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LIGHT-DEPENDENT INCORPORATION OF SELENITE AND SULPHITE INTO SELENOCYSTEINE AND CYSTEINE BY ISOLATED PEA CHLOROPLASTS

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Key Word Index—*Pisum sativum*; peas; *Trifolium repens*; white clover; Leguminosae; selenite reduction; sulphite reduction; selenocysteine synthesis; cysteine synthesis; sulphide synthesis; cysteine synthase; glutathione reductase; sulphite reductase; chloroplasts.

Abstract—Illuminated intact pea chloroplasts in the presence of *O*-acetylserine (*O*AS) catalysed incorporation of SeO_3^{2-} and SO_3^{2-} into selenocysteine and cysteine at rates of *ca* 0.36 and 6 μmol/mg Chl per hr respectively. Sonicated chloroplasts catalysed SeO_3^{2-} and SO_3^{2-} incorporation at *ca* 3.9 and 32% respectively of the rates of intact chloroplasts. Addition of GSH and NADPH increased the rates to *ca* 91 and 98% of the intact rates, but SeO_3^{2-} incorporation under these conditions was essentially light-independent. In the absence of *O*AS, intact chloroplasts catalysed reduction of SO_3^{2-} to S^{2-} at rates of *ca* 5.8 μmol/mg Chl per hr. In the presence of *O*AS, S^{2-} did not accumulate. Glutathione (GSH) reductase was purified from peas and was inhibited by $ZnCl_2$. This enzyme, in the presence of purified clover cysteine synthase, *O*AS, GSH and NADPH, catalysed incorporation of SeO_3^{2-} into selenocysteine (but not SO_3^{2-} into cysteine). The reaction was inhibited by $ZnCl_2$. Incorporation of SeO_3^{2-} into selenocysteine by illuminated intact chloroplasts and sonicated chloroplasts (with NADPH and GSH) was also inhibited by $ZnCl_2$ but not by KCN. Conversely, incorporation of SO_3^{2-} into cysteine was inhibited by KCN but not by $ZnCl_2$. It was concluded that SeO_3^{2-} and SO_3^{2-} are reduced in chloroplasts by independent light-requiring mechanisms. It is proposed that SeO_3^{2-} is reduced by light-coupled GSH reductase and that the Se^{2-} produced is incorporated into selenocysteine by cysteine synthase.

INTRODUCTION

Purified cysteine synthases from Se-accumulator and non-accumulator plants catalyse the incorporation of Se^{2-} into selenocysteine. Trace amounts of Se^{2-} can be readily incorporated since the enzyme from plants has a higher affinity for Se^{2-} than for S^{2-} [1]. Chloroplasts, which contain most of the cysteine synthase activity associated with leaf tissue [2, 3], also incorporate Se^{2-} into selenocysteine [1]. This suggests that chloroplasts have the potential to synthesize selenocysteine provided that they possess mechanism(s) for the formation of Se^{2-}. Since plants readily incorporate SeO_3^{2-} and SeO_4^{2-} into various selenoamino acids [4–6], this implies that mechanisms must exist for the reduction of the more oxidized forms of Se to oxidation state −2. The most likely site for these reduction processes is the chloroplast since this organelle is the primary site of production of reducing equivalents for use in the reduction of SO_4^{2-} [7], NO_2^- [8] and CO_2. Any Se^{2-} produced could be readily assimilated via the cysteine synthase activity associated with chloroplasts.

The form of inorganic Se most likely to be available to plants is uncertain and is probably dependent on many factors (e.g. soil type) [9]. SeO_3^{2-}, however, is known to be more readily assimilated than SeO_4^{2-} or elemental Se (Se^0) [10, 11]. SeO_3^{2-} could be reduced either by the bound assimilatory SO_4^{2-} reduction pathway [12] by exchange with carrier-S-SO_3^-, or, by free (unbound) SO_3^{2-} reductase. Both these possibilities require that Se is metabolized by processes analogous to those for inorganic S. Alternatively SeO_3^{2-} could be reduced by an independent pathway. The possibility that SeO_3^{2-} is reduced by the bound pathway has not been investigated. However, SeO_3^{2-} is reduced by an NADPH-specific SO_3^{2-} reductase (EC 1.8.1.2) from *Escherichia coli* [13]. The maximum rate of SeO_3^{2-} reduction catalysed by the *E. coli* enzyme was *ca* 10-fold faster than for SO_3^{2-} but the very high K_m value for SeO_3^{2-} (90 mM) suggests that this mechanism is unlikely to be important *in vivo*. In plants, SO_3^{2-} reductase activity is associated with chloroplasts [14, 15] but reduction of SeO_3^{2-} by this enzyme has not been reported.

Hsieh and Ganther [16] have described an alternative pathway for SeO_3^{2-} reduction in yeast. They reported that yeast GSH reductase (EC 1.6.4.2), in the presence of NADPH and GSH, catalyses the reduction of SeO_3^{2-} to Se^{2-}. Since at least some of the GSH reductase activity of plants is associated with chloroplasts [17, 18] and reduction of oxidized GSH (GSSG) is coupled to photosynthetic electron transport [18], this raises the possibility that SeO_3^{2-} could be reduced to Se^{2-} in chloroplasts in a light-dependent reaction involving GSH reductase.

This paper describes a comparative study of light-dependent incorporation of SeO_3^{2-} into selenocysteine and SO_3^{2-} into cysteine by isolated pea chloroplasts. We propose that light-dependent SeO_3^{2-} reduction in chloroplasts proceeds via GSH reductase. Purified pea glutathione reductase, in the presence of *O*AS, GSH,

Table 1. Incorporation of $^{75}SeO_3^{2-}$ into selenocysteine by illuminated chloroplasts

Chloroplasts	Treatment Incubation conditions	Selenocysteine synthesized (nmol/mg Chl per hr) Expt 1	Expt 2	Expt 3	Expt 4
Intact	Complete mixture*	351	356	239	145
	Complete mixture minus light	7	nd†	nd	nd
	Complete mixture minus *O*AS	0	0	0	0
	Complete mixture plus DCMU (2 μM)	25	33	nd	nd
	Complete mixture plus Na_2SO_3 (0.5 mM)	220	231	nd	nd
Sonicated	Complete mixture	14	11	nd	nd
	Complete mixture plus GSH (4 mM)	178	203	nd	nd
	Complete mixture plus GSH (4 mM) and NADPH (1 mM)	323	402	nd	nd
	Complete mixture plus GSH (4 mM) and NADPH (1 mM) minus light	241	378	nd	nd
	Complete mixture plus GSSG (4 mM) and NADPH (1 mM)	nd	219	nd	nd
	Complete mixture plus GSSG (4 mM) and NADPH (1 mM) minus light	nd	200	nd	nd

* Incubation conditions for the complete mixture were as described in Experimental and were performed in the light unless specified otherwise. Chloroplasts, prior to sonication, for expts 1–4 were 82, 86, 73 and 55% intact respectively. The Chl concentrations for expts 1–4 were 146, 146, 224 and 174 μg/ml respectively.

† nd: Not determined.

NADPH and purified clover cysteine synthase also catalysed the incorporation of SeO_3^{2-} into selenocysteine.

RESULTS

Incorporation of $^{75}SeO_3^{2-}$ into selenocysteine by illuminated chloroplasts

When intact illuminated chloroplasts were incubated in the presence of *O*AS and $^{75}SeO_3^{2-}$ and the reaction terminated with *N*-ethylmaleimide (*N*EM), two ^{75}Se-labelled compounds were detected as judged by PC in solvents I and II. The two products, which ran with R_f values of 0.37 and 0.62 in solvent I, were almost invariably synthesized in the ratio 3:1 respectively. The major metabolite (R_f 0.37 in solvent I) was indistinguishable from selenocysteine-*N*EM and its identity was confirmed by paper electrophoresis (PE) in buffers I and II. The minor compound, which was not identified, was apparently uncharged at pH 2.5 as it remained on the starting line during PE in buffer I; it was not selenocystine, Se-methylselenocysteine, selenocystathionine or selenomethionine. The rate of incorporation of SeO_3^{2-} into selenocysteine by intact illuminated chloroplasts varied from 0.15 to 0.55 μmol/mg Chl per hr; the rates appeared to be correlated with the intactness of the chloroplast preparations (Table 1). For a given chloroplast preparation, the rate of SeO_3^{2-} incorporation at 30° was constant for *ca* 60 min and thereafter decreased steadily. Standard assays of selenocysteine synthesis were therefore conducted at 30° for 30 min. The rate of synthesis of the minor ^{75}Se-labelled product was constant for *ca* 2 hr.

Incorporation of SeO_3^{2-} into selenocysteine by intact chloroplasts was dependent on light and *O*AS and was inhibited by 2 μM DCMU and 0.5 mM Na_2SO_3 (92 and 26% inhibition respectively) (Table 1). The rate of selenocysteine synthesis increased with SeO_3^{2-} concentration up to *ca* 0.2 mM; the rate was essentially independent of SeO_3^{2-} concentration from 0.2 to 1 mM (Fig. 1). The effect of SeO_3^{2-} concentration on the synthesis of the minor incorporation product was similar to that for selenocysteine except that the minor product was not detected at 0.04 mM SeO_3^{2-} (Fig. 1).

Illuminated sonicated chloroplasts in the presence of *O*AS did not catalyse incorporation of $^{75}SeO_3^{2-}$ into selenocysteine at appreciable rates. Addition of 4 mM GSH, however, effected a large increase in the rate of $^{75}SeO_3^{2-}$ incorporation (typically 13- to 20-fold) and this was further enhanced by addition of 1 mM NADPH (Table 1). In the presence of GSH and NADPH, sonicated chloroplasts also catalysed SeO_3^{2-} incorporation in the dark and the rate was only 7–17% less than that observed in the light. Oxidized GSH (GSSG) could serve in place of GSH. Like the system containing GSH, SeO_3^{2-} incorporation by sonicated chloroplasts in the presence of 1 mM NADPH and 4 mM GSSG was essentially light-independent (Table 1).

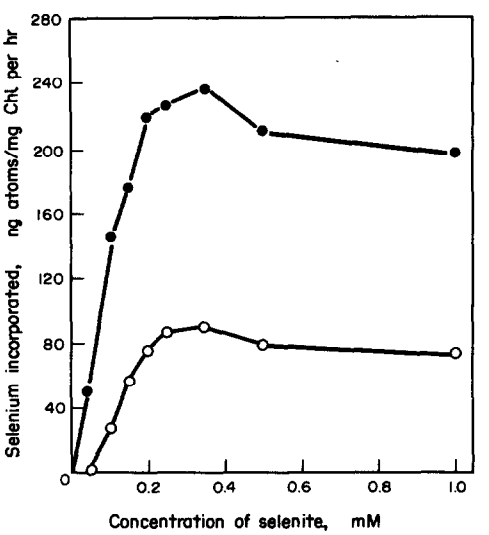

Fig. 1. Effect of concentration of $Na_2{}^{75}SeO_3$ on incorporation of ^{75}Se-label into (●) selenocysteine and (○) an unidentified compound by illuminated intact chloroplasts. Conditions were as described in Experimental except that the concentration of $Na_2{}^{75}SeO_3$ was altered as specified. Details of the unidentified compound are described in the text. Chl concentration, 214 μg/ml; chloroplast intactness, 72%.

Table 2. Reduction of SO_3^{2-} to S^{2-} by illuminated chloroplasts

Treatment		S^{2-} formed (μmol/mg Chl/hr)
Chloroplasts	Incubation mixture	
Intact	Complete mixture*	2.61
	Complete mixture minus light	0.01
	Complete mixture plus DCMU (2 μM)	0.01
	Complete mixture plus Na_2SeO_3 (0.1 mM)	0.09
Sonicated	Complete mixture	0.60
	Complete mixture minus light	0.01
	Complete mixture plus GSH (1 mM)	1.18
	Complete mixture plus GSH (1 mM) and NADPH (0.5 mM)	2.24
	Complete mixture plus GSH (1 mM) and NADH (0.5 mM)	1.02
	Complete mixture plus GSH (1 mM) and NADPH (0.5 mM) minus light	2.18

* Incubation conditions for the complete mixture were as described in Experimental and were conducted in the light. All incubations were performed with 0.25 mM Na_2SO_3 and 130 μg Chl/ml. Chloroplasts, prior to sonication, were 82% intact. The concentration of SO_3^{2-} used for the experiments described in this table was not optimal (see Fig. 2).

Reduction of SO_3^{2-} to S^{2-} by illuminated chloroplasts

In the absence of any other additions, illuminated intact chloroplasts catalysed SO_3^{2-}-dependent synthesis of S^{2-} at optimum rates of 5.5–6.3 μmol/mg Chl per hr.

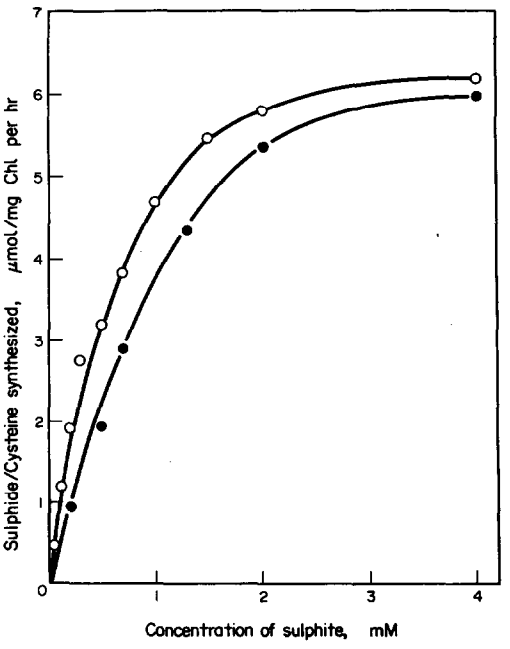

Fig. 2. Effect of concentration of Na_2SO_3 on (○) S^{2-} synthesis (in the absence of *O*AS) and (●) cysteine synthesis (in the presence of *O*AS) by illuminated intact chloroplasts. Conditions were as described in Experimental except that the concentration of Na_2SO_3 was altered as specified. Cysteine synthesis was determined by the colorimetric method. Chl concentration, 132 μg/ml; chloroplast intactness, 85%.

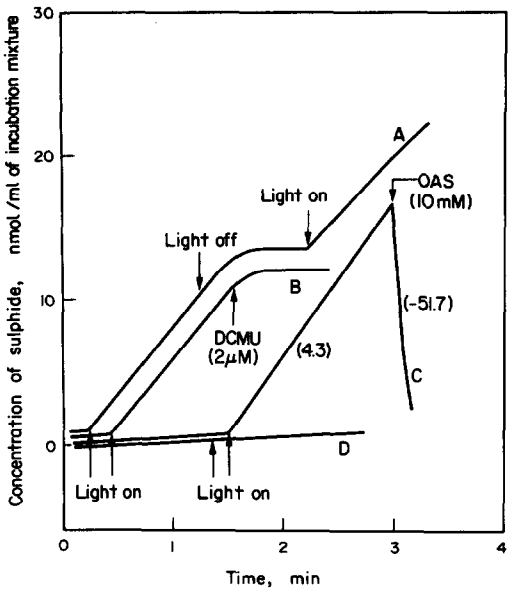

Fig. 3. Effect of *O*AS, DCMU and light on SO_3^{2-}-dependent synthesis of S^{2-} by intact chloroplasts as determined with a sulphide ion electrode. Conditions were as described in Experimental for the determination of SO_3^{2-} reductase in chloroplasts except that S^{2-} concentration was monitored continuously and additions were made as shown. The results of four experiments (A–D) are illustrated: A–C, with 1 mM Na_2SO_3; D, without Na_2SO_3. All experiments were initiated in the dark and illuminated as shown. Values beside the curves represent the rate of S^{2-} production or consumption in μmol/mg Chl per hr. Chl concentration, 130 μg/ml; chloroplast intactness 85%.

The reaction, which could be readily monitored with a sulphide ion electrode, did not occur in the dark nor in the presence of 2 μM DCMU. SeO_3^{2-} (0.1 mM) strongly inhibited the reaction (Table 2). The rate of S^{2-} synthesis increased with SO_3^{2-} concentration up to *ca* 2 mM (Fig. 2); higher concentrations (up to 4 mM) were not inhibitory. A concentration of *ca* 0.56 mM SO_3^{2-} supported $V_{max}/2$. When intact chloroplasts were illuminated in the presence of SO_3^{2-}, and S^{2-} allowed to accumulate, subsequent addition of *O*AS caused a rapid depletion of the accumulated S^{2-} (Fig. 3) implying that the S^{2-} produced could be readily incorporated into cysteine.

Sonicated chloroplasts catalysed SO_3^{2-}-dependent synthesis of S^{2-} in the light at *ca* 23% of the rate of intact chloroplasts (Table 2). The rate was enhanced *ca* 2-fold by 1 mM GSH. Addition of 0.5 mM NADPH further enhanced the rate to values which approximated those for intact chloroplasts (Table 2). NADH would not substitute for NADPH in the reaction.

Incorporation of $^{35}SO_3^{2-}$ into cysteine by illuminated chloroplasts

Intact illuminated chloroplasts, in the presence of *O*AS, catalysed incorporation of $^{35}SO_3^{2-}$ into cysteine at optimum rates of 5.6–6.0 μmol/mg Chl per hr. The formation of free S^{2-}, as measured by the sulphide ion electrode, could not be detected in the presence of *O*AS. The identity of cysteine was confirmed by the procedures described in [3]. Cysteine synthesis (in the presence of *O*AS) increased with the concentration of SO_3^{2-} in much

Table 3. Incorporation of $^{35}SO_3^{2-}$ into cysteine by illuminated chloroplasts

Chloroplasts	Treatment Incubation mixture	Cysteine synthesized (μmol/mg Chl/hr)
Intact	Complete mixture*	3.23
	Complete mixture minus light	0
	Complete mixture minus *O*AS	0
	Complete mixture plus DCMU (2 μM)	0
	Complete mixture plus Na_2SeO_3 (0.5 mM)	1.63
Sonicated	Complete mixture	1.01
	Complete mixture plus GSH (1 mM) and NADPH (0.5 mM)	3.15
	Complete mixture plus GSH (1 mM) and NADPH (0.5 mM) minus light	3.02

* Incubation conditions for the complete mixture were as described for the radiochemical method in Experimental and were conducted in the light. All incubations contained 130 μg Chl/ml. Chloroplasts, prior to sonication, were 86% intact.

the same manner as S^{2-} synthesis by illuminated intact chloroplasts (in the absence of *O*AS) (Fig. 2). Collectively, these observations are consistent with the production of S^{2-} from SO_3^{2-} and incorporation of S^{2-} into cysteine via cysteine synthase. $^{35}SO_3^{2-}$ was not incorporated into cysteine in the dark nor in the absence of *O*AS (Table 3). DCMU (2 μM) and SeO_3^{2-} (0.5 mM) inhibited the reaction (*ca* 100 and 50% inhibition respectively).

Sonicated chloroplasts in the presence of *O*AS also catalysed incorporation of $^{35}SO_3^{2-}$ into cysteine. Like SO_3^{2-}-dependent synthesis of S^{2-} in the absence of OAS, cysteine synthesis by sonicated chloroplasts was enhanced *ca* 3-fold by GSH and NADPH (Table 3).

Effect of $ZnCl_2$ *and* KCN *on incorporation of* SeO_3^{2-} *and* SO_3^{2-} *into selenocysteine and cysteine by illuminated chloroplasts in the presence of* OAS

Incorporation of SO_3^{2-} into cysteine by intact chloroplasts and sonicated chloroplasts (in the presence of GSH and NADPH) was completely inhibited by 1 mM KCN but 1 mM $ZnCl_2$ had no significant effect (Table 4).

Table 5. Typical purification of GSH reductase from pea-shoots

Treatment*	Protein (mg)	GSH reductase (units/mg protein)
Crude extract	2250	0.17
$(NH_4)_2SO_4$ fraction	296	1.96
Acetone (34–48% v/v)	85	2.79
DEAE-cellulose	22.4	12.37

* Purification treatments were as described in Experimental.

Conversely incorporation of SeO_3^{2-} into selenocysteine by intact chloroplasts and sonicated chloroplasts (with GSH and NADPH) was not significantly affected by 1 mM KCN but 1 mM $ZnCl_2$ was inhibitory; the inhibition was greater with sonicated chloroplasts (*ca* 72%) than with intact chloroplasts (*ca* 24%). Since 1 mM $ZnCl_2$ and 1 mM KCN did not inhibit the activity of purified clover cysteine synthase (results not shown), the results imply that $ZnCl_2$ and KCN inhibit the reduction of SeO_3^{2-} and SO_3^{2-} respectively.

Incorporation of SeO_3^{2-} *into selenocysteine in the presence of* GSH *reductase and cysteine synthase*

The simple three-step purification procedure for pea-shoot GSH reductase increased the specific activity *ca* 73-fold (Table 5). Substantial cysteine synthase activity was associated with freshly purified enzyme preparations but was decreased to relatively insignificant levels by storage at −15° for at least 2 weeks in the absence of DTT (see Experimental). The pH optimum for the partially purified enzyme was 8.0 in 95 mM Tris–HCl buffer. The enzyme catalysed reaction was strongly inhibited by $ZnCl_2$; 0.01 mM and 0.1 mM caused 78 and 100% inhibition respectively. $MgCl_2$ was a less effective inhibitor (23% inhibition at 0.1 mM). KCN (1 mM) had no significant effect on enzyme activity (less than 0.5% inhibition).

Hsieh and Ganther [16] reported that yeast GSH reductase, in the presence of NADPH and GSH, catalyses the reduction of SeO_3^{2-} to Se^{2-}. The ability of the purified pea enzyme to catalyse this reaction was examined by flushing a reaction vial (90 × 13 mm) containing $^{75}SeO_3^{2-}$, GSH, NADPH, Tris–HCl buffer pH 8 and enzyme (1 ml) with O_2-free N_2 into a second vial containing 0.1 M $AgNO_3$. Incubations were maintained

Table 4. Effect of $ZnCl_2$ and KCN on incorporation of $^{35}SO_3^{2-}$ and $^{75}SeO_3^{2-}$ into cysteine and selenocysteine by illuminated chloroplasts

Chloroplasts	Treatment Incubation mixture	Cysteine synthesized (μmol/mg Chl/hr)	Selenocysteine synthesized (μmol/mg Chl/hr)
Intact	Complete mixture*	2.41	0.55
	Complete mixture plus $ZnCl_2$ (1 mM)	2.33	0.42
	Complete mixture plus KCN (1 mM)	0	0.53
Sonicated	Complete mixture plus NADPH (1 mM) and GSH (4 mM)	2.25	0.60
	Complete mixture plus NADPH (1 mM), GSH (4 mM) and $ZnCl_2$ (1 mM)	2.32	0.17
	Complete mixture plus NADPH (1 mM), GSH (4 mM) and KCN (1 mM)	0	0.56

* The complete mixture for SeO_3^{2-} incorporation was as described in Experimental. The complete mixture for SO_3^{2-} incorporation was as described for the radiochemical method except that 0.5 μmol $Na_2^{35}SO_3$ was used. All incubations contained 184 μg Chl/ml and were conducted in the light at 30°. Chloroplasts prior to sonication were 94% intact.

at 35° and terminated after 30 min with 1 ml 11 M HCl. Flushing with O_2-free N_2 was continued for a further 10 min. ^{75}Se-Label was not detected in the $AgNO_3$ trap over a wide range of reaction conditions including 0.1–5 mM $Na_2{}^{75}SeO_3$ (up to 2 Ci/mol), 1–20 mM GSH, (GSH:SeO_3^{2-} ratio, 1:5–200:1), 2–10 mM NADPH, 5–50 units GSH reductase and reaction times from 5 to 120 min. Similar experiments to detect the production of $H_2{}^{75}Se$ from $Na_2{}^{75}SeO_3$ by illuminated chloroplasts were also unsuccessful.

The ability of purified pea GSH reductase to reduce SeO_3^{2-} to Se^{2-} in the presence of GSH and NADPH was also examined by monitoring the synthesis of selenocysteine in the presence of *O*AS and purified clover cysteine synthase (hereafter referred to as the coupled enzyme system). This method was used in view of the extreme instability of Se^{2-} which is readily oxidized to Se°. When $^{75}SeO_3^{2-}$ was supplied to the coupled enzyme system and the reaction terminated with NEM, a ^{75}Se-labelled product was detected which had an R_f value identical to that of selenocysteine-*N*EM when subjected to PC in solvent I. The product was confirmed as selenocysteine by PE in systems I and II. Synthesis of selenocysteine did not occur in the absence of *O*AS or GSH but GSSG could be substituted for GSH (Table 6); presumably it was reduced to GSH in the presence of GSH reductase and NADPH. Some selenocysteine was synthesized in the absence of clover cysteine synthase but this could be quantitatively attributed to residual cysteine synthase associated with the GSH reductase preparation. In the absence of GSH reductase, the rate of selenocysteine synthesis was *ca* 30–40% of the complete system. Since GSH reductase was not associated with the purified cysteine synthase preparation, this implies that SeO_3^{2-} was also reduced to Se^{2-} non-enzymically. The GSH reductase-dependent synthesis of selenocysteine was completely inhibited by 0.25 mM $ZnCl_2$ but addition of 1 mM KCN had little effect. Synthesis of cysteine was not detected when $^{35}SO_3^{2-}$ replaced $^{75}SeO_3^{2-}$ in the standard assay.

Synthesis of selenocysteine from SeO_3^{2-} *in the presence of* DTT, *O*AS *and cysteine synthase*

When clover cysteine synthase was incubated in Tris–HCl buffer pH 8 containing 5 mM *O*AS, 0.5 mM Na_2SeO_3 and 3 mM DTT for 20 min at 25°, a product was formed which reacted with Gaitonde reagent-2 [1]. The reaction did not occur in the absence of SeO_3^{2-}, *O*AS, DTT or cysteine synthase, suggesting that the product was selenocysteine [1]. The product was confirmed as selenocysteine by PC in solvents I and II of the *N*EM-derivative of the ^{75}Se-labelled product formed from $^{75}SeO_3^{2-}$. In the presence of 0.2 mM SeO_3^{2-}, the effect of DTT concentration on selenocysteine synthesis was sigmoidal; maximum synthesis occurred at 2–4 mM, none at 0.5 mM or less, and intermediate synthesis from 1 to 2 mM.

Table 6. Incorporation of $^{75}SeO_3^{2-}$ into selenocysteine by the GSH reductase/cysteine synthase coupled enzyme system

Treatment	Selenocysteine synthesized (nmol/min/ml incubation mixture)
Complete mixture*	15.7
Complete mixture minus *O*AS	0
Complete mixture minus GSH	0
Complete mixture minus cysteine synthase	0.3
Complete mixture minus GSH reductase	6.0
Complete mixture minus NADPH	4.8
Complete mixture but with GSSG (2 mM) in place of GSH	8.3
Complete mixture plus $ZnCl_2$ (0.25 mM)	4.5
Complete mixture plus KCN (1 mM)	15.5

* The complete mixture was as described for the coupled enzyme assay in Experimental.

In the absence of cysteine synthase and *O*AS, DTT reduced SeO_3^{2-} to Se°. This was monitored spectrophotometrically at 380 nm [19] in reaction mixtures (1 ml) containing 2 mM DTT and 100 mM Tris–HCl pH 8 at 25°; reactions were initiated with Na_2SeO_3 (0.1–0.5 mM). In the presence of cysteine synthase (2 units) and *O*AS (10 mM), the total amount of Se° produced from 400 nmol SeO_3^{2-} over 10 min was 146 nmol less than in incubation mixtures lacking these additions. Incubations lacking cysteine synthase and *O*AS failed to synthesize selenocysteine, but in the presence of these reagents 115 nmol selenocysteine was synthesized. The inhibition of Se° synthesis and recovery (79%) of Se in selenocysteine implies that Se^{2-} was an intermediate in the synthesis of Se°.

Formation of Se° was also observed in incubation mixtures containing 0.2–2 mM GSH instead of DTT. Cysteine synthase and *O*AS caused a small inhibition (*ca* 15%) of Se° synthesis under these conditions but the theoretical production of selenocysteine was too small to detect by the colorimetric method.

DISCUSSION

Illuminated intact chloroplasts, in the presence of *O*AS, catalysed the synthesis of two ^{75}Se-labelled products from $^{75}SeO_3^{2-}$. The major product was identified by PC and PE as selenocysteine. A more rigorous identification of this compound is described in [1]. The minor product, which was not identified, was presumed to be a product formed either enzymically or non-enzymically from cysteine (e.g. by reaction with cysteine [20]) since it was only detected when selenocysteine was also formed. Furthermore, the rate of synthesis of the minor compound remained constant after the rate of selenocysteine synthesis began to decrease after 60 min (Fig. 1). Accordingly no further studies of the minor product were attempted.

The requirement for *O*AS for selenocysteine synthesis by intact chloroplasts suggests that cysteine synthase is involved and that Se is assimilated as Se^{2-}. This in turn implies that SeO_3^{2-} is first reduced to Se^{2-}. Since the cysteine synthase reaction is light-independent [1, 3], the light-requirement for incorporation of SeO_3^{2-} into selenocysteine and the sensitivity of this reaction to DCMU (Table 1) implies that the reduction of SeO_3^{2-} to Se^{2-} is coupled to photosynthetic electron transport. The maximum rate of selenocysteine synthesis occurred at relatively low concentrations of SeO_3^{2-} (Fig. 1). This suggests that both the SeO_3^{2-} reduction mechanism and the Se^{2-} assimilation mechanism must have relatively high affinities for their respective substrates. This is consistent with the relatively high affinity of cysteine synthase for Se^{2-} [1].

Sonicated chloroplasts, in the presence of GSH, NADPH and *O*AS catalysed the incorporation of SeO_3^{2-} into selenocysteine at rates which approximated those for illuminated intact chloroplasts (Table 1). This reaction, however, was largely independent of light, suggesting that the light reaction was serving to produce GSH and NADPH for use in SeO_3^{2-} reduction.

In the absence of *O*AS, illuminated intact chloroplasts reduced SO_3^{2-} to S^{2-}. This reaction was light-dependent and DCMU sensitive (Table 2) indicating that light-dependent electron transport was involved in SO_3^{2-} reduction. This confirms the previous studies of Trebst and coworkers [7, 21] although the rates we report are *ca* 6-fold greater than those in [21]. In the presence of *O*AS, SO_3^{2-} was incorporated into cysteine and accumulation of S^{2-} was not observed. Cysteine synthesis, like S^{2-} synthesis, was light-dependent and DCMU sensitive. These results suggest that light-dependent SO_3^{2-} reduction is coupled to S^{2-} assimilation into cysteine in the presence of *O*AS. The rapid consumption of S^{2-}, produced from SO_3^{2-}, when *O*AS was supplied (Fig. 3) is consistent with this proposal. The latter experiment (Fig. 3) also demonstrates that the light-independent consumption of S^{2-} by cysteine synthase [3] is much more rapid than the light-dependent reduction of SO_3^{2-} by sulphite reductase. The approximately similar rates of SO_3^{2-} reduction to S^{2-} (without *O*AS) and SO_3^{2-} incorporation into cysteine (with *O*AS) by illuminated chloroplasts at all concentrations of SO_3^{2-} examined (Fig. 2) suggests that the rate of SO_3^{2-} incorporation is a function of the kinetics of the light-coupled SO_3^{2-} reductase. This implies that the affinity of SO_3^{2-} reductase for SO_3^{2-} (K_m estimated at *ca* 0.56 mM) is less than the affinity of cysteine synthase for S^{2-}. The very high activity of cysteine synthase in chloroplasts and the high affinity of the enzyme for S^{2-} reported previously [3] are consistent with this conclusion.

Sonicated chloroplasts catalysed reduction of SO_3^{2-} to S^{2-} at *ca* 23% of the rate catalysed by intact chloroplasts. However, in the presence of NADPH and GSH, the rate approximated that of intact chloroplasts (Table 2) and implies that GSH and NADPH were involved in the reduction of free SO_3^{2-} by SO_3^{2-} reductase. At this stage we are unable to comment on whether GSH serves as a sulphonyl-carrier, as proposed for adenosine phosphosulphate transferase and thiosulphonate reductase [22, 23], or serves some other function. The requirement for NADPH is consistent with some reports that it serves as the reductant for SO_3^{2-} reductase [24, 25]. However, the effect of ferredoxin on SO_3^{2-} reduction [21] was not investigated. The effect of GSH and NADPH on incorporation of SO_3^{2-} into cysteine by sonicated chloroplasts (Table 3) is consistent with their effect on SO_3^{2-} reduction (Table 2).

The effect of KCN and $ZnCl_2$ on the incorporation of SeO_3^{2-} and SO_3^{2-} into selenocysteine and cysteine is relevant to the mechanisms of SeO_3^{2-} and SO_3^{2-} reduction. The complete inhibition of SO_3 incorporation into cysteine by KCN in intact and sonicated chloroplasts (Table 4) is consistent with the sensitivity of the SO_3^{2-} reductases from various sources to KCN [15, 25, 26]. Since KCN does not inhibit photosynthetic electron transport from H_2O to NADP [27, 28] or purified clover cysteine synthase, this implies that KCN specifically inhibited SO_3^{2-} reductase in the systems examined (Table 4). Since incorporation of SeO_3^{2-} into selenocysteine was not significantly affected by KCN this suggests that reduction of SeO_3^{2-} by SO_3^{2-} reductase is unlikely. On the other hand, $ZnCl_2$ inhibited the incorporation of SeO_3^{2-} into selenocysteine but had no significant effect on the incorporation of SO_3^{2-} into cysteine. As $ZnCl_2$ does not significantly inhibit the uncoupled rate of ferricyanide-dependent O_2 evolution by osmotically shocked chloroplasts (P. P. Jablonski, personal communication) or light-dependent incorporation of SO_3^{2-} by intact chloroplasts (Table 4), this demonstrates that $ZnCl_2$ does not inhibit light-dependent electron flow from H_2O to NADP. Since clover cysteine synthase was also insensitive to $ZnCl_2$, then $ZnCl_2$ must specifically inhibit reduction of SeO_3^{2-} to Se^{2-}. The greater inhibition of SeO_3^{2-} incorporation in sonicated chloroplasts relative to intact chloroplasts (Table 4) is presumably a function of entry of $ZnCl_2$ through the outer membranes of intact chloroplasts.

Another line of evidence which suggests that SeO_3^{2-} is reduced by a different mechanism to SO_3^{2-} is the relative effects of NADPH and GSH on the reduction and incorporation of SeO_3^{2-} and SO_3^{2-} by sonicated chloroplasts. The rates of SO_3^{2-} reduction to S^{2-} (without *O*AS) and SO_3^{2-} incorporation (with *O*AS) were enhanced *ca* 3- to 4-fold by GSH and NADPH (Tables 2 and 3) whereas SeO_3^{2-} incorporation was enhanced *ca* 17- to 34-fold (Table 1). This suggests that the roles of GSH and NADPH were different for the two substrates.

If SO_3^{2-} and SeO_3^{2-} reduction proceed by independent mechanisms then, in theory, it should be possible to demonstrate that SeO_3^{2-} does not inhibit the reduction and incorporation of $^{35}SO_3^{2-}$, and that SO_3^{2-} does not interfere with the incorporation of $^{75}SeO_3^{2-}$. The results of these investigations (Tables 1, 2 and 3) are inconsistent with the proposal for independent pathways but the inhibitions observed could be due to the formation of selenotrisulphide [29], which would serve to lower the concentration of SO_3^{2-} and SeO_3^{2-} and thus cause an apparent inhibition of both activities. Further, as discussed by Shrift [9], SeO_3^{2-} and SO_3^{2-} are chemically the most different of the inorganic Se/S analogues and would not be expected to act biologically as a pair of competitive antagonists.

The properties of partially purified pea GSH reductase are consistent with previous reports of the enzyme from plant sources [17, 30–32]. The pea enzyme was highly sensitive to $ZnCl_2$ (78% inhibition at 10 μM) and insensitive to 1 mM KCN. In the presence of GSH, NADPH, *O*AS and clover cysteine synthase, GSH reductase catalysed the incorporation of $^{75}SeO_3^{2-}$ into selenocysteine (Table 6). The enzyme-dependent reaction was inhibited by $ZnCl_2$. These results demonstrate that pea GSH reductase, in the presence of GSH and NADPH, catalyses the reduction of SeO_3^{2-} to Se^{2-} as described for the yeast enzyme [16]. Failure to detect the formation of H_2Se under anaerobic conditions in the absence of *O*AS and cysteine synthase was attributed to the inhibitory effect of enzyme protein on volatilization of H_2Se [16]. Similar comments apply to experiments with illuminated intact chloroplasts without *O*AS which were conducted in the presence of BSA.

The synthesis of selenocysteine by the coupled enzyme system in the absence of GSH reductase could result from non-enzymic reduction by GSH of the GSSeSG and GSSeH intermediates proposed by [16] in the reduction of SeO_3^{2-} to Se^{2-} (Fig. 4). Non-enzymic reduction of

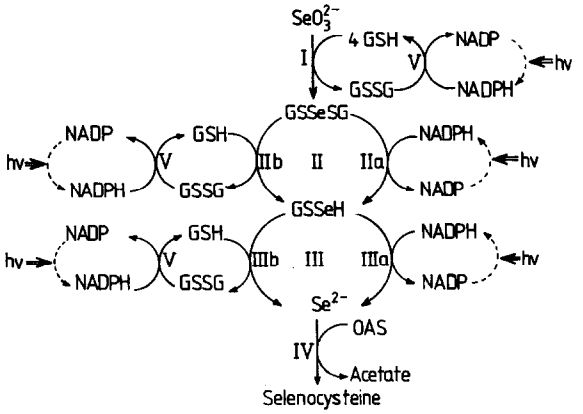

Fig. 4. Proposed pathway for the incorporation of SeO_3^{2-} into selenocysteine by illuminated chloroplasts. Reactions I–III are as proposed by Hsieh and Ganther [16] and reaction IV as in [1]. Reaction I is non-enzymic. Reactions II and III can proceed enzymically in the presence of GSH reductase using NADPH as reductant (IIa and IIIa) or non-enzymically using GSH as reductant (IIb and IIIb). Light-coupled GSH reductase [18] (reaction V) catalyses reduction of GSSG formed in reaction I or by non-enzymic reduction in reactions IIb and IIIb. Light is also required for the production of NADPH for use in the enzyme-catalysed reactions in IIa and IIIa.

SeO_3^{2-} by DTT, a non-physiological thiol, was demonstrated by monitoring Se°. Se^{2-} was shown to be an intermediate in the formation of Se° since cysteine synthase and *O*AS inhibited the production of Se° with the concomitant production of selenocysteine.

We propose that the mechanism shown in Fig. 4 provides one explanation for the light-dependent incorporation of SeO_3^{2-} into selenocysteine in chloroplasts. Although *O*AS has not yet been reported in chloroplasts, all the other conditions required for the scheme occur in this organelle. They include light-coupled reduction of NADP, an internal pool of GSH (estimated at 3.5 mM [33]), cysteine synthase [2, 3] and GSH reductase [17, 18, 32]. The scheme requires that the internal pool of GSSG produced in reaction I be reduced to GSH. This could be accomplished by light-dependent GSH-reductase [18]. The latter reaction, together with the requirement for NADPH for reactions II and III, would explain the requirement for light. Alternatively, if reactions I–III proceed non-enzymically using GSH as reductant, light would be required for the reduction of GSSG by GSH reductase [18]. Since none of the component reactions, including the light reaction, are inhibited by KCN, and since GSH reductase is inhibited by $ZnCl_2$, the inhibition studies (Table 4) are consistent with the proposed scheme.

EXPERIMENTAL

Plant material. Pea seedlings (*Pisum sativum* cv Massey Gem) and white clover (*Trifolium repens*) were raised as in [3].

Chemicals. NADPH and GSH were obtained from Sigma and GSSG from Boehringer. All other chemicals were synthesized or purchased as described in [1, 3].

Chloroplasts were prepared from pea shoots by method B described in [3] and incubations conducted in the appropriate iso-osmotic medium for method B chloroplasts. Sonication and determination of intactness were as in [3].

Cysteine synthase (*O*AS acetate lyase adding H_2S, EC 4.2.99.8) was purified from white clover leaf tissue as in [3] except that all procedures were conducted in the absence of DTT and the enzyme used shortly after purification. Enzyme activity was monitored by the colorimetric method in [3]. Purified enzyme was free from GSH reductase activity and typically exhibited 1.5 units/mg protein.

Purification and assay of pea shoot GSH *reductase.* The procedure described in [3] for the purification of pea shoot cysteine synthase was used. The purified prepn was stored for at least 2 weeks at −15° in the absence of DTT prior to use in the coupled enzyme assay. The cysteine synthase associated with the prepn decreased by *ca* 95% during storage whereas GSH reductase activity decreased by *ca* 5%. Typical activities of GSH reductase and cysteine synthase after storage were 11.8 and 0.39 units/mg protein respectively.

GSH reductase activity was determined by GSSG-dependent oxidation of NADPH at 340 nm. Reaction mixtures (1 ml) containing (in μmol) GSSG (0.3), Tris–HCl buffer pH 8 (95) and GSH reductase were pre-incubated at 25° for 5 min and the reaction initiated with 50 nmol NADPH. Enzyme activity is expressed as μmol NADPH oxidized/min (GSH reductase units); sp. act. is expressed as units/mg of protein.

Assay of SO_3^{2-} *reductase in illuminated chloroplasts.* Enzyme activity was monitored by S^{2-} production using a sulphide ion electrode (Model No. 94–16A, Orion Research Inc., Cambridge, Mass., U.S.A.) coupled to a mV-meter and recorder. Incubation mixtures contained 40 μmol HEPES buffer pH 7.6, Na_2SO_3 (amount specified for each expt) and chloroplasts (Chl and intactness specified for each expt) in 1 ml of the iso-osmotic medium used for resuspending method B chloroplasts [3]. Incubations were conducted at 28° for 10 min in the light (14 mEi/sec/m^2). SO_3^{2-} reductase activity is expressed as μmol S^{2-} produced/mg Chl/hr.

Incorporation of SO_3^{2-} *into cysteine by illuminated chloroplasts.* Two methods were used. *Colorimetric.* Reaction mixtures contained (in μmol) *O*AS (10) Na_2SO_3 (2) HEPES buffer pH 7.6 (45) and chloroplasts (Chl and intactness specified for each expt) in 1 ml of iso-osmotic medium for method-B chloroplasts [3]. Incubations were conducted at 30° for 30 min and illuminated at 14 mEi/sec per m^2. Reactions were terminated with 0.2 ml 1.5 M TCA and precipitated material removed by centrifugation. Cysteine was determined in samples of the supernatant soln by the colorimetric method in [3]. Activity is expressed as μmol cysteine synthesized/mg Chl/hr. *Radiochemical.* Reaction mixtures were as described for the colorimetric method except that 1 μmol $Na_2{}^{35}SO_3$ (1 Ci/mol) was used. Incubation conditions were as described above but reactions were terminated with 0.2 ml hot (50°) EtOH (80%) containing 0.1 M *N*EM. Samples were subjected to PC in solvent I and the dried chromatograms monitored for radioactivity with a gas-flow strip scanner.

Incorporation of SeO_3^{2-} *into selenocysteine by illuminated chloroplasts.* Incubations were conducted at 30° for 30 min and contained (in μmol) HEPES buffer pH 7.6 (45), *O*AS (10), $Na_2{}^{75}SeO_3$ (0.2, 5 Ci/mol) and chloroplasts (Chl and intactness specified for each expt) in 1 ml of iso-osmotic medium for method B chloroplasts [3]. Reaction mixtures were illuminated at 14 mEi/sec per m^2 and terminated with 0.2 ml hot (50°) EtOH (80%) containing 0.1 M *N*EM. Samples were subjected to PC in solvent I and PE in buffer I. Dried chromatograms and electrophoretograms were monitored for ^{75}Se-selenocysteine-*N*EM with a gas-flow scanner and the labelled portions cut out and ^{75}Se-label determined in a Nuclear Chicago analyser at 405 keV.

Incorporation of SeO_3^{2-} *into selenocysteine in the presence of purified pea* GSH *reductase and clover cysteine synthase (coupled enzyme system).* Incubations (1 ml) were conducted under N_2

at 35° for 20 min and contained (in μmol) *O*AS (10), GSH (4), NADPH (2), $Na_2{}^{75}SeO_3$ (0.2), K-Pi buffer pH 7.8 (100), *ca* 5 units of purified pea GSH reductase (stored for at least 2 weeks, containing *ca* 0.04 units cysteine synthase per unit GSH reductase) and *ca* 5 units cysteine synthase (free from GSH reductase). Reactions were terminated with 0.2 ml hot (50°) EtOH (80%) containing 0.1 M *N*EM and thereafter the procedure was as described for SeO_3^{2-} incorporation by chloroplasts. Activity of the coupled system is expressed as nmol selenocysteine synthesized/min/ml.

PC and electrophoresis. PC was conducted on Whatman 3 MM paper using one of the following solvents: (I) *n*-BuOH–HOAc–H_2O (4:1:5), 16 hr [34]; (II) *n*-BuOH–Py–H_2O (1:1:1), 16 hr [4]. Electrophoresis was performed on Whatman 3 MM paper at 4° in one of the following buffer systems: (I) 1 M HOAc buffer, pH 2.5 for 6 hr at 22 V/cm [34]; (II) 0.2 M NaH_2PO_4/0.1 M citrate pH 2.7 for 4 hr at 20 V/cm [4]. Markers were detected with 0.1% ninhydrin in *n*-BuOH.

The colorimetric methods for the estimation of cysteine and selenocysteine were as in [3] and [1] respectively. Protein and Chl were determined as in [3].

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