

Selenium Metabolism in *Neptunia amplexicaulis*¹

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ABSTRACT

ATP sulfurylase (EC 2.7.7.4), cysteinyl-tRNA synthetase (EC 6.1.1.16), and methionyl-tRNA synthetase (EC 6.1.1.10) from *Neptunia amplexicaulis* have been purified approximately 162-, 140- and 185-fold, respectively. Purified ATP sulfurylase in the presence of purified inorganic pyrophosphatase catalyzed the incorporation of sulfate into adenosine 5'-phosphosulfate; evidence of an analogous reaction with selenate is presented. Crude extracts catalyzed both the sulfate- and the adenosine 5'-phosphosulfate-dependent NADH oxidation in the adenosine 5'-phosphosulfate kinase assay of Burnell and Whatley (1977 *Biochim Biophys Acta* 481: 266-278), but an analogous reaction with selenate could not be detected. Both purified cysteinyl-tRNA synthetase and methionyl-tRNA synthetase used selenium-containing analogs as substrates in both the ATP-pyrophosphate exchange and the aminoacylation assays.

It seems that selenium-containing amino acids are excluded from proteins by a mechanism(s) other than substrate discrimination at the amino acid activation stage of protein synthesis.

Administration of selenate to a variety of plants and microorganisms results in the appearance of selenium in various amino acids, peptides, and proteins (19). These studies led to the conclusion that selenate was metabolized via the assimilatory sulfate reduction pathway. This was supported by reports that yeast ATP sulfurylase catalyzed the synthesis of adenosine 5'-phosphoselenate from selenate (13). Shaw and Anderson (32) also reported that spinach leaf ATP sulfurylase catalyzed selenate-dependent ATP-PPi exchange. Further support for the above proposal has been provided by a number of reports that the cysteine synthetase from bacteria (10) and both selenium-accumulating and nonaccumulating plants (22) utilize sulfide and selenide for the synthesis of cysteine and selenocysteine, respectively. Finally, a number of studies with both plants and microorganisms have shown that methionyl-tRNA synthetase (3, 11, 15, 16, 25) and cysteinyl-tRNA synthetase (8, 9, 11, 12, 40) can use the corresponding selenium analogs as substrates.

There is now growing evidence to indicate that there are parts of the assimilatory sulfate reduction pathway from which selenium is excluded. Shaw and Anderson (34) reported that purified ATP sulfurylase from both selenium-accumulators and nonaccumulators could synthesize adenosine 5'-phosphosulfate from ATP and sulfate, but they were unable to synthesize adenosine 5'-phosphoselenate from ATP and selenate. Burnell and Whatley (9) were unable to demonstrate 3'-phosphoadenosine 5'-phosphoselenate synthesis in spinach leaf extracts. And finally, in the sequence of sulfate activation, Burnell *et al.* (J. N. Burnell, W. H. Shaw, and J. W. Anderson, unpublished results) were unable to detect the

incorporation of selenate into sulfolipid in both selenium-accumulators and nonaccumulators, confirming the earlier results of Nissen and Benson (24).

More recently, Ng and Anderson (23) have demonstrated that sulfite and selenite are reduced to sulfide and selenide, respectively, via different pathways.

This paper reports that purified ATP sulfurylase from *Neptunia amplexicaulis* (a selenium-accumulator) catalyzes the synthesis of adenosine 5'-phosphoselenate, that crude extracts catalyze the synthesis of 3'-phosphoadenosine 5'-phosphosulfate but do not catalyze the synthesis of 3'-phosphoadenosine 5'-phosphoselenate, and that purified cysteinyl- and methionyl-tRNA synthetase are unable to distinguish between sulfate-containing substrates and the selenium-containing analogs.

A mechanism for the exclusion of selenium-containing amino acids from incorporation into proteins is proposed, together with a pathway for selenium metabolism in *N. amplexicaulis*.

MATERIALS AND METHODS

PLANT MATERIALS

Seeds of *N. amplexicaulis* *F. richmondii* (family, Mimosaceae) were collected from seleniferous areas of Northwest Queensland, Australia. Seeds were scored with a razor blade to render the hard seed coat permeable to water and germinated on filter paper soaked in water, and the seedlings were raised in a glasshouse. Seeds from mature plants were used as sources of seed tissue.

CHEMICALS

All chemicals were obtained from sources described by Burnell and Anderson (4) and Burnell and Shrift (8).

METHODS

Selenocysteine was prepared by reducing commercially available DL-selenocystine; the purity of selenocysteine was checked by paper chromatography as described previously (8). Homocysteine was prepared by heating 1 eq homocysteine thiolactone-HCl with 2 eq NaOH at 100 C for 3 min (14). tRNA was isolated from *Neptunia* seed meal by the phenol method of Holley *et al.* (17). Both labeled and unlabeled selenate was purified immediately prior to use by the method of Dilworth and Bandurski (13).

Analytical disc polyacrylamide gel electrophoresis was conducted by the method of Shrift *et al.* (35) with 7% acrylamide and stacking gel in Tris-glycine buffer (pH 8.5). The gels were run in a Canalco electrophoresis apparatus at a current of 5 mamp/tube. Proteins were determined by the method of Lowry *et al.* (21) after removing the β -mercaptoethanol by heating (36). Apparent values of K_m and V_{max} , with their associated standard errors, were computed by the method of Wilkinson (39). Appropriate replots of the apparent constants were used to obtain K_m , V_{max} , and K_i values for the various substrates and inhibitors.

The data presented here are calculated from the initial rates of the reactions.

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PURIFICATION OF ATP SULFURYLASE, CYSTEINYL-tRNA SYNTHETASE, AND METHIONYL-tRNA SYNTHETASE

All steps of the purification procedures were conducted at 0 to 4 C and the buffer used throughout the purification contained 100 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 10% (w/v) glycerol, and 25 mM β -mercaptoethanol; the β -mercaptoethanol was added to the buffer solutions immediately prior to use.

PREPARATION OF CRUDE ENZYME EXTRACT AND PRELIMINARY FRACTIONATION

N. amplexicaulis seeds, milled to a powder, were homogenized for 4 min (eight 30-s bursts) in a blender with a tissue to buffer ratio of 1 g to 8 ml. The homogenate was centrifuged at 12,000g for 20 min and the supernatant was treated with protamine sulfate. Protamine sulfate (0.1%, w/v) was added to the crude extract at a ratio of 1 ml protamine sulfate to 5 ml crude extract. The suspension was stirred for 10 min and then centrifuged at 30,000g for 20 min. The supernatant was decanted and the precipitate was discarded. Solid (NH₄)₂SO₄ was added to the supernatant to give 33% saturation. The precipitate was removed by centrifugation at 30,000g and the supernatant was brought to 45% saturation by the addition of (NH₄)₂SO₄. Following centrifugation at 30,000g for 30 min, the supernatant was brought to 70% saturation by the addition of (NH₄)₂SO₄ and the precipitate was recovered by centrifugation. The precipitate was dissolved in a minimal volume of buffer and dialyzed for 18 h.

SEPHADEX G-200 COLUMN CHROMATOGRAPHY

The dialyzed (NH₄)₂SO₄ fraction was applied to a column of Sephadex G-200 (3.5 × 75 cm) equilibrated with buffer and eluted at a flow rate of 0.42 ml/min. Fractions of 5 ml were collected and assayed for ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase. Fractions containing the bulk of the activities were pooled.

DEAE-CELLULOSE (DE52) COLUMN CHROMATOGRAPHY

The pooled G-200 fractions were applied to a column of DEAE-cellulose (DE52) (3.5 × 12 cm) and the column was washed with 150 ml buffer. The enzymes were eluted with a 500-ml linear KCl gradient from 0 to 0.3 M KCl at a flow rate of 0.45 ml/min. Fractions (5 ml) were collected and assayed for ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase activities. Methionyl-tRNA synthetase was separated from ATP sulfurylase and cysteinyl-tRNA synthetase, and the fractions containing methionyl-tRNA synthetase activity were pooled and dialyzed. Those fractions containing ATP sulfurylase and cysteinyl-tRNA synthetase activity were pooled and dialyzed against buffer for 12 h.

HYDROXYAPATITE COLUMN CHROMATOGRAPHY

The dialyzed DE52 fractions containing ATP sulfurylase and cysteinyl-tRNA synthetase activity were applied to a column of hydroxyapatite (2.0 × 10.0 cm) equilibrated with buffer. The enzymes were separated by elution with a linear KCl gradient from 0 to 0.3 M KCl. Those fractions containing only ATP sulfurylase activity were pooled, concentrated by pressure dialysis and dialyzed against buffer for 12 h. Similarly, those fractions containing only cysteinyl-tRNA synthetase activity were pooled, concentrated by pressure dialysis, and dialyzed against buffer for 12 h.

The dialyzed DE52 fractions containing methionyl-tRNA synthetase activity were purified on hydroxyapatite as described for the purification of ATP sulfurylase and cysteinyl-tRNA synthe-

tase. Only those fractions with high specific activity were pooled, concentrated by pressure dialysis, and dialyzed against buffer for 12 h.

ENZYME ASSAYS

Assays of ATP Sulfurylase. In each of the assays the reaction mixture was at pH 8.0 in 0.2 M Tris buffer. In the assays measuring the production of [³²P]ATP, this was identified by paper chromatography (32).

Sulfate-dependent ATP-PPi Exchange. This was conducted as described by Burnell and Roy (6). The reaction mixture contained 4 mM Na₂K₂ATP, 2 mM [³²P]Na₄P₂O₇, and 40 mM K₂SO₄ in buffer. Control reaction mixtures contained H₂O in place of K₂SO₄. The reaction was started by adding 0.15 mg enzyme and stopped by adding 2 ml 7.5% (w/v) trichloroacetic acid. [³²P]ATP was separated from [³²P]PPi by adsorption to charcoal and washing as described by Berg (2) before the radioactivity adsorbed on the charcoal was counted using a Nuclear Enterprises Ltd. end-window planchette counter. ATP sulfurylase activity is expressed as the sulfate-dependent ATP-PPi exchange in nmol/min.

Reverse Reaction: Formation of ATP. The reaction mixture contained 0.2 mM adenosine 5'-phosphosulfate, 0.4 mM [³²P]-Na₄P₂O₇, and 10 mM MgCl₂ in buffer. The reaction was started by adding 30 μ g enzyme and stopped by adding 2 ml 7.5% (w/v) trichloroacetic acid. [³²P]ATP was separated and determined as above. ATP sulfurylase activity is expressed as the APS²-dependent ATP synthesis in μ mol/min.

Forward Reaction: Formation of APS. The reaction mixture contained 4 mM ATP, 10 mM MgCl₂, 40 mM Na₂³⁵SO₄ (5 Ci/mol), and 4 units inorganic pyrophosphatase/100 mM Tris-HCl buffer (pH 8.0). The reaction was started by adding 0.5 mg enzyme and was stopped by adding 4 ml ice-cold 100 mM Tris-HCl buffer (pH 8.0)/100 mM Na₂SO₄. The [³⁵S]adenosine 5'-phosphosulfate was separated from [³⁵S]sulfate by the ethanol precipitation technique of Reuveny and Filner (30), and the radioactivity was measured in a Packard Tri-Carb 3255 scintillation counter, or separated by adsorption to charcoal, and the radioactivity was measured in a planchette counter. Assays were performed in duplicate. ATP sulfurylase activity is expressed as nmol APS synthesized/h.

Selenate-dependent Elemental Selenium Formation. This was conducted as described by Dilworth and Bandurski (13). Reaction mixtures contained 0.2 M Tris-HCl (pH 8.0), 10 mM MgCl₂, 10 mM ATP, 10 mM GSH, 0.2 ml (4%, w/v) CM-cellulose, 10 mM Na₂SeO₄, 2 units purified inorganic pyrophosphatase, and 10 to 30 units ATP sulfurylase in a total volume of 1.0 ml. The reaction was started by the addition of ATP sulfurylase and the A₃₈₀ was continually monitored. A standard curve was constructed as described previously (13).

Selenate-dependent Phosphate Release. Reaction mixtures were as described for measuring selenate-dependent elemental selenium formation. Reactions were run for 30 min and terminated by the addition of 2 ml (10%, w/v) trichloroacetic acid. Phosphate was determined by the method of Anderson and Rowan (1) for measuring phosphate in the presence of a thiol.

Assay of APS Kinase. APS kinase was assayed as described previously (9). The reaction mixture contained 10 μ mol MgCl₂, 0.5 μ mol P-enolpyruvate, 5.0 μ mol ATP, 200 μ mol Tris-HCl (pH 8.0), 10 μ mol NaF, 5 μ mol KCN, 10 units lactate dehydrogenase, 10 units pyruvate kinase, 0.3 μ mol NADH, and 0.1 ml of crude extract in a final volume of 0.95 ml. The reaction was started by the addition of 20 nmol adenosine 5'-phosphosulfate or 40 μ mol sulfate in 0.05 ml. APS kinase activity is expressed as the adenosine 5'-phosphosulfate-dependent oxidation of NADH in μ mol/h.

Cysteinyl- and Methionyl-tRNA Synthetases.

² Abbreviation: APS kinase, adenosine 5'-phosphosulfate kinase.

ATP-PPi Exchange. Cysteinyl- and methionyl-tRNA synthetases were assayed by cysteine- and methionine-dependent ATP-PPi exchange, respectively, as described previously (8). Reaction mixtures contained, in a total of 1 ml, 100 μmol Tris-HCl (pH 8.0), 10 μmol MgCl_2 , 4 μmol $\text{Na}_2\text{K}_2\text{ATP}$ (disodium salt with KOH to pH 8.0), 2.0 μmol [^{32}P]PPi (0.25 Ci/mol), 5 μmol DTT, 0.1 μmol L-cysteine (or 10 μmol L-methionine), and a limiting amount of enzyme; control reactions contained no L-cysteine or L-methionine. Reactions were run for 20 min at 37 C and were terminated by the addition of 2 ml 7.5% (w/v) trichloroacetic acid. [^{32}P]ATP was separated and determined as above. Enzyme activity is expressed as the difference in the ATP-PPi exchange rates determined for assay mixtures with and without added amino acid substrates, i.e. L-cysteine (or L-methionine)-dependent ATP-PPi exchange.

Aminoacylation of tRNA. The aminoacylation of tRNA by methionine and cysteine was assayed as described previously (3).

Assay of Inorganic Pyrophosphatase, ATPase, and 3'-Nucleotidase. Inorganic pyrophosphatase and ATPase were measured by the methods of Shaw and Anderson (31). 3'-Nucleotidase was measured by the method of Burnell and Anderson (4).

RESULTS

Purification of ATP Sulfurylase, Cysteinyl-tRNA Synthetase, and Methionyl-tRNA Synthetase. Crude extracts of *N. amplexicaulis* leaf tissue contained low amounts of ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase activity; partial purification of the crude extract did not increase enzyme activity. Since *N. amplexicaulis* seeds contained about 400 times the activity of both amino acid-activating enzymes and about 250 times the activity of ATP sulfurylase, compared with leaf tissue from fresh leaves of growing plants, all three enzymes were purified from seed material.

The purification procedure for the three enzymes completely removed both alkaline pyrophosphatase and ATPase activities; these enzymes interfere with the determination of enzymes which catalyze substrate-dependent ATP-PPi exchange (32).

Methionyl-tRNA synthetase was separated from ATP sulfurylase and cysteinyl-tRNA synthetase by ion-exchange chromatography on DEAE-cellulose, and ATP sulfurylase was separated from cysteinyl-tRNA synthetase by ion-exchange chromatography on hydroxyapatite.

ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase were purified 162-, 140-, and 185-fold, respectively (Table I). All three enzymes were separated from pyrophosphatase, ATPase, APS kinase, and ADP sulfurylase activities and were free of cross-contamination.

None of the other amino acids commonly found in proteins could replace cysteine or methionine as substrates of cysteinyl- or methionyl-tRNA synthetase, respectively, indicating that the purified enzymes were free of the other aminoacyl-tRNA synthetases. Rechromatography of the purified enzymes on Sephadex G-200 gave single, sharp, symmetrical peaks of enzyme activity with constant specific activity across the peaks. Electrophoresis in polyacrylamide gel gave single protein bands for ATP sulfurylase and cysteinyl-tRNA synthetase and one major and one minor band for methionyl-tRNA synthetase.

Properties. Both cysteinyl- and methionyl-tRNA synthetases were unstable in the absence of either a sulfhydryl-group reducing agent or glycerol; both enzymes were inactivated by extensive dialysis. Both purified ATP sulfurylase and purified cysteinyl-tRNA synthetase were stable for at least 6 months at -20 C , whereas purified methionyl-tRNA synthetase lost about 30% of its activity in 3 months at -20 C .

The kinetic properties of the enzymes were determined from ATP- ^{32}P PPi exchange data with the use of purified enzymes. Sulfate-, cysteine-, and methionine-dependent ATP-PPi exchange

was proportional to enzyme concentration and was linear with time for at least 40 min at 37 C. When a series of buffers was used to study the pH optimum of the enzymes, the pH optimum of ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase was between 7.0 and 9.5, 7.0 and 8.5, and 7.8 and 8.5, respectively.

The cysteinyl-tRNA synthetase purified from *N. amplexicaulis* seeds was similar in many of its properties to the enzyme from mung bean and *Astragalus* seeds; the substrate specificity, pH optimum, sensitivity to inhibitors, and kinetic constants were similar to those reported previously (8, 9). The K_m for ATP was calculated to be 1.11 mM; this value is of the same order of magnitude as the K_m for the cysteinyl-tRNA synthetase from other sources (8, 11, 18).

The methionyl-tRNA synthetase purified from *N. amplexicaulis* seeds was also similar in many of its properties to the enzyme from other sources; the substrate specificity, pH optimum, and sulfhydryl-group reagent sensitivity were similar to those reported previously (11, 12, 15). The K_m for ATP was calculated to be 1.18 mM; this value is of the same order of magnitude as the value obtained for the methionyl-tRNA synthetase from *Sarcina lutea* (15) but is higher than the K_m value obtained for *Paracoccus denitrificans* (11), lupin seeds (20), and rat liver (12).

Effect of Inhibitors. To facilitate the study of the effect of sulfhydryl-group reagents on cysteinyl-tRNA synthetase, the analog α -aminobutyric acid was used as substrate as it lacks the sulfhydryl group and does not react directly with the inhibitor.

ATP sulfurylase was virtually insensitive to sulfhydryl-group reagents: *p*-chloromercuribenzoate (1–10 μM), *N*-ethylmaleimide (0.1–5.0 mM), and iodoacetamide (0.1–5.0 mM) caused less than 10% inhibition. In contrast, ATP sulfurylase was inhibited by phenylhydrazine. Both cysteinyl- and methionyl-tRNA synthetases were sulfhydryl-group reagent-sensitive and carbonyl-group reagent-insensitive. All three enzymes were virtually inactive in the absence of Mg^{2+} . Unlike the ATP sulfurylase from spinach, the ATP sulfurylase from *N. amplexicaulis* was not inhibited by high concentrations of Mg^{2+} when assayed by the reverse reaction.

The ATP-PPi exchange reaction catalyzed by purified ATP sulfurylase was inhibited by chlorate, nitrate, AMP, and ADP; K_i values are summarized in Table II. The kinetics of the inhibition of sulfate-dependent ATP-PPi exchange by ClO_3^- , NO_3^- , AMP, and ADP was studied in factorial experiments with sulfate and ATP. The inhibition by ClO_3^- and NO_3^- was competitive with respect to sulfate and uncompetitive with respect to ATP. The inhibition of ATP-PPi exchange by AMP and ADP was competitive with respect to ATP and noncompetitive with respect to sulfate. Using the ATP-PPi exchange assay ATP sulfurylase activity was not affected by the PPi concentration between 0.05 and 4.0 mM, i.e. ATP-PPi exchange is independent of PPi concentration.

Reverse Reaction. The effect of concentration of APS (0.005–0.2 mM) and PPi (0.004–0.4 mM) on the activity of the reverse reaction catalyzed by ATP sulfurylase was studied in a factorial experiment in the presence of 10 mM MgCl_2 . The slopes and the intercepts of double-reciprocal plots of activity against concentration of APS were dependent on the concentration of PPi. The slopes and intercepts of double-reciprocal plots of activity against concentration of PPi were dependent on the concentration of APS. These studies indicated that the reaction mechanism for the reverse reaction was sequential.

Substrate Specificity. A number of substances structurally and physiologically related to sulfate, cysteine, and methionine were tested to see if they could serve as substrates for ATP sulfurylase, cysteinyl-tRNA synthetase, and methionyl-tRNA synthetase, respectively, using the ATP-PPi exchange assay.

ATP Sulfurylase. Purified ATP sulfurylase catalyzed selenate-dependent ATP-PPi exchange; the K_m (selenate) was 4-fold less

Table I. Purification of ATP Sulfurylase, Cysteinyl-tRNA Synthetase, and Methionyl-tRNA Synthetase from *N. amplexicaulis*All three enzymes were purified from 40 g of *N. amplexicaulis* seeds

Fraction	Total Protein	Specific Activity	Total Enzyme Activity	Recovery	Purification
	mg	nmol min ⁻¹ mg ⁻¹ protein	units	%	-fold
ATP sulfurylase					
Crude extract	12,426	3.4	42,248	100	1
(NH ₄) ₂ SO ₄ fractionation	780	19.7	15,366	36.4	5.8
Sephadex G-200	275	38.5	10,587	25.1	11.3
DE52	42	121.3	5,095	12.1	35.7
Hydroxyapatite	3.8	550.8	2,093	5.0	162.0
Cysteinyl-tRNA synthetase					
Crude extract	12,426	2.4	29,822	100	1
(NH ₄) ₂ SO ₄	780	15.3	11,934	40.0	6.4
Sephadex G-200	192	57.2	10,982	36.8	23.8
DE52	31	121.3	3,760	12.6	50.5
Hydroxyapatite	2.4	336.0	806	2.7	140.2
Methionyl-tRNA synthetase					
Crude extract	12,426	1.3	16,154	100	1
(NH ₄) ₂ SO ₄	780	9.2	7,176	44.4	7.1
Sephadex G-200	215	22.8	4,902	30.3	17.5
DE52	27	52.3	1,412	8.7	40.2
Hydroxyapatite	1.9	240.5	457	2.8	185.1

Table II. Kinetic Constants for ATP Sulfurylase of *N. amplexicaulis*

ATP sulfurylase was assayed by either the ATP-PPi exchange method or by the reverse reaction, APS-dependent synthesis of ATP, as described under "Materials and Methods."

Substrate or Inhibitor	K_m	K_i	Reaction
	mM		
SO ₄ ²⁻	25		Exchange
ATP	0.87		Exchange
PPi	0.037		Reverse
APS	0.013		Reverse
SeO ₄ ²⁻	6.06	3.81	Exchange
AMP		2.92	Exchange
ADP		1.57	Exchange
NO ₃ ⁻		3.50	Exchange
ClO ₃ ⁻		0.24	Exchange

than the K_m (sulfate) but the V_{max} (selenate) was 3-fold less than the V_{max} (sulfate). The enzyme kinetics of the effect of selenate on sulfate-dependent ATP-PPi exchange was consistent with both substrates competing for a single site (29) (Fig. 1). Using the forward reaction assay, ATP sulfurylase, in the presence of purified inorganic pyrophosphatase and Mg²⁺, catalyzed the synthesis of [³⁵S]adenosine 5'-phosphosulfate from ATP and [³⁵S]sulfate; [³⁵S]adenosine 5'-phosphosulfate was the only ³⁵S-labeled compound formed in the assay as determined by paper chromatography. Synthesis of [³⁵S]adenosine 5'-phosphosulfate could not be detected in the absence of any of the assay components. Addition of selenate to reaction mixtures used to study the synthesis of [³⁵S]adenosine 5'-phosphosulfate inhibited the rate at which [³⁵S]sulfate was incorporated into [³⁵S]adenosine 5'-phosphosulfate. A series of factorial experiments varying the selenate concentration and the [³⁵S]sulfate concentration indicated that the inhibition was competitive. When [³⁵S]sulfate was replaced with [⁷⁵Se]selenate in the assay of the forward reaction, a ⁷⁵Se-labeled compound was formed which was adsorbed to charcoal; the formation of this compound was dependent on the presence of all components of the assay (Table III). Attempts to elute the ⁷⁵Se-

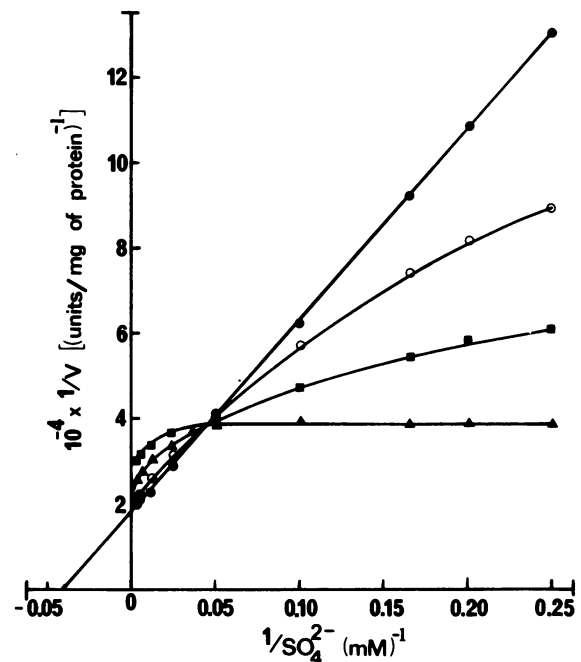


FIG. 1. Double-reciprocal plot of the effect of sulfate concentration on ATP sulfurylase activity in the presence and absence of selenate. ATP sulfurylase activity was measured by ATP-PPi exchange and reaction mixtures were as described under "Materials and Methods" except that selenate was added at the concentrations specified. (●), no selenate; (○), 5 mM selenate; (■), 20 mM selenate; (▲), 40 mM selenate.

labeled compound and identify it by paper chromatography were unsuccessful. Addition of sulfate to the reaction mixtures caused a decrease in the radioactivity adsorbed to the charcoal (Table III).

Purified ATP sulfurylase from *N. amplexicaulis* also catalyzed the ATP sulfurylase-dependent formation of elemental selenium in the assay of Dilworth and Bandurski (13). Unlike the formation of a ⁷⁵Se-labeled charcoal absorbable compound, the formation of

Table III. Effects of Omitting Components of Reaction Mixture and Adding Sulfate to Reaction Mixtures on Synthesis of a Charcoal-adsorbable ^{76}Se -labeled Compound

Reaction mixtures contained 38 units ATP sulfurylase, 5 units purified inorganic pyrophosphatase, 10 mM ATP, 10 mM MgCl_2 , 10 mM ^{76}Se -selenate ($4.8 \mu\text{mol}/\mu\text{Ci}$) and 0.2 M Tris-HCl (pH 8.0) in a total of volume of 0.5 ml. Reactions were run for 45 min at 37 C and terminated by heating in a boiling water bath for 60 s. Following the addition of 1 ml of a charcoal suspension [25 mg/ml Norit A charcoal, 100 mM sodium selenate and 50 mM acetate buffer (pH 4.0)] reaction mixtures were filtered and washed with a solution of 100 mM sodium selenate in 50 mM acetate buffer (pH 4.0), and the radioactivity adsorbed onto the charcoal was measured with an end-window planchette counter.

Reaction Mixtures	Synthesis in Experiments		
	1	2	3
	<i>cpm</i>		
Complete	4973	3892	4467
Complete minus ATP	409	373	366
Complete minus Mg^{2+}	432	511	478
Complete minus inorganic pyrophosphatase	497	549	461
Complete minus ATP sulfurylase	362	419	385
Complete plus 10 mM sulfate	913		1098
Complete plus 100 mM sulfate	564		473

Table IV. Effect of Reaction Components on Production of Elemental Selenium by ATP Sulfurylase

Reaction mixtures were as described under "Materials and Methods" except for the exclusion of the components stated.

Reaction Mixture	Rate of Formation of Elemental Selenium in Experiments	
	1	2
	$\mu\text{mol}/\text{h}$	
Complete	0.64	0.73
Complete minus pyrophosphatase	0.19	0.21
Complete minus ATP	0	0
Complete minus Mg^{2+}	0	0
Complete minus selenate	0	0
Complete minus ATP sulfurylase	0	0

elemental selenium was not dependent on the presence of inorganic pyrophosphatase. However, the presence of inorganic pyrophosphatase did increase the rate of formation of elemental selenium (Table IV). All other reaction components were essential for the formation of elemental selenium.

The *N. amplexicaulis* ATP sulfurylase also catalyzed the selenate-dependent release of phosphate from ATP; this required the presence of all reaction components. Experiments conducted to determine the stoichiometry of the reaction gave an average ratio of 1.98 ± 0.11 mol phosphate/mol elemental selenium formed (Fig. 2). Addition of increasing concentrations of sulfate to reaction mixtures decreased both the elemental selenium formed and the release of phosphate (Fig. 3).

When ^{35}S sulfate was replaced by ^{76}Se selenate in the forward reaction assay and the end products of the reaction were separated by the ethanol precipitation method of Reuveny and Filner (30), no radioactivity was detected in the ethanol-soluble fraction.

APS Kinase. When APS kinase activity was measured in crude enzyme preparations using the APS-dependent NADH oxidation method of Burnell and Whatley (9), NADH was oxidized at a rate of 6.8 nmol/min·ml crude extract; NADH was oxidized at a rate of 2.7 nmol/min·ml crude extract when APS was replaced with

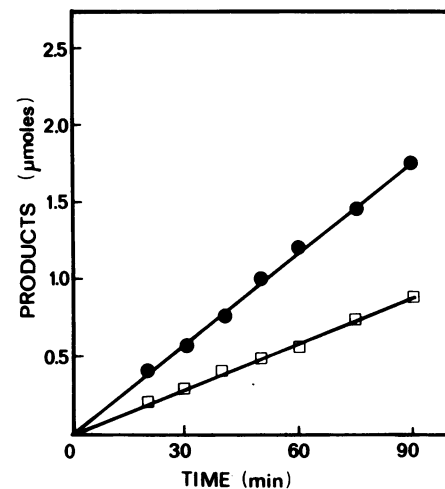


FIG. 2. Enzymic production of elemental selenium and phosphate as a function of time. (●), phosphate formed; (□), elemental selenium formed.

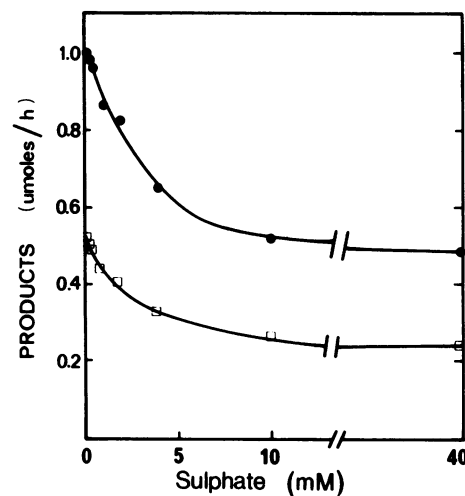


FIG. 3. Effect of sulfate concentration on the production of phosphate and elemental selenium. Reaction mixtures were as described under "Materials and Methods" except that sulfate was added at the concentration specified. (●), phosphate; (□), elemental selenium.

sulfate. No selenate-dependent NADH oxidation was detected when APS was replaced with selenate (2–40 mM). Extensive dialysis of the crude enzyme preparation to remove endogenous thiols did not affect the rate of sulfate- or adenosine 5'-phosphosulfate-dependent NADH oxidation. No selenate-dependent NADH oxidation was detected in assays containing dialyzed crude enzyme.

Cysteinyl- and Methionyl-tRNA Synthetase. Only a few compounds were alternative substrates for *N. amplexicaulis* cysteinyl-tRNA synthetase. In the presence of DTT, *S*-ethyl cysteine, α -aminobutyric acid, and Se-methylselenocysteine were 50% active or less. Considering the effect of Se-methylselenocysteine and DTT previously reported (5), DTT was removed from enzyme preparations by dialysis; in the absence of DTT, Se-methylselenocysteine did not serve as a substrate for cysteinyl-tRNA synthetase (Table V). L-Selenocysteine, however, was fully active. The K_m of the enzyme for selenocysteine was about 3-fold greater than for cysteine and the V_{max} (selenocysteine) was approximately the same as the V_{max} (cysteine). The K_m of the enzyme for α -aminobutyric acid was 12.5 mM and the V_{max} (α -aminobutyric acid) was approximately 15% of the V_{max} (cysteine).

A large number of compounds acted as alternative substrates for methionyl-tRNA synthetase from *N. amplexicaulis* (Table VI).

Table V. Substrate Specificity of Cysteinyl-tRNA Synthetase from *N. amplexicaulis*

The substrate specificity of cysteinyl-tRNA synthetase was determined using the ATP-PPi exchange assay as described under "Materials and Methods" except that cysteine was replaced by alternative substrates at the concentrations shown in the table.

Substrate	Structure	Activity	
		+DTT	-DTT
		% control	
L-Cysteine, 1 mM	HS-CH ₂ -CHNH ₂ -COOH	100	100
L-Selenocysteine, 10 mM	HSe-CH ₂ -CHNH ₂ -COOH	110.6	n.d. ^a
α -L-Aminobutyric acid, 10 mM	CH ₃ -CH ₂ -CHNH ₂ -COOH	15.6	15.9
S-Ethyl-L-cysteine, 10 mM	CH ₃ CH ₂ S-CH ₂ -CHNH ₂ -COOH	10.6	10.8
Se-methyl-L-selenocysteine, 10 mM	CH ₃ -Se-CH ₂ -CHNH ₂ -COOH	49.8	0
Cysteine sulfinic acid, 10 mM	HO ₂ S-CH ₂ -CHNH ₂ -COOH	0	0
Cysteic acid, 10 mM	HO ₃ S-CH ₂ -CHNH ₂ -COOH	0	0
S-Methylcysteine sulfoxide, 10 mM	CH ₃ -SO-CH ₂ -CHNH ₂ -COOH	0	0
D-Cysteine, 10 mM	HS-CH ₂ -CHNH ₂ -COOH	0	0

^a not determined.

Table VI. Substrate Specificity of Methionyl-tRNA Synthetase from *N. amplexicaulis*

The substrate specificity of methionyl-tRNA synthetase was determined using the ATP-PPi exchange assay. Reaction mixtures were as described under "Materials and Methods" except that methionine was replaced by alternative substrates at a final concentration of 10 mM.

Substrate	Structure	K_m	Relative
			V_{max}
		mm	%
Methionine	CH ₃ -S-CH ₂ -CH ₂ -CHNH ₂ -COOH	0.167	100
Selenomethionine	CH ₃ -Se-CH ₂ -CH ₂ -CHNH ₂ -COOH	1.56	122
L-Homocysteine	HS-CH ₂ -CH-CHNH ₂ -COOH	5.55	84
S-Methyl cysteine sulfoxide	CH ₃ -SO-CH ₂ -CHNH ₂ -COOH	n.d. ^a	34
L-Norleucine	CH ₃ -CH ₂ -CH ₂ -CH ₂ -CHNH ₂ -COOH	11.11	12
α -Methyl-L-methionine	CH ₃ -S-CH ₂ -CH ₂ -C(CH ₃)NH ₂ -COOH	1.0	11
Seleno-DL-ethionine	CH ₃ -CH ₂ -Se-CH ₂ -CH ₂ -CHNH ₂ -COOH	n.d.	8.4
L-Cystathionine	HOOC-H ₂ NHC-CH ₂ -CH ₂ -S-CH ₂ -CHNH ₂ -COOH	n.d.	3.4
S-Ethyl-cysteine	CH ₃ -CH ₂ -S-CH ₂ -CHNH ₂ -COOH	n.d.	0
L-Methionine sulfone	CH ₃ -SO ₂ -CH ₂ -CHNH ₂ -COOH	n.d.	0
L-Methionine sulfoxide	CH ₃ -SO-CH ₂ -CH ₂ -CHNH ₂ -COOH	n.d.	0

^a not determined.

The V_{max} (selenomethionine) was significantly higher than the V_{max} (methionine) but the K_m methionyl-tRNA synthetase for methionine was 1 order of magnitude lower than the K_m of the enzyme for selenomethionine and almost every other alternative substrate. The ³²P-labeled products synthesized by the purified methionyl-tRNA synthetase in assays containing either methionine or any of the alternative substrates were identified by paper chromatography as [³²P]ATP. The ATP-PPi exchange of standard incubation mixtures containing methionine catalyzed by methionyl-tRNA synthetase was inhibited by the addition of selenomethionine, norleucine, selenoethionine, and homocysteine. The kinetics of the inhibition of ATP-PPi exchange by the alternative substrates was similar to that described for mung bean seed cysteinyl-tRNA synthetase (7) and was consistent with the kinetics of two substrates competing for one enzyme (29). The purified methionyl-tRNA synthetase catalyzed the synthesis of both [³H]methionyl-tRNA and [⁷⁶Se]selenomethionyl-tRNA. The formation of [³H]methionyl-tRNA was inhibited by the addition of selenomethionine to reaction mixtures and the formation of [⁷⁶Se]selenomethionyl-tRNA was inhibited by the addition of methionine to reaction mixtures (Figs. 4 and 5). A molar ratio of 1:1 of selenomethionine to methionine reduced the formation of methionyl-tRNA by 50%, a molar ratio of 1:1 of selenomethionine to methionine also reduced the formation of [⁷⁶Se]selenomethionyl-tRNA by 50% (Figs. 4 and 5).

Purified cysteinyl-tRNA synthetase catalyzed the synthesis of [³H]cysteinyl-tRNA; addition of selenocysteine to reaction mixtures reduced the rate of formation of [³H]cysteinyl-tRNA. Figure 6 indicates that an inhibition of 50% was found at a molar ratio of 15:1 (selenocysteine to cysteine).

High background counts associated with proteins have so far prevented the study of the inhibition by cysteine of [⁷⁶Se]selenocysteine charging of tRNA.

DISCUSSION

Early reports on nutrition experiments involving feeding selenium compounds to plants indicated that selenate is the preferred form of selenium taken up by the roots (37). More recently, Ng and Anderson (23) have reported that plant extracts catalyze the reduction of selenite to selenide in a light-dependent reaction which is distinguishable from sulfite reduction. The question now remains as to the mechanism of selenate reduction to selenite.

A recent report of the formation catalyzed by purified yeast ATP sulfurylase, of an "active selenate" with properties similar to adenosine 5'-phosphosulfate (13), prompted a reinvestigation of the enzymic properties of plant ATP sulfurylase with respect to selenate. Dilworth and Bandurski (13) proposed a mechanism for the ATP sulfurylase-catalyzed conversion of selenate to selenite which is based upon the thiolitic cleavage of adenosine 5'-phosphoselenate by glutathione, a compound found in tissues in con-

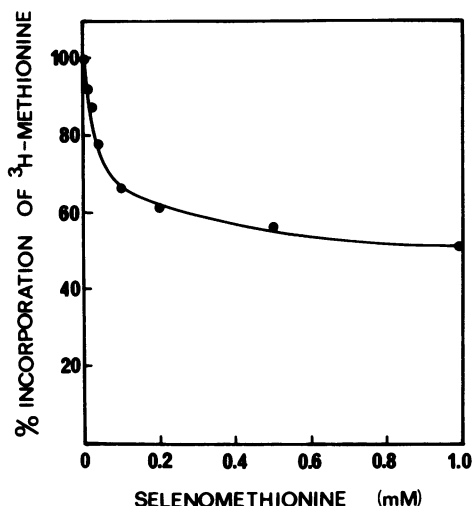


FIG. 4. Per cent aminoacylation of tRNA by methionine in the presence of varying concentrations of selenomethionine. Reaction mixtures were as described under "Materials and Methods" except that selenomethionine was added at the concentrations specified. The activity of the purified *Neptunia* methionyl-tRNA synthetase used in this experiment and assayed in the absence of selenomethionine was 19.6 nmol methionyl-tRNA formed/h.

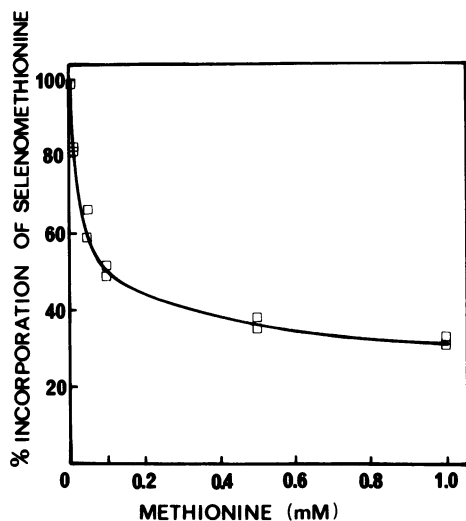


FIG. 5. Per cent aminoacylation of tRNA by selenomethionine in the presence of varying concentrations of methionine. Reaction mixtures were as described under "Materials and Methods" except that [³H]methionine was replaced with 0.1 mM [⁷⁵Se]selenomethionine and methionine was added at the concentrations specified. The activity of the methionyl-tRNA synthetase was 21.2 nmol selenomethionine-tRNA formed/h.

centrations adequate for the proposed mechanism.

The ATP sulfurylase purified from *Neptunia* seeds was similar in many of its properties to the enzyme purified from spinach leaf tissue (32, 33) and from *Astragalus* leaf tissue (34). The pH optimum, sensitivity to inhibitors, the reaction mechanism, and the substrate specificity were similar. The ATP sulfurylase from *Neptunia* catalyzed both sulfate- and selenate-dependent ATP-PPi exchange, was inhibited by ClO₃⁻, NO₃⁻, ADP, and AMP, and the inhibition patterns were quantitatively similar to those reported for spinach-leaf ATP sulfurylase (32, 33). In contrast, however, the K_m values of the enzyme for both sulfate and selenate were almost 10 times higher than those for the ATP sulfurylase from spinach and *Astragalus* leaf tissue, although the K_m (selenate)/ K_m (sulfate) and the V_{max} (selenate)/ V_{max} (sulfate) ratios were

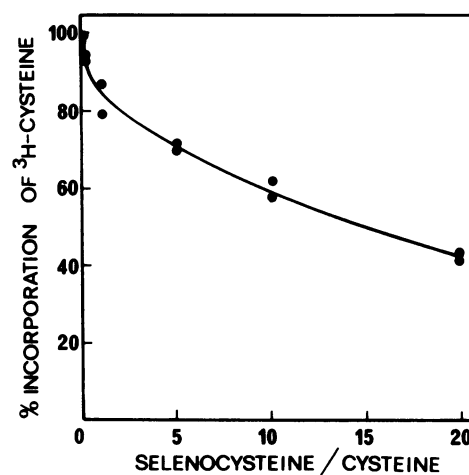


FIG. 6. Selenocysteine inhibition of aminoacylation of tRNA by cysteine. Reaction mixtures were as described under "Materials and Methods" except that selenocysteine was added at the concentrations specified. The activity of the cysteinyl-tRNA synthetase was 14.3 nmol cysteinyl-tRNA formed/h.

approximately the same.

The results indicate that the ATP sulfurylase from *Neptunia* also has an ordered reaction mechanism and that ATP is the first substrate to react with the enzyme and that APS is the last product released in the reaction. The kinetics of sulfate/selenate competition studies not only demonstrated that sulfate and selenate compete for the same enzyme but that they also compete for the same site on the enzyme. These results suggested that, under suitable experimental conditions, *N. amplexicaulis* ATP sulfurylase catalyzes the synthesis of adenosine 5'-phosphoselenate.

Results from a number of experiments provide evidence to support the formation of adenosine 5'-phosphoselenate from ATP and selenate in the presence of purified ATP sulfurylase: (a) competitive inhibition by selenate of the formation of [³⁵S]adenosine 5'-phosphosulfate in the forward reaction assay; (b) the detection of the formation of a charcoal-adsorbable ⁷⁵Se-labeled compound in the forward reaction assay when [³⁵S]sulfate was replaced by [⁷⁵Se]selenate (Table III); (c) the ATP sulfurylase-dependent reduction of selenate to elemental selenium in the presence of glutathione (Table IV); (d) the selenate-dependent release of Pi from ATP in the spectrophotometric assay of the formation of elemental selenium with a stoichiometric ratio of 2 mol Pi/mol selenium formed (Fig. 2); (e) competitive inhibition by sulfate of the formation of elemental selenium and Pi in the spectrophotometric assay of the formation of elemental selenium (Fig. 3).

No accumulation of adenosine 5'-phosphoselenate could be detected using paper chromatographic separation of reaction products when [⁷⁵Se]selenate was substituted for [³⁵S]sulfate in the forward reaction assay. This may have been due to the instability of the selenophosphate anhydride for the chromatograms were run at 25 C for 16 h. Furthermore, no ethanol-soluble ⁷⁵Se-labeled nucleotide product was detected in the forward reaction assay; this may be due to a difference in ethanol solubility between adenosine 5'-phosphosulfate and adenosine 5'-phosphoselenate.

Although sulfate- and adenosine 5'-phosphosulfate-dependent NADH oxidation was observed using crude extracts in the APS-kinase assay of Burnell and Whatley (9), no selenate-dependent NADH oxidation was detected. The apparent inability of APS kinase to use adenosine 5'-phosphoselenate as a substrate may be due to either the substrate discrimination by APS kinase or the instability of adenosine 5'-phosphoselenate. The methionyl-tRNA synthetase purified from *Neptunia* seeds was similar to the enzyme from mung bean seeds in its ability to catalyze the formation of

[^{76}Se]selenomethionyl-tRNA (3). The enzyme catalyzed the formation of selenomethionyl-tRNA at a rate similar to the rate of formation of methionyl-tRNA and the competitive inhibition patterns shown with methionine and selenomethionine in the aminoacylation of tRNA experiments (Figs. 4 and 5) indicate that selenomethionine is as good a substrate as methionine for *Neptunia* methionyl-tRNA synthetase.

The *Neptunia* cysteinyl-tRNA synthetase was similar to other plant cysteinyl-tRNA synthetases in its ability to catalyze both cysteine- and selenocysteine-dependent ATP-PP_i exchange. Although the enzyme from *Neptunia* catalyzed the formation of cysteinyl-tRNA experiments to study the charging of tRNA by selenocysteine were hindered by the high background counts obtained due to the high affinity of selenium for proteins, a problem encountered previously (40). However, provided an amino acid analog is able to support ATP-PP_i exchange, the ability of an analog to inhibit the charging of tRNA with its true amino acid substrate can be used as a measure of determining the substrate specificity of an aminoacyl-tRNA synthetase (3). The fact that selenocysteine supported ATP-PP_i exchange and also inhibited the formation of cysteinyl-tRNA strongly suggests that selenocysteine is an alternative substrate for the enzyme from *Neptunia*.

Therefore, on the condition that both selenocysteinyl-tRNA and selenomethionyl-tRNA can participate in protein synthesis, the extent of incorporation of selenium-containing amino acid analogs into proteins would largely depend upon the intracellular cysteine to selenocysteine and methionine to selenomethionine ratios as suggested previously (16). It seems that selenocysteine and selenomethionine are not excluded from proteins at the amino acid-activation stage.

Considering the results presented here and elsewhere, over-all pathway of selenium in *Neptunia* can now be examined.

Evidence is presented here in support of the conversion of selenate to selenite by a reaction catalyzed by ATP sulfurylase. The reduction of selenite to selenide by plant extracts and the incorporation of selenide into selenocysteine by extracts of *Neptunia* have been reported previously (22, 23). However, it has been reported that *Neptunia*, grown on selenium-containing soils, do not incorporate selenium-substituted amino acids but, instead, accumulate their selenium in the form of nonprotein amino acids; the main selenium-containing compounds found in seleniferous plants being Se-methylselenocysteine and selenocystathionine (26, 27, 28, 38). Realizing that alkyl derivatives of selenocysteine do not act as substrates of cysteinyl-tRNA synthetase, immediate methylation of selenocysteine following selenocysteine synthesis

would therefore exclude selenocysteine incorporation into proteins. On the other hand, methionyl-tRNA synthetase has a relatively loose substrate specificity so selenomethionine is probably not excluded from incorporation into proteins at the amino acid-activation stage but is excluded because selenomethionine is not synthesized in *N. amplexicaulis* and other selenium accumulators. This view is strongly supported by a number of reports. Firstly, seleniferous plants do not contain selenium in either free selenomethionine or in protein-bound selenomethionine (39). Secondly, neither Se-methylselenomethionine nor selenohomocysteine have been detected in seleniferous plants (28, 38). And thirdly, the fact that selenocystathionine is found in such high concentrations (27) and that selenohomocysteine, selenomethionine, or Se-methylselenomethionine are not found in *N. amplexicaulis* suggests strongly that β -cystathionase, the enzyme which catalyzes the hydrolysis of cystathionine to homocysteine in the methionine biosynthetic pathway, does not catalyze the hydrolysis of selenocystathionine, thereby preventing the conversion of selenocysteine to selenomethionine via the transsulfurylation pathway. This block in the methionine biosynthetic pathway to selenium analogs would explain why selenium is not incorporated into not only proteins but also in selenium-containing analogs of homocysteine and methionine derivatives.

A proposed metabolic pathway for selenium in both selenium-accumulator and nonaccumulator plants based on the evidence presented here is shown in Figure 7.

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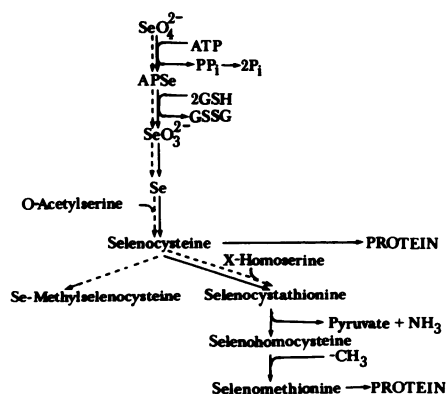


FIG. 7. Proposed pathway for selenium metabolism in selenium accumulator and nonaccumulator plants. (---), metabolic pathway for selenium accumulators; (—), metabolic pathway for selenium nonaccumulators. X ~Homoserine, activated formed of homoserine; ~CH₃, methyl donor.

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