*Phytochemistry*, 1978, Vol 17, p 2069–2074 © Pergamon Press Ltd. Printed in England

0031-9422/78/1201-2069 $02 00.0

# SYNTHESIS OF SELENOCYSTEINE BY CYSTEINE SYNTHASES FROM SELENIUM ACCUMULATOR AND NON-ACCUMULATOR PLANTS

B. HOCK NG and JOHN W. ANDERSON

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

(*Revised received* 20 *April* 1978)

**Key Word Index**—*Trifolium repens*, white clover; *Pisum sativum* (cv Massey Gem); peas; *Astragalus* spp.; *Neptunia amplexicaulis*; Leguminosae; cysteine synthase (*O*-acetylserine sulphhydrylase); selenocysteine synthesis; selenide assimilation; sulphide assimilation.

**Abstract**—Cysteine synthases were partially purified from leaf tissue of 3 selenium-accumulator species (*Neptunia amplexicaulis*, *Astragalus racemosus* and *A. bisulcatus*) and 4 non-accumulators (peas, white clover, *A. sinicus* and *A. hamosus*). The properties of all 7 enzymes with respect to cysteine synthesis from $S^{2-}$ and *O*-acetylserine (*O*AS) were similar. All of the enzymes also catalysed the synthesis of selenocysteine when $S^{2-}$ was replaced with $Se^{2-}$. There were no distinct differences between the properties of the enzymes from selenium-accumulator and non-accumulator plants with respect to selenocysteine synthesis. $Se^{2-}$ inhibited the synthesis of cysteine and $S^{2-}$ inhibited the synthesis of selenocysteine implying competition between $S^{2-}$ and $Se^{2-}$ for the enzyme. The affinities of the enzymes for $Se^{2-}$ were substantially greater than for $S^{2-}$, and $V_{max}$ (selenocysteine) was *ca* 7–48% of $V_{max}$ (cysteine). Isolated pea chloroplasts catalysed the synthesis of selenocysteine from *O*AS and $Se^{2-}$ at a rate of *ca* 22–26 μmol/mg Chl/hr. Sonicating the chloroplasts slightly enhanced the rate.

## INTRODUCTION

Various Se-accumulator and non-accumulator plants synthesize the Se analogues of intermediates of the metabolic pathway leading from cysteine to methionine [1, 2]. In accumulators, they include selenocystathionine (*Neptunia amplexicaulis* and *Stanleya pinnata*) and selenohomocystine (*Astragalus crotalariae*) [3–5]. Peterson and Butler [6] reported that the non-accumulators *Trifolium repens* and *Lolium perenne* incorporate $^{75}SeO_3^{2-}$ into selenocystine, selenocysteic acid and selenomethionine, although the identity of some of these products is rather tentative. Some species, including *Phaseolus lunatus* (non-accumulator) [7] and various accumulator and non-accumulator species of *Astragalus* [1, 2] also synthesize the Se-methylated derivatives of selenocysteine and/or selenomethionine.

Little is known about the pathway for the assimilation of inorganic Se in plants. By analogy with methionine biosynthesis, the occurrence of Se analogues of the pathway would suggest that selenocysteine is the precursor of these compounds although free selenocysteine itself has not been demonstrated in plants [2]. Labelling studies [8, 9] suggest that selenocysteine is also the precursor of Se-methylselenocysteine in *Astragalus bisulcatus*. One mechanism which might account for the synthesis of selenocysteine from inorganic Se could be that the cysteine synthases (*O*AS sulphhydrylase, EC 4.2.99.8) of plants catalyse the following reaction:

$$Se^{2-} + OAS \rightarrow \text{selenocysteine} + \text{acetate}.$$

In this paper we report that purified cysteine synthases from several Se-accumulator and non-accumulator species catalyse this reaction and that the kinetics of the enzymes from the two types of plants are similar.

## RESULTS

### *Synthesis of selenocysteine by purified clover and pea cysteine synthases*

When clover enzyme was incubated in buffered medium containing 20 mM *O*AS and gassed with $H_2Se$ (method A) a product was formed which gave a positive reaction with reagent-2 of Gaitonde [10]. The product did not form in the absence of enzyme, *O*AS, or $H_2Se$, nor when the enzyme was pretreated at 100° for 10 min. Samples of the reaction mixture were gassed with $O_2$ and subjected to PC. The oxidation product, which gave a positive reaction with ninhydrin, was indistinguishable from cystine and selenocystine. Samples of the reaction mixture were also treated with *N*-ethylmaleimide (NEM) and subjected to PC in solvents I and II. The product was indistinguishable from the NEM-adduct of selenocysteine and ran with a slightly lower $R_f$ than the NEM adduct of cysteine in solvents I and II. The oxidation product of reaction mixtures gassed with $H_2^{75}Se$ (method A) was confirmed as selenocysteine by PC and paper electrophoresis (PE). A labelled oxidation product, indistinguishable from cystine and selenocystine, was also detected by PE when the clover enzyme was incubated with *O*AS-[$^{14}$C] and unlabelled $H_2Se$.

The procedure of Burnell and Whatley [11] for the isolation of selenocystine was used to prepare the reaction product from a combined pool of 20 individual reaction mixtures containing clover enzyme and $H_2Se$ generated by method B. The isolated product and authentic selenocystine were examined by UV spectrophotometry ($\lambda_{max}$ 300 nm for both compounds, $\lambda_{min}$ for authentic selenocystine and isolated product, 265 and 273 nm, respectively). On reduction with $NaBH_4$ the

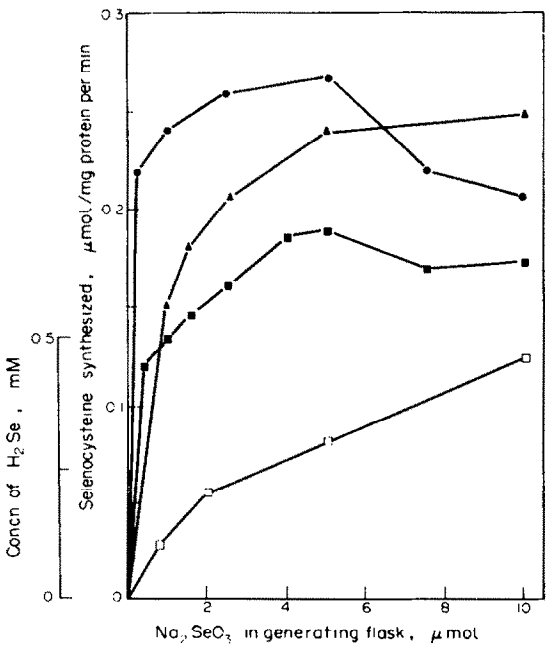


Fig. 1. Effect of $Na_2SeO_3$ on selenocysteine synthesis by purified clover cysteine synthase. $H_2Se$ was generated from $Na_2SeO_3$ by method A and selenocysteine was measured colorimetrically (method 1). The results of 3 independent experiments are shown (●, ▲, ■). The approximate mean concentration of $H_2Se$ in the incubation mixtures for 5 min assays (calculated from data similar to those shown in Fig. 1) is also shown (□).

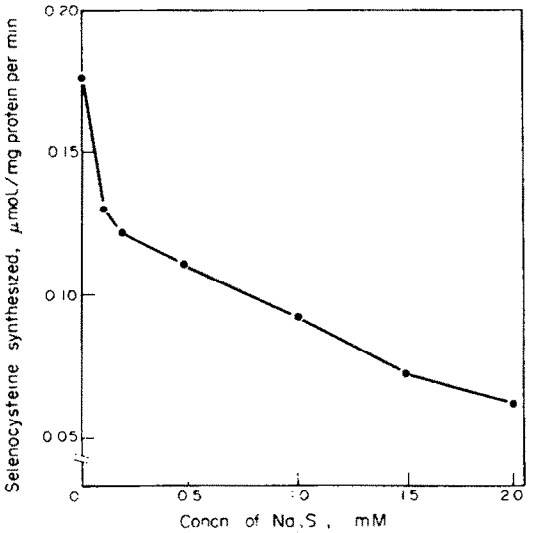


Fig. 2. Effect of $Na_2S$ on the incorporation of $H_2{}^{75}Se$ into selenocysteine by purified cysteine synthase from *A. sinicus*. $H_2{}^{75}Se$ was generated from $Na_2{}^{75}SeO_3$ (5 μmol) by method A and selenocysteine determined by method 2.

peak at 300 nm disappeared and a shoulder was detected at *ca* 250 nm for both compounds; the spectrum of the reduced form is consistent with that described for selenocysteine by Huber and Criddle [12]. The amount of selenocysteine present in the isolated sample as determined by the absorbance at 300 nm (using the authentic sample as reference) was, after correcting for stoichiometry, in good agreement (90%) with the amount of selenocysteine present after reduction with $NaBH_4$ as measured by the colorimetric method.

Some simple properties of the enzyme-catalysed synthesis of selenocysteine were examined by the Gaitonde reaction using purified clover enzyme. The rate of the reaction at 35° was constant for *ca* 10 min suggesting that the $H_2Se$ generating system (method A using 5 μmol $Na_2SeO_3$) maintained a saturating concentration of $H_2Se$ in the reaction mixture for this time. After 15 min at 35°, however, the rate of synthesis of selenocysteine decreased steadily. The reaction rate was proportional to enzyme concentration up to 30 μg protein/ml. Clover cysteine synthase did not catalyse the synthesis of selenocysteine when *O*AS was replaced with *O*-acetyl-L-homoserine or L-serine (each 10 mM).

The effect of $H_2Se$ concentration on the rate of selenocysteine synthesis was investigated by method A by varying the amount of $Na_2SeO_3$. These experiments (Fig. 1) show that 5 μmol $Na_2SeO_3$ produced sufficient $H_2Se$ in the incubating flask (*ca* 0.3 mM) to saturate the enzyme. Method A, however, was unsatisfactory for establishing a stable concentration of $H_2Se$ at low levels of $Na_2SeO_3$. When the effect of $H_2Se$ concentration was re-examined by adding aliquots of a stock solution of $H_2Se$ prepared by method B, optimum activity occurred at 0.1 mM; at 30 μM the activity was *ca* 81% of the rate at 0.1 mM indicating that the $K_m$ ($Se^{2-}$) was less than 30 μM.

Purified pea cysteine synthase also catalysed the reactions described for the clover enzyme, though 1 μmol $Na_2SeO_3$ produced sufficient $H_2Se$ (method A) to saturate the pea enzyme, larger amounts being slightly inhibitory. The observed maximum rates using methods A and B for the production of $H_2Se$ were similar. The rates of synthesis of selenocysteine by the pea enzyme as determined by the radiochemical and colorimetric procedures were also similar (Table 1). This provides further evidence that the Gaitonde reaction is satisfactory for estimating selenocysteine.

The absorbance of the Gaitonde reaction products of cysteine and selenocysteine are additive (see Experimental). This, together with the 10-fold lower sensitivity of selenocysteine with respect to cysteine, permits an investigation of competition between $S^{2-}$ and $Se^{2-}$ for cysteine synthase. In the presence of 20 mM *O*AS and 1 mM $S^{2-}$, the $A_{560}$ catalysed by the *A. sinicus* enzyme decreased with increasing concentration of $H_2Se$ (*ca* 80%

Table 1. Synthesis of selenocysteine by partially purified pea-shoot cysteine synthase

| Method for generating $H_2Se$ | Method for measuring selenocysteine | Selenocysteine synthesized (μmol/mg protein/min) |
|---|---|---|
| Method A (1 μmol $Na_2SeO_3$) | Colorimetric | 3.97 |
| Method A (1 μmol $Na_2{}^{75}SeO_3$) | Paper electrophoresis | 3.65 |
| Method B | Colorimetric | 3.76 |

Table 2. Typical purification of cysteine synthases from leaf tissue of some *Astragalus* species and *Neptunia amplexicaulis*

| Species | Treatment | Protein (mg) | Specific activity (μmol/min/mg protein) | Yield (%) | Relative purification |
|---|---|---|---|---|---|
| *A. sinicus* | | | | | |
| | Acetone powder crude extract | 2304 | 0.15 | 100 | 1 |
| | Heat (55° for 2 min) | 1027 | 0.31 | 92.1 | 2.1 |
| | $(NH_4)_2SO_4$ fraction | 367.5 | 0.80 | 85.1 | 5.3 |
| | DEAE–cellulose (first elution) | 85.9 | 1.57 | 39 | 10.5 |
| | Sepharose-2B | 39.1 | 1.58 | 17.9 | 10.5 |
| | DEAE–cellulose (second elution) | 14.0 | 3.18 | 12.9 | 21.2 |
| *A. hamosus* | | | | | |
| | Acetone powder crude extract | 263.2 | 0.48 | 100 | 1 |
| | $(NH_4)_2SO_4$ fraction | 90.8 | 1.07 | 76.9 | 2.2 |
| | Sephadex G-200 | 13 | 2.85 | 29.3 | 5.9 |
| | DEAE–cellulose | 3 | 9.60 | 22.8 | 20 |
| *A. racemosus* | | | | | |
| | Acetone powder crude extract | 532 | 0.40 | 100 | 1 |
| | $(NH_4)_2SO_4$ fraction | 85 | 2.08 | 83.1 | 5.2 |
| | Sephadex G-200 | 16.6 | 3.59 | 28.1 | 8.9 |
| | DEAE–cellulose | 2.9 | 6.27 | 8.5 | 15.7 |
| *A. bisulcatus* | | | | | |
| | Acetone powder crude extract | 417.1 | 0.14 | 100 | 1 |
| | $(NH_4)_2SO_4$ fraction | 58.5 | 0.54 | 54.1 | 3.9 |
| | Sephadex G-200 | 16.9 | 1.49 | 43.1 | 10.6 |
| | DEAE–cellulose | 2.6 | 1.87 | 8.3 | 13.4 |
| *N. amplexicaulis* | | | | | |
| | Acetone powder crude extract | 314 | 0.31 | 100 | 1 |
| | Heat (55° for 2 min) | 272 | 0.33 | 94.2 | 1.1 |
| | $(NH_4)_2SO_4$ fraction | 38.4 | 1.44 | 58.1 | 4.6 |
| | DEAE–cellulose | 3.2 | 5.08 | 17.3 | 16.4 |
| | Sepharose 2B | 1.7 | 5.67 | 10.1 | 18.3 |

inhibition at 0.3 mM). Conversely, the incorporation of $H_2^{75}Se$ into selenocysteine catalysed by purified *A. sinicus* enzyme was inhibited by $S^{2-}$ (Fig. 2).

*Synthesis of selenocysteine by the cysteine synthases from* Astragalus *and* Neptunia *spp.*

The procedure for purifying cysteine synthase from *Trifolium repens* [11] was used, with minor modifications, to purify the enzymes from leaf tissue of *Astragalus bisulcatus*, *A. racemosus*, *Neptunia amplexicaulis* (Se accumulators), *A. sinicus* and *A. hamosus* (non-accumulators). The enzymes were purified *ca* 10–20-fold (Table 2). All of these enzymes exhibited a pH optimum pf 7.5–7.9 in K-Pi buffer and had similar $K_m$ values for *O*AS (3.0–6.5 mM) and $S^{2-}$ (0.15–0.25 mM) as determined by the colorimetric assay for cysteine synthesis (Table 3). The cysteine synthases from all of the species examined (including clover and peas) catalysed the synthesis of selenocysteine from $Se^{2-}$ and *O*AS as determined by the Gaitonde reaction. The rate of selenocysteine synthesis was measured for each enzyme over a range of $H_2Se$ concentrations (method B) to determine the optimum rate. All of the enzymes exhibited rates of *ca* 75% of the optimum rate at $H_2Se$ concentrations less than 50 μM

Table 3. Some properties of cysteine synthases from leaf tissue of several selenium-accumulator and non-accumulator species

| Species | pH optimum† | $K_m$(*O*AS) (mM) | $K_m$ $S^{2-}$ (mM) | $V_{max}$ (selenocysteine) / $V_{max}$ (cysteine) |
|---|---|---|---|---|
| Non accumulators | | | | |
| *Trifolium repens* | 7.8* | 3.5 (0.51)* | 0.51 (0.13)* | 0.21 |
| *Pisum sativum* | 7.5* | 3.1 (0.13)* | 0.24 (0.06)* | 0.48 |
| *Astragalus hamosus* | 7.6 | 4.0 | 0.15 | 0.07 |
| *A. sinicus* | 7.6 | 3.9 | 0.21 | 0.20 |
| Selenium accumulators | | | | |
| *A. racemosus* | 7.5 | 3.0 | 0.19 | 0.18 |
| *A. bisulcatus* | 7.6 | 3.6 | 0.25 | 0.13 |
| *Neptunia amplexicaulis* | 7.9 | 6.5 | 0.19 | 0.47 |

All estimations of cysteine synthase and selenocysteine synthesis were made by the colorimetric assay.

* Values abstracted from [11] using colorimetric assay. $K_m$ values in parentheses were determined with a sulphide ion electrode [11].

† pH optima determined in K-Pi buffer.

Table 4. Synthesis of selenocysteine by isolated pea chloroplasts

| Treatment | | Selenocysteine synthesized (μmol/mg Chl/hr) | |
|---|---|---|---|
| Chloroplasts | Incubation mixture | Experiment 1 | Experiment 2 |
| Intact | Complete | 26.4 | 22.2 |
| Intact | Without light | 25.9 | n.d.* |
| Intact | Without *OAS* | 0.1 | 0 |
| Sonicated | Complete | 29.6 | 26.5 |
| Stroma | Complete | 32.7 | 27.7 |
| Nil | Complete | 0 | 0 |

For experiment 1, the complete system contained (in μmol) HEPES buffer pH 7.6 (40), *OAS* (20), $H_2^{75}Se$ and chloroplasts (68% intact, 180 μg Chl) in 1 ml of iso-osmotic medium (see Experimental). Incubations were conducted at 28° for 20 min in the light (14 mEin/m²/sec) under $N_2$. $H_2^{75}Se$ was generated from $Na_2^{75}SeO_3$ (5 μmol, 1 Ci/mol) by method A and selenocysteine determined by method 2. The conditions for experiment 2 were as for experiment 1 except that $H_2Se$ (0.15 μmol) was prepared by method B and chloroplasts (200 μg Chl) were 56% intact. Selenocysteine for experiment 2 was determined by method 1.

* n.d. = not determined.

indicating that $K_m(Se^{2-})$ was less than this value. The optimum rate of synthesis of selenocysteine relative to $V_{max}$ for cysteine synthesis, varied for the different enzymes (Table 3). No consistent differences in the relative rates of selenocysteine: cysteine synthesis between enzymes from Se accumulator and non-accumulator species were detected.

*Synthesis of selenocysteine by isolated pea chloroplasts*

Isolated chloroplasts (56–68% intact) catalysed the synthesis of selenocysteine in the presence of *OAS* and $H_2Se$ at rates of *ca* 22–26 μmol/mg Chl/hr. The reaction was *OAS*-dependent and independent of light. Sonicating the chloroplasts caused a small increase in the rate (Table 4). Following sonication, the selenocysteine synthesis activity was associated with the soluble fraction.

## DISCUSSION

The results reported in this paper demonstrate that the cysteine synthases from Se-accumulator and non-accumulator plants utilize $Se^{2-}$ as an alternative substrate to $S^{2-}$ to form selenocysteine in lieu of cysteine. Our results are consistent with the preliminary studies of Burnell and Whatley [11] on the synthesis of selenocysteine by the cysteine synthase from *Paracoccus denitrificans*. The UV spectra of the oxidized and reduced forms of the reaction product, the positive reaction with the Gaitonde reagent, and the results of labelling studies with $^{75}Se^{2-}$ and *OAS*-[$^{14}$C] indicate that the reaction product was selenocysteine. The optimum rate of synthesis of selenocysteine was *ca* 2–14-fold less than for cysteine for most of the enzymes examined. The $K_m$ ($Se^{2-}$) values, estimated by the colorimetric assay to be less than 50 μM for all enzymes, are substantially less than the $K_m$ ($S^{2-}$) for the enzymes from *Neptunia amplexicaulis* and *Astragalus* species (Table 3) and other plants [13–16]. This implies that $Se^{2-}$ competes favourably with $S^{2-}$ for the enzyme. The inhibitory effect of $H_2Se$ on cysteine synthesis (using *OAS* and $S^{2-}$ as substrates) when measured as $A_{560}$ is consistent with this proposal. (Any selenocysteine synthesized from *OAS* and $H_2Se$ will make only a small contribution to $A_{560}$). The inhibition of selenocysteine synthesis by $H_2S$ (Fig. 2) also supports this possibility. Thus, whilst we are unable to cite values for $K_m$ ($Se^{2-}$) it is apparent that cysteine synthase, like ATP sulphurylase [17, 18], has a higher affinity and a lower $V_{max}$ for the Se analogue of the sulphur substrate than it has for the sulphur substrate itself. Our data on the use of $S^{2-}$ and $Se^{2-}$ by cystine synthase provide a further example of the property of some enzymes of sulphur metabolism to catalyse analogous reactions with the appropriate Se analogues. Some other examples include cysteinyl-*t*RNA synthetase (cysteine/selenocysteine) [19], ATP sulphurylase ($SO_4^{2-}/SeO_4^{2-}$) [17, 19, 20] and methionyl-*t*RNA synthetase (methione/selenomethionine) [21, 22].

Although free selenocysteine has not been demonstrated in plants, the incorporation of $Se^{2-}$ into selenocysteine by plant cysteine synthases is consistent with the proposed role of selenocysteine as a precursor of many of the selenoamino acids found in various Se-accumulator and non-accumulator plants. Our results, however, do not provide any information which might explain differences in Se metabolism in Se-accumulator and non-accumulator plants. We did not find any consistent differences in the properties of the cysteine synthases between the two groups of plants with respect to enzyme activity per g fr. wt, the affinity for $S^{2-}$ and $Se^{2-}$, and the $V_{max}$ ($S^{2-}$)/$V_{max}$ ($Se^{2-}$) ratio. Thus, if inorganic Se enters intermediary metabolism via the reaction catalysed by cysteine synthase *in vivo*, our results imply that other mechanistic differences must be responsible for the different patterns of Se assimilation observed in various accumulator and non-accumulator plants. Some differences of this kind have been observed with respect to the activation of selenocysteine by the cysteinyl-*t*RNA synthetases [J. N. Burnell and A. Shrift, personal communication].

Cysteine synthase is associated with chloroplasts [13, 23]. 'Intact' pea chloroplasts catalyse selenocysteine synthesis at rates of *ca* 22–26 μmol/mg Chl/hr (Table 4). These rates are in fair agreement with those predicted from the $V_{max}$ (selenocysteine)/$V_{max}$ (cysteine) ratio for purified pea cysteine synthase (Table 3) and the rate of cysteine synthesis (*ca* 75 μmol/mg Chl/hr) for intact pea chloroplasts as determined by the colorimetric method [13] (predicted rate for selenocysteine, 36 μmol/mg Chl/hr). The slightly enhanced rates of selenocysteine synthesis following sonication and the association of activity with the chloroplast soluble fraction following this treatment (Table 4) are consistent with the results for cysteine synthesis [13]. Here again, we presume that the enhanced rate following sonication is because the chloroplast membrane of intact chloroplasts limits the rate of entry of *OAS* and/or $Se^{2-}$.

If inorganic Se is assimilated via cysteine synthase *in vivo*, then plants must possess mechanisms for the reduction of $SeO_4^{2-}$ and $SeO_3^{2-}$ to $Se^{2-}$ since the more oxidized forms of Se are readily incorporated into selenoamino acids [6, 7, 24]. The mechanism for the reduction of $SeO_4^{2-}$ remains controversial [18, 25]. However, Hsieh and Ganther [26] have demonstrated that yeast glutathione reductase, in the presence of glutathione and NADPH, catalyses the reduction of $SeO_3^{2-}$ to $Se^{2-}$. Since glutathione reductase is associated with chloroplasts [27, 28] and the reduction of oxidized

glutathione is coupled to photosynthetic electron transport [27], it is possible therefore that $SeO_3^{2-}$ is reduced to $Se^{2-}$ in chloroplasts in a light-dependent reaction involving glutathione reductase. $Se^{2-}$ so generated, could be assimilated into selenocysteine by the chloroplast cysteine synthase.

## EXPERIMENTAL

White clover (*Trifolium repens*) and peas (*Pisum sativum* cv Massey Gem) were raised as in ref. [13]. Seeds of *Astragalus* spp. were obtained and raised as in ref. [18]. Seeds of *Neptunia amplexicaulis f. richmondii* were collected from seleniferous areas of N.W. Queensland, Australia (voucher, Queensland Herbarium BRI 169865) and plants raised as for *Astragalus* [18]. $Me_2CO$ powders were prepared from leaf-tissue of *N. amplexicaulis* and *Astragalus* spp. and stored in a desiccator at $-15°$. The powders, which were used as the enzyme source for these spp., showed no loss of activity during storage for 6 months.

*Chemicals.* DL-Selenocystine and NEM were obtained from Sigma (St. Louis, Mo., USA), $Al_2Se_3$ from Alfa Products (Danvers, MA., USA) and $Na_2{}^{75}SeO_3$ from The Radiochemical Centre (Amersham, Bucks., U.K.). All other compounds were synthesized or purchased as described in [13].

*Colorimetric estimation of selenocysteine.* Selenocysteine was prepared by reacting DL-selenocystine with a 10-fold excess of dithiothreitol (DTT) in 0.1 M Tris–HCl buffer pH 8 at 30° for 1 hr. The product formed a pink pigment ($A_{max}$ 560–565 nm) when reacted with Gaitonde reagent-2 for the estimation of cysteine [10]. Neither DTT nor selenocystine gave a positive reaction. Accordingly, selenocysteine was measured using Gaitonde reagent-2 [10] except that $A_{560}$ was measured within 5 min as the pigment rapidly deteriorated. The reaction with selenocysteine, however, was 10-fold less sensitive than that for cysteine and at 560 nm only complied with the Beer–Lambert law up to an $A$ of *ca* 0.25. Nevertheless, for a path length of 1 cm the reaction could be used to detect up to 250 nmol selenocysteine in a 0.5 ml sample (final vol., 3.5 ml). Various attempts to improve the sensitivity of the reaction and to stabilize the pigment (e.g. replacing HCl with $H_3PO_4$ and using various organic solvents as diluents were unsuccessful. When cystine was reduced with DTT, it gave the same reaction with Gaitonde reagent-2 as a 2-fold amount of cysteine. Cystine, homocystine, Se-methylselenocysteine, 2-methylcysteine, homocysteine, homocystine, *O*AS, $S^{2-}$ and $Se^{2-}$ did not give a reaction with the Gaitonde reagent-2.

When $A_{560}$ was determined for mixtures of cysteine and selenocysteine, the value was equal to the sum of the $A$ for the 2 substrates when measured alone.

*Generation of* $H_2Se$. Method A was used to supply $H_2Se$ or $H_2{}^{75}Se$ directly to incubation mixtures and was performed in the apparatus described in ref. [29]. $Na_2SeO_3$ (1–5 μmol) or $Na_2{}^{75}SeO_3$ (1–5 μmol, 1 Ci/mol) in 0.5 ml was placed in the generating flask and 350 mg Zn dust added; the amount of $Na_2SeO_3$ used is specified for each expt. The apparatus was flushed with $O_2$-free $N_2$ for 10 min and 1 ml 11 M HCl added rapidly to initiate production of $H_2Se$. Flushing with $N_2$ was continued for a further 5 min. This method did not permit direct and accurate control of the $H_2Se$ concn in the incubation flasks. This was due to non-enzymic oxidation of $H_2Se$ to $Se^0$ in various parts of the apparatus, volatilization of the $H_2Se$ from the incubation flasks and presumably variations in the rate of displacement and the proportion of $H_2Se$ released from $Na_2SeO_3$. Method A was examined for simulated incubations in the absence of enzyme. When $H_2Se$ was generated from 1 μmol $Na_2{}^{75}SeO_3$ only 11.4% of the label was recovered in the incubation flask after 5 min; the remainder was recovered in the $AgNO_3$ trap or as $Se^0$ in the generating flask and connecting tubing. Time studies of the concn of $H_2Se$ in the incubation flasks showed that, for any one level of $Na_2SeO_3$ supplied, the concn of $H_2Se$ gradually decreased (e.g. for 5 μmol $Na_2SeO_3$, $H_2Se$ concn fell from 0.31 mM at 1 min to 0.23 mM at 15 min). The proportion of $H^2Se$ lost was greatest at low levels of $Na_2SeO_2$ (e.g. for 5, 2 and 1 μmol of $Na_2SeO_3$, the $H_2Se$ concn decreased by 25, 36 and 73% between 1 and 15 min, respectively). The concn of $H_2Se$ in the incubation flasks at 3 min was used as an approximate estimate of the $H_2Se$ concn produced by a given amount of $Na_2SeO_3$ for enzyme incubations lasting 5 min.

Method B was used to prepare a stock soln of $H_2Se$, samples of which were transferred to incubation tubes. Method B was conducted in the apparatus of [29] except that the tube containing the reaction mixture was replaced with one containing 2 ml $H_2O$ (collecting tube). $Al_2Se_3$ (200 mg) was placed in a dry reaction flask which was swept with $O_2$-free $N_2$ at a rapid rate for 10 min. The flow rate was then decreased to 200 ml/min and $H_2O$ added drop by drop to displace $H_2Se$. After 5 min, the collecting tube was disconnected, sealed and samples analysed for $Se^{2-}$ by titrating with $AgNO_3$ with an $Ag^+$ electrode (model 94-16A, Orion Research Inc., Cambridge, Mass., U.S.A.). This method permitted more accurate control of the $H_2Se$ concn in the incubation mixture and losses during the incubation period were much less (6.6% decrease over 10 min). Method B was also more convenient for varying the $H_2Se$ concn in incubation mixtures although it was found that the amount of $H_2Se$ transferred was not strictly proportional to the vol. of the sample, the losses increasing with the vol. of the sample.

*Chloroplasts* were prepared by method B described in [13] and incubations conducted in iso-osmotic medium. Sonication and determination of intactness were as in ref. [13].

*Extraction and purification of cysteine synthases.* The enzymes from peas and clover were prepared as in ref. [13]. Crude extracts of *N. amplexicaulis* were prepared by blending 15 g $Me_2CO$ powder in 200 ml of 100 mM K-Pi buffer pH 7 containing 1 mM $Na_2EDTA$ and 1 mM DTT (medium 1). Thereafter the procedure was as for the clover enzyme [13]. Cysteine synthease from *A. sinicus* was prepared as for *N amplexicaulis* except that, following gel filtration on Sepharose 2B, the active fractions were pooled, dialysed against medium 2 (as for medium 1 but K-Pi buffer at 50 mM) and chromatography on DEAE–cellulose (described for the clover enzyme [13]) repeated. Crude extracts of *A. hamosus* were prepared as for *N. amplexicaulis*. The supernatant soln was recovered, heat treated and subjected to the first $(NH_4)_2SO_4$ treatment as described for the clover enzyme [13]. Precipitated protein was discarded and additional $(NH_4)_2SO_4$ (0.319 g/ml) was added. Precipitated protein was recovered, dissolved in medium 2 and dialysed against the same medium. The enzyme soln was passed through a Sephadex G-200 column (60 × 2.5 cm) equilibrated with medium 2 and active fractions subjected to chromatography on DEAE-cellulose as for the clover enzyme [13]. The cysteine synthases from *A. bisulcatus* and *A. racemosus* were extracted and purified as described for *A. hamosus*. All operations were performed at 2°. With the exception of the pea enzyme [13], all purified prepns were stored in medium 2 but with DTT increased to 5 mM [13].

*Assay of cysteine synthase and synthesis of selenocysteine.* Cysteine synthase, using $Na_2S$ as substrate, was measured colorimetrically as per method 1 in [13]. Two methods were used to measure selenocysteine. Method 1 (colorimetric): Incubation mixtures were prepared in small tubes (12 × 75 mm) and contained (in μmol) K-Pi buffer pH 7.8 (200), *O*AS (20), DTT (1) and enzyme in 1 ml. When method A was used for generating $H_2Se$, the incubation tube was connected to the $H_2Se$ generating flask and after flushing the apparatus with $O_2$-free $N_2$, the reaction was initiated by addition of HCl to the $Na_2SeO_3$. Flushing with $O_2$-free $N_2$ (introduced via the generating flask) was continued for a further 5 min after initiating the reaction, at a rate of 200 ml/min. The incubation mixtures were maintained at 35° for 5 min, terminated with 0.1 ml 1.5 M TCA and samples analysed for selenocysteine by the colorimetric method. When method B was used to supply $H_2Se$, reaction mixtures, described above, were made up in a smaller vol. such that the vol. after addition of the $H_2Se$ soln was 1 ml. The reaction tubes containing the incubation mixture (without $H_2Se$) were bubbled with $N_2$ for 5 min, and the reaction initiated by

addition of $H_2Se$ soln. The head space in the tube was flushed with $N_2$ and sealed. Thereafter the procedure was as described above.

Method 2 for the synthesis of selenocysteine involved the incorporation of $H_2{}^{75}Se$. Incubation mixtures were prepared as for method 1 and reactions initiated by supplying $H_2{}^{75}Se$ generated from $Na_2{}^{75}SeO_3$ by method A. Incubations were conducted at 35° for 5 min and terminated with 0.2 ml hot 80% EtOH (50°) containing 100 mM NEM. Samples were subjected to PC in solvent I or PE in HOAc buffer. After detecting selenocysteine-[$^{75}$Se]-NEM with a gas-flow strip detector the labelled portions of the chromatogram were cut out and $^{75}$Se-label determined in a Nuclear Chicago gamma analyser at 405 keV.

*PC and electrophoresis.* PC was conducted on Whatman 3MM paper using one of the following solvents: (I) *n*-BuOH–HOAc–$H_2O$ (4:1:5) [30]: (II) *t*-BuOH–$HCO_2H$–$H_2O$ (14:3:3) [31]. Electrophoresis was performed on Whatman 3MM paper for 6 hr at 22 V/cm in HOAc buffer, pH 2.5 [30]. Chromatograms and electrophoretograms were monitored for radioactivity with a gas flow scanner. NEM-markers were made by mixing solns of cysteine or selenocysteine with a 20% molar excess of NEM. Markers were detected with 0.1% ninhydrin in *n*-BuOH.

Protein and chlorophyll were determined as described in [13].

*Acknowledgements*—This work was supported by a grant from The Austrialian Research Grants Committee. B.H.N. was the holder of a La Trobe University Research Scholarship.

## REFERENCES

1. Shrift, A. (1969) *Annu. Rev. Plant Physiol.* **24**, 381.
2. Shrift A. (1973) in *Organic Selenium Compounds: Their Chemistry and Biology* (Klayman, D. L. and Gunther, W. H. H., eds.) p. 763. Wiley, New York.
3. Peterson, P. J. and Butler, G. W. (1967) *Nature* **213**, 599.
4. Virupaksha, T. K. and Shrift, A. (1963) *Biochim. Biophys. Acta* **74**, 791.
5. Virupaksha, T. K., Shrift, A. and Tarver, H. (1966) *Biochim. Biophys. Acta* **130**, 45.
6. Peterson, P. J. and Butler, G. W. (1962) *Aust. J. Biol. Sci.* **15**, 126.
7. Nigam, S. N. and McConnell, W. B. (1973) *Phytochemistry* **12**, 359.
8. Chen, D. M., Nigam, S. N. and McConnell, W. B. (1970) *Can. J. Biochem.* **48**, 1278.
9. Chow, C. M., Nigam, S. N. and McConnell, W. B. (1972) *Biochim. Biophys. Acta* **273**, 91.
10. Gaitonde, M. K. (1967) *Biochem. J.* **104**, 627.
11. Burnell, J. N. and Whatley, F. R. (1977) *Biochim. Biophys. Acta* **481**, 246.
12. Huber, R. E. and Criddle, R. S. (1967) *Arch Biochem. Biophys.* **122**, 164.
13. Ng, B. H. and Anderson, J. W. (1978) *Phytochemistry* **17**, 879.
14. Ascaño, A. and Nicholas, D. J. D. (1976) *Phytochemistry* **16**, 889.
15. Ngo, T. T. and Shargool, P. D. (1973) *Analyt. Biochem.* **54**, 247.
16. Bertagnolli, B. L. and Wedding, R. T. (1977) *Plant Physiol.* **60**, 115.
17. Shaw, W. H. and Anderson, J. W. (1972) *Biochem. J.* **127**, 237.
18. Shaw, W. H. and Anderson, J. W. (1974) *Biochem. J.* **139**, 37.
19. Shrift, A., Bechard, D., Harcup, C. and Fowden, L. (1976) *Plant Physiol.* **58**, 248.
20. Wilson, L. G. and Bandurski, R. S. (1958) *J. Biol. Chem.* **233**, 975.
21. Hoffman, J. L., McConnell, K. P. and Carpenter, D. R. (1970) *Biochim. Biophys. Acta* **71**, 459.
22. Coch, E. H. and Greene, R. C. (1971) *Biochim. Biophys. Acta* **230**, 223.
23. Fankhauser, H., Brunold, C. and Erismann, K. H. (1976) *Experientia* **32**, 1494.
24. Martin, J. L., Shrift, A. and Gerlach, M. L. (1971) *Phytochemistry* **10**, 945.
25. Dilworth, G. L. and Bandurski, R. S. (1977) *Biochem. J.* **163**, 521.
26. Hsieh, H. S. and Ganther, H. E. (1975) *Biochemistry* **14**, 1632.
27. Jablonski, P. P. and Anderson, J. W. (1978) *Plant Physiol.* **61**, 221.
28. Schaedle, M. and Bassham, J. A. (1977) *Plant Physiol.* **59**, 1011.
29. Diplock, A. T., Caygill, C. P. J., Jeffrey, E. H. and Thomas, C. (1971) *Biochem J.* **123**, 721.
30. Ellis, R. J. (1963) *Phytochemistry* **2**, 129.
31. Gutcho, M. and Laufer, L. (1954) in *Glutathione* (Colowick, S., Lazarow, A., Racker, E., Schwartz, D. R., Stadtman, E. and Waetsch, H., eds.). p. 79. Academic Press, New York.