrolled oxidative side reactions with [^{75}Se]selenocystathionine and [^{75}Se]selenocysteine Alternative procedures need to be elucidated and in this regard the use of alkylating agents could prove useful (Fig 4)

Selenocystathionine and cystathionine synthesis as determined by [^{14}C]PHS incorporation exhibit some properties which indicate that the two products are synthesized by a common mechanism Crude extracts support the two reactions at similar rates and both reactions are inhibited at cysteine and selenocysteine concentrations greater than *ca* 0 5 mM (Fig 1) Also, both reactions exhibit similar sensitivity to propargylglycine (Fig 2) and the rates of [^{14}C]PHS incorporation with cysteine and selenocysteine are not additive (Table 3) The kinetics of selenocysteine inhibition of [^{3}H]cysteine incorporation at low total amino acid concentrations (Fig 6) also suggest that cysteine and selenocysteine compete for a common reaction site though the data do not make clear whether selenocysteine is a purely competitive inhibitor or exhibits mixed competitive/non-competitive kinetics [20, 23]. Interestingly, spinach extracts exhibit a higher affinity for selenocysteine (K_m = 70 μM) than cysteine (K_m = 240 μM) Given the similar V_{max} values for the two substrates it follows from the higher affinity for selenocysteine that CγS would metabolize more selenocysteine than cysteine in equimolar mixtures at non-saturating concentrations of both substrates This could contribute to the large inhibition of [^{3}H]cysteine incorporation by selenocysteine (Table 2) The data provide no evidence in support of a selenocysteine-specific selenocystathionine γ-synthase in spinach extracts Rather, the data suggest that CγS, like several other enzymes of the sulphate assimilation pathway, supports a reaction with the selenium isologue of the pathway, other examples include ATP sulphurylase [24–26], cysteine synthase [19] and β-cystathionase [27]

Plants contain the enzymes glutathione reductase and cysteine synthase which catalyse the incorporation of SeO_3^{2-} into selenocysteine [19, 28, 29] Since the β-cystathionases from various plant species reportedly metabolise both cystathionine and selenocystathionine with similar affinity [27], then it follows from the present study that the transsulphuration pathway of plants has the capacity to incorporate selenocysteine into selenohomocysteine, the presumed precursor of selenomethionine, an important incorporation product in many non-accumulator plants when supplied with inorganic selenium [30, 31] The localization of homoserine kinase, CγS and most of the β-cystathionase activity in chloroplasts [10] together with the capacity of these organelles to incorporate SeO_3^{2-} into selenocysteine [28] suggests that illuminated chloroplasts have the capacity to incorporate inorganic selenium into selenocystathionine

Although the CγS of spinach has the capacity to incorporate selenocysteine into selenocystathionine, presumably non-accumulators such as spinach normally produce selenocysteine at very slow rates due to the low levels of SeO_3^{2-} in most soils Reports that many non-

accumulator species readily incorporate applied selenium into protein (mostly as selenomethionine) suggest that the transsulphuration pathway is open to selenium and that selective removal of selenium isologues of the pathway into metabolically inert compartments is unlikely.

EXPERIMENTAL

Plant material Me_2CO powders were prepared from freshly harvested leaves of field grown spinach (*Spinacia oleracea*) and stored in a dessicator at -10°

Chemicals L-[3,3'-^{3}H]Cystine dihydrochloride, L-[U-^{14}C]homoserine and L-[^{75}Se]selenocystine were purchased from the Radiochemical Centre (Amersham, Bucks, U K) AHS, *O*-acetyl-L-serine and L-cystathionine were obtained from Calbiochem (Los Angeles, Ca, U S A.). L-Selenocystathionine was synthesized as described in ref. [27] Samples of PHS and L-serine-*O*-sulphate were gifts from Dr J Giovanelli (Bethesda) and Dr I Murakoshi (Chiba) respectively Dowex 1-Cl^- and 50-H^+ were obtained under the respective designations of AG- × 10, 100-200 mesh and AG 50W- × 4, 200-400 mesh (Bio-Rad Labr, Richmond, Ca., U S A) All other chemicals were purchased from Sigma (U S A)

Preparation of enzyme extracts Crude extracts of spinach were prepared by blending 5 g of spinach Me_2CO powder in 50 ml of 0 1 M K-Pi buffer, pH 7 2 Thereafter the procedure was as described in ref [2]

Assay of CγS Four methods based on the methods described in refs [2, 7] were used Method A ([^{3}H]cysteine incorporation) incubations (50 μl) were conducted under N_2 at 30° in small capped tubes and contained (in μmol), L-cystathionine (0 007), L-[^{3}H]cysteine (0.025, 0 2 μCi), MOPS (30) pH 7 7, PHS (0.25) and crude extract (0.025–0 15 mg protein) Reactions were terminated after 1 hr by addition of 0 5 ml of a cold soln of 6 8% TCA containing L-cysteine (126 μmol) and L-cystathionine (0 34 μmol) Terminated reaction mixtures were then treated as described in ref [2] with the modifications detailed in ref. [7]. Method B ([U-^{14}C]PHS incorporation): incubations (50 μl) were conducted under N_2 at 30° in small capped tubes essentially as described for method A except that unlabelled L-cysteine and [U-^{14}C]PHS (0 25 μmol, 0 063 μCi) were used Reactions were terminated after 1 hr with 0 5 ml of a cold soln of 6 8% TCA containing L-cystathionine (0 34 μmol) only Insoluble material was removed by centrifugation (3000 g, 10 min) and radioactive cystathionine was resolved from [^{14}C]PHS (and any labelled homoserine formed non-enzymically from it) by ion-exchange chromatography on a column of Dowex 50-H^+ [32]. The aq NH_3 eluate from the column was evapd and the residue dissolved in 1 05 ml of H_2O. A sample of this soln (1 ml) was added to 9 ml of aq counting scintillant and the cystathionine produced was determined from the radioactivity Method C ([^{75}Se]selenocysteine incorporation) reactions were essentially as described for method A except that [^{75}Se]seleno-L-cysteine (0 025 μmol, 1 μCi) replaced L-[^{3}H]cysteine In some preliminary studies SHS replaced PHS as the α-aminobutyryl donor 2-Mercaptoethanol (10 mM) was present in all solns used for washing and eluting Dowex 50-H^+ columns Method D ([U-^{14}C]PHS incorporation) reactions were as described for method B except that unlabelled seleno-DL-cysteine (0 05 μmol) replaced L-cysteine. CγS activity is expressed as nmol of cystathionine or (where appropriate) selenocystathionine synthesized/min/mg protein

Preparation of PHS ^{14}C-Labelled and unlabelled PHS was synthesized enzymically from homoserine and ATP catalysed by homoserine kinase essentially as described in refs [2, 33] together with some modifications suggested in ref [34] Homoserine kinase was purified from bakers yeast [2, 27] after disrupting the cells with liquid N_2 [35]. The thawed material was suspended in an equal vol of 0 1 M K_2CO_3 buffer (pH 8 5) containing 14 mM 2-mercaptoethanol and stirred for 48 hr while maintaining the pH at 8 5 with aq NH_3 After removing particulate matter by centrifugation, solid $(NH_4)_2SO_4$ was added and the protein fraction precipitating between 32 and 44% saturation was dissolved in 50 mM Tris–HCl (pH 7 4) containing 14 mM 2-mercaptoethanol This extract was dialysed overnight against Tris–HCl buffer (as above) and employed as the source of homoserine kinase for the synthesis of PHS

Unlabelled PHS was synthesized in the reaction mixture described in ref [33] except that $MgCl_2$ was substituted for $MgSO_4$ and KCl (50 mM) was included [34] The reaction mixture (600 ml) contained 16 mmol DL-homoserine and a trace of L-[U-^{14}C]homoserine (5 μCi, 0 125 μmol) to monitor purification together with homoserine kinase derived from 500 g of yeast. After 8 hr at 20° the reaction was terminated by heating (100°) After filtration, PHS was purified as in ref [2] The yield was 45% with respect to L-homoserine The product contained no UV-absorbing material and no Pi [36] PE in buffer B revealed one major radioactive component which reacted with ninhydrin and co-migrated with an authentic sample of PHS No [^{14}C]homoserine was detected

[U-^{14}C]PHS was synthesized from L-[U-^{14}C]homoserine (50 μCi, 20 μmol) in a reaction (4 ml at 30°C) containing dialysed homoserine kinase derived from 20 g of yeast. All other incubation conditions were as in ref [34] The reaction was terminated after 3.5 hr by heating (100°) and insoluble material removed by centrifugation and washed twice with H_2O (1 ml) The supernatant soln and washings were combined, diluted to 66 ml with H_2O and applied to a column of Dowex 1-Cl^- (0 8 × 7.8 cm) The column was washed with 7.8 ml of H_2O and then with 7 5 mM HCl The radioactive material eluting between 12 5-47.0 ml HCl was collected Charcoal (1 g) was added to remove UV-absorbing material After stirring for 30 min the charcoal was recovered by filtration and washed twice with 10 ml of H_2O The combined filtrate was lyophilized, dissolved in 3 ml of H_2O and applied to a column of Dowex 50-H^+ (0 8 × 3.6 cm) The column was washed with 12 ml of H_2O The washings were lyophilized and the residue dissolved in 0 42 ml of H_2O The yield of [U-^{14}C]PHS from L-[U-^{14}C]homoserine was 77% and was at least 96% radiopure as judged by PE in buffers B and C.

Electrophoresis and chromatography PE was conducted at 4° on Whatman 3 MM paper in the following buffers A, 0 46 M HCO_2H, pH 1 9, for 75 min at 2 5 kV [2, 3], B, 1% Pyr adjusted to pH 3 5 with glacial HAc [37, 38] for 45 min at 3.3 kV, C, 125 mM triethanolamine–HCl, pH 8 0, for 45 min at 2 5 kV [2] Descending PC was performed on Whatman 3 MM paper at room temp using the following solvents A, PrOH–conc HCl–H_2O (6 2 1) [39], B, MeOH–pyridine–1 25 M HCl (37 4 8) [40] Amino acids were visualized with ninhydrin Radioactivity on chromatograms and electrophoretograms was determined by cutting strips into 1 cm sections and counting in 5 ml of aq counting scintillant

Preparation and estimation of cysteine L-Cystine, either unlabelled or as L-[3, 3'-^{3}H]cystine dihydrochloride was dissolved in 0 1 M HCl and adjusted to pH 8 with 0.8 M-Tricine (pH 9) prior to incubation under N_2 at 30° for 30 min with a 50-fold excess of DTT [41] Solns of L-cysteine were prepared immediately prior to use and estimated with reagent 2 as described in ref [42]

Preparation and estimation of selenocysteine. Seleno-DL-cystine or [^{75}Se]seleno-L-cystine was dissolved in 1 M HCl and reduced with DTT immediately prior to use as described for the prepar-

ation of cysteine Stock solns of selenocysteine were not estimated by the method described for cysteine [42] since this procedure has low sensitivity towards selenocysteine and the resulting chromophore is not stable [19, 41] It was assumed that reduction of selenocystine went to completion

Other methods Evapn was carried out at 25° with a rotary vacuum evaporator Enzyme kinetic data were plotted by the Hanes–Woolf method [23] and K_m values were determined by the least squares method [23] Protein was measured as in ref [43] using bovine serum albumin as standard

Acknowledgements—We wish to thank Dr A R Scarf for preparing authentic L-selenocystathionine This work was funded by the Australian Research Grants Scheme J. C Dawson was the holder of a Commonwealth Postgraduate Scholarship

REFERENCES

1 Giovanelli, J, Mudd, S H and Datko, A H (1980) in *The Biochemistry of Plants* (Miflin, B J, ed) Vol 5, p 453 Academic Press, New York
2 Datko, A H, Giovanelli, J and Mudd, S H (1974) *J Biol Chem* **249**, 1139
3 Giovanelli, J and Mudd, S H (1966) *Biochem Biophys Res Commun* **25**, 366
4 Giovanelli, J and Mudd, S H (1971) *Biochim Biophys Acta* **227**, 671
5 Madison, J T and Thompson, J F (1976) *Biochem Biophys Res Commun* **71**, 684
6 Thompson, G A, Datko, A H and Mudd, S H (1982) *Plant Physiol* **70**, 1347
7 Thompson, G A, Datko, A H, Mudd, S H and Giovanelli, J (1982) *Plant Physiol* **69**, 1077
8 Thompson, G A, Datko, A H and Mudd, S H (1983) *Plant Physiol* **71**, 241
9 Aarnes, H (1986) *Plant Sci Letters* **19**, 81
10 Wallsgrove, R M, Lea, P J and Miflin, B J (1983) *Plant Physiol* **71**, 780
11 Brown, T A and Shrift, A (1982) *Biol Rev* **57**, 59
12 Anderson, J W and Scarf, A R (1983) in *Metals and Micronutrients Uptake and Utilization by Plants* (Robb, D A. and Pierpoint, W S, eds), p 241 Academic Press, London
13 Datko, A H and Mudd, S H (1982) *Plant Physiol* **69**, 1070
14 Jenkins, C L D, Rogers, L J and Kerr, M W (1983) *Phytochemistry* **22**, 19
15 Jenkins, C L D, Rogers, L J and Kerr, M W (1983) *Phytochemistry* **22**, 347
16 Peterson, P J and Butler, G W (1962) *J Chromatogr* **139**, 391
17 Scala, J and Williams, H H (1964) *J Chromatogr* **15**, 546
18 Meens, G E and Feeney, R E (1971) in *Chemical Modification of Proteins*, p 218 Holden-Day, San Francisco
19 Ng, B H and Anderson, J W (1978) *Phytochemistry* **17**, 2069
20 Dixon, M and Webb E C (1971) *Enzymes* 2nd Edn Longman, London
21 Johnston, M, Marcotte, P, Donovan, J and Walsh, C (1979) *Biochemistry* **18**, 1729
22 Johnston, M, Janowski, D, Marcotte, P, Tanaka, H, Esaki, N, Soda, K and Walsh, C (1979) *Biochemistry* **18**, 4690
23 Segel, I H (1975) *Enzyme Kinetics* John Wiley, New York
24 Wilson, L G and Bandurski, R S (1958) *J Biol Chem* **233**, 975
25 Shaw, W H and Anderson, J W (1972) *Biochem J* **127**, 237
26 Burnell, J N (1981) *Plant Physiol* **67**, 316
27 McCluskey, T J, Scarf, A R and Anderson, J W (1986) *Phytochemistry* **25**, 2063
28 Ng, B H and Anderson, J W (1979) *Phytochemistry* **18**, 573.
29 Jablonski, P P and Anderson, J W (1982) *Phytochemistry* **21**, 2179
30 Peterson, P J and Butler, G W (1962) *Aust J Biol Sci* **15**, 126
31 Olson, O E, Novacek, E J, Whitehead, E I and Palmer, I S (1970) *Phytochemistry* **9**, 1181
32 Mudd, S H, Finkelstein, J D, Irreverre, F and Laster, L (1965) *J Biol Chem* **240**, 4382
33 Flavin, M and Slaughter, C (1960) *J Biol Chem* **235**, 1103
34 Thoen, Å, Rognes, S E and Aarnes, H (1978) *Plant Sci Letters* **13**, 113
35 Black, S (1955) in *Methods in Enzymology* (Colowick, S P and Kaplan, N O, eds) Vol 1, p 509 Academic Press, New York
36 Taussky, H H and Shorr, E (1953) *J Biol Chem* **202**, 675
37 Flavin, M (1962) *J Biol Chem* **237**, 768
38 Flavin, M and Slaughter, C (1964) *Biochemistry* **3**, 885
39. Fischer, G A (1957) *Biochim Biophys Acta* **25**, 50
40 Datko, A H, Giovanelli, J and Mudd, S H (1975) *Anal Biochem* **64**, 80
41 Burnell, J N, Karle, J A and Shrift, A (1980) *J Inorg Biochem* **12**, 343
42 Gaitonde, M K (1967) *Biochem J* **104**, 627
43 Peterson, G L (1977) *Anal Biochem* **83**, 346