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Activation of Selenate by Adenosine 5′-Triphosphate Sulphurylase from *Saccharomyces cerevisiae*

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In the presence of ATP and Mg^{2+}, ATP sulphurylase from *Saccharomyces cerevisiae* catalysed the conversion of selenate into a compound with the electrophoretic and acid-lability properties of adenosine 5′-sulphatophosphate. Structural characterization, involving extensive purification of adenosine 5′-selenophosphate, proved impossible. However, we showed ATP-, Mg^{2+}- and ATP sulphurylase-dependent, and inorganic pyrophosphatase-stimulated, production of elemental selenium from selenate in the presence of GSH (reduced glutathione). Since selenate was not reduced by GSH, this reaction proved that ATP sulphurylase had formed an active selenate. The enzyme-catalysed formation of elemental selenium had the same kinetics and GSH-dependency as the non-enzymic reduction of selenite to elemental selenium by GSH. In the presence of inorganic pyrophosphatase, 2mol of P_i was released for each mol of 'active selenate' formed. This was shown by a spectrophotometric assay for elemental selenium. The observed reactivity with thiols and the instability of the enzymic product were those predicted for selenium anhydrides. By analogy with the chemistry of sulphur, the product of the thiolytic cleavage of a selenium anhydride would be converted into selenite. The selenite would then be reduced by the thiol to elemental selenium. We conclude that ATP sulphurylase can catalyse the formation of adenosine 5′-selenophosphate. The anhydride can be reduced by thiols in a manner similar to the reduction of selenite. These results probably explain the ability of mammals, lacking a sulphate reductase system, to incorporate selenium from selenate into seleno-amino acids.

There is increasing awareness of the biological importance of the element selenium. Animal diseases have been related to the toxic effects of selenium-containing compounds (Rosenfeld & Beath, 1964) and the role of selenium as an essential trace element in animal and bacterial nutrition has been established (Frost & List, 1975; Stadtman, 1974). Early studies on selenium compounds focused on the ability of other compounds to protect livestock from the toxic effects of selenium. The ability of chemically analogous sulphur compounds to protect against selenium toxicity became apparent. This, together with knowledge that most of the naturally occurring selenium compounds were analogues or derivatives of naturally occurring sulphur compounds, led to the concept of a relationship between the metabolism of sulphur and selenium.

This concept has been expanded to suggest that the assimilation of inorganic selenium compounds might utilize the same enzymes as those used in inorganic sulphur assimilation (cf. Shrift, 1973). The ability of various forms of inorganic selenium to be metabolized to reduced organic forms has been shown in animals (McConnell & Portman, 1952), bacteria (Tuve & Williams, 1961) and plants (Fleming & Alexander, 1972). Whether the enzymes of the sulphur-assimilatory system are responsible for these biological transformations is not known. There is evidence supporting common metabolic routes for sulphur and selenium metabolism, and contradictory evidence indicating that they follow independent routes. Evidence suggesting that the pathways of selenium and sulphur assimilation are independent, at least in some organisms, is the fact that animals cannot reduce sulphate or sulphite to the sulphide level, yet selenate and selenite can be reduced. Also, there are classes of naturally occurring sulphur compounds for which no analogous selenium compounds exist (Shrift, 1973), and a pathway has been proposed (Hsieh & Ganther, 1975) for the reduction of selenite to selenide in animals, but which would not reduce sulphite. However, it should be noted that selenite and sulphite are chemically the most different of the inorganic selenium–sulphur analogues.

The alternative possibility, that inorganic sulphur and selenium are metabolized by the same enzymes, is supported by the following evidence. There is competition between inorganic sulphate and selenate for biological uptake (Leggett & Epstein, 1956). The

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enzyme sulphite reductase (EC 1.8.1.2) from *Escherichia coli* can reduce selenite (Kemp *et al.*, 1963), although the low affinity for selenite makes a role *in vivo* as a selenite reductase unlikely. The first enzyme of the sulphur-assimilatory pathway, ATP sulphurylase (EC 2.7.7.4), has been reported to activate selenate (Wilson & Bandurski, 1958). And, most convincingly, mutants of *Aspergillus* lacking ATP sulphurylase are insensitive to normally inhibitory concentrations of selenate (Arst, 1968). The evidence for differing pathways mentioned above would not rule out sulphurylase activation of selenate, as animals can activate sulphate, and the branch point in the pathway that leads to the compounds for which no selenium analogues are known occurs after the activation step.

Bandurski *et al.* (1956) and Wilson & Bandurski (1958) concluded that the ATP sulphurylase from baker's yeast catalysed the formation of adenosine 5′-selenophosphate on the basis of the following evidence. (1) Several anions catalysed the release of pyrophosphate from ATP in the presence of the enzyme, but only sulphate and selenate catalysed the exchange of [^{32}P]pyrophosphate into unlabelled ATP. (2) The enzyme catalysed formation of charcoal-adsorbable [^{75}Se]selenate in the presence of ATP and Mg^{2+}. Since charcoal does not adsorb selenate but would adsorb a selenate nucleotide, this was used as an assay for adenosine 5′-selenophosphate synthesis. (3) The enzyme catalysed formation of a selenium-containing compound with the electrophoretic and acid-lability properties of adenosine 5′-sulphatophosphate. Shaw & Anderson (1974) confirmed the [^{32}P]pyrophosphate exchange but, in their hands, a purified plant ATP sulphurylase appeared not to form a selenium-containing compound with the electrophoretic mobility of adenosine 5′-sulphatophosphate. It was thus necessary to reinvestigate selenate activation by ATP sulphurylase. We confirm the earlier studies and, further, have proved the formation of 'active selenate' by sulphurylase by trapping and reducing the product with GSH*. Further, this non-enzymic reduction of adenosine 5′-selenophosphate by GSH provides a reasonable mechanism for selenate reduction by animals lacking a sulphate reductase. An abstract of these results has been published (Dilworth & Bandurski, 1976), and these data are a portion of research done for a doctoral dissertation (Dilworth, 1974).

Experimental

Materials

Dry baker's yeast (*Saccharomyces cerevisiae*) (Red Star) was obtained from Universal Foods, Milwaukee, WI 53201, U.S.A. [^{75}Se]Selenate and [^{35}S]sulphate were supplied by New England Nuclear Corp., Boston, MA, U.S.A. Radioactive selenate and unlabelled carrier were purified immediately before use. The selenate was mixed (10:1, v/v) with 30% (w/v) H_2O_2 and heated at 100°C for 10min. The selenate solution was neutralized with 2M-Tris (free base), catalase (8000 units; all enzyme activities are in EC units, 1 μmol of substrate converted/min) was added and the reaction mixture boiled for 5min after foaming subsided. Na_2SeO_4 and Na_2SeO_3 were obtained from Alfa Inorganics, Beverly, MA, U.S.A. (GSH, ATP, AMP, 3′-AMP, inorganic pyrophosphatase and catalase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.). CM-cellulose was supplied by Hercules Inc., Wilmington, DE, U.S.A. DEAE-Sephadex A-25 was a product of Pharmacia, Uppsala, Sweden.

Methods

Enzyme preparations. Diethyl ether-treated yeast sulphate-activating enzymes were prepared by the method of Robbins (1962) and corresponded to his fraction III. Yeast sulphate-activating enzymes from dried yeast were prepared by placing dry yeast and sea sand (4:1, w/w) in a Waring blender and grinding for 10min. The mixture was then suspended in 4 times its weight of 0.05M-K_2HPO_4 and stirred at 4°C for 16h. The remaining steps were as for the preparation of the ether-treated sulphate-activating enzyme. Yeast ATP sulphurylase was prepared by taking the preparation analogous to Robbins's (1962) fraction III from dry yeast through the steps leading to fraction IV. These preparations were assayed by the molybdolysis reaction of Wilson & Bandurski (1958), as described by Robbins (1962), and using the same units of activity.

Spectrophotometric assay of elemental selenium formation. The reaction mixture contained (in μmol): Tris/HCl, pH 8.0, 100; $MgCl_2$, 10; ATP, 10; Na_2SeO_4, 10; GSH, 10; inorganic pyrophosphatase, 0.2 unit; 0.2ml of 4% (w/v) CM-cellulose; ATP sulphurylase, 27 units; in a total volume of 1.0ml. The reaction was started by the addition of enzyme while monitoring the A_{380}. A standard curve was made by adding various amounts of Na_2SeO_3 to a reaction mixture containing all components except Na_2SeO_4. The A_{380} was converted into equivalents of elemental selenium by using a standard curve. Beer's law was obeyed except for a discontinuity in the curve and care was used to avoid concentrations in the region of non-linearity. The selenate-dependent phosphate release from ATP was determined in a manner similar to that previously described (Wilson & Bandurski, 1958), except that phosphorus was determined by the assay method of Sumner (1944). The amount of phosphate formed in the presence of selenate minus

* Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

that formed without selenate was the selenate-dependent phosphate release.

Electrophoresis. Electrophoresis studies were performed on an E-C electrophoresis apparatus with a bed length of 45cm. Whatman 3MM paper strips (6cm wide) were used with buffers of either 0.1M-sodium acetate, pH4.5, or 0.1M-Tris/HCl, pH7.6, at 10V/cm and at 7–9°C. The strips were air-dried and counted for radioactivity on a gas-flow strip counter.

Column chromatography. DEAE-Sephadex A-25 was left to swell in 0.5M-ammonium formate, pH7.0 (adjusted with aq. NH_3), for several days. The gel was then equilibrated with the loading buffer, 0.05M-ammonium formate, pH7.0. The other column parameters are presented in the Figure legends.

The [^{35}S]sulphur and [^{75}Se]selenium were counted for radioactivity in Bray's (1960) solution by using gain and window settings appropriate to ^{14}C and ^{3}H respectively. Phosphorus was determined with a micro-adaptation of the method of Ames (1966), with AMP as a standard. Adenine was measured by the A_{245}, A_{260} and A_{275} and using the formula $A_T = A_{260} - \frac{1}{2}(A_{245} + A_{275})$. The A_T is converted into adenine concentration by using a molar absorption coefficient of 7470 litre·mol^{-1}·cm^{-1}. This method, previously used for other compounds (Ueda & Bandurski, 1969), yields a baseline correction for compounds having a broad maximum.

Results

The inability of Shaw & Anderson (1974) to reproduce the electrophoretic data of Wilson & Bandurski (1958) using a purified enzyme preparation from a higher plant prompted a reinvestigation of the formation of a selenium-containing compound with the electrophoretic mobility of adenosine 5′-sulphatophosphate by using a yeast sulphate-activating enzyme. Fig. 1 confirms the enzymic formation of a ^{75}Se-labelled compound with the mobility of adenosine 5′-sulphatophosphate. This compound was destroyed by keeping the incubation mixture at pH 1.0 for 20min before electrophoresis, conditions that completely hydrolyse adenosine 5′-sulphatophosphate. The lack of a radioactive peak in the region of adenosine 3′-phosphate 5′-sulphatophosphate confirms the earlier report that a compound corresponding to adenosine 3′-phosphate 5′-selenophosphate was not formed (Wilson & Bandurski, 1958). The radioactive selenium peak in the region of adenosine 5′-sulphatophosphate could not be eluted without destruction of the putative adenosine 5′-selenophosphate, as a second electrophoresis showed only selenate.

An alternative method of separating adenosine 5′-sulphatophosphate and adenosine 3′-phosphate 5′-sulphatophosphate from inorganic sulphate and other nucleotides was attempted with the objective of determining stoicheiometry of adenine/phosphate/sulphate (or selenate) in the putative adenosine 5′-selenophosphate. A DEAE-Sephadex A-25 column system developed by Wilson & Bierer (1976) was used for these experiments. Fig. 2 shows the elution profile of incubation mixtures containing ATP sulphurylase and either [^{35}S]sulphate or [^{75}Se]selenate. The identity of adenosine 5′-sulphatophosphate was established by the coincident peaks of adenine, sulphur and phosphorus in the molar proportions 0.96:1:1.05. As shown in Fig. 2, no selenium-containing compound was obtained in the region of adenosine 5′-sulphatophosphate. When the yeast sulphate-activating enzymes were used in these experiments, adenosine 3′-phosphate 5′-sulphatophosphate, with the adenine/sulphur/phosphate proportions 1:1:2.3, was the only sulphur-containing nucleotide detected. There was no adenine-containing selenium compound. Thus the formation of an adenosine 5′-sulphatophosphate-like selenium compound could be shown with electrophoresis but not with a DEAE-Sephadex column eluted with ammonia-

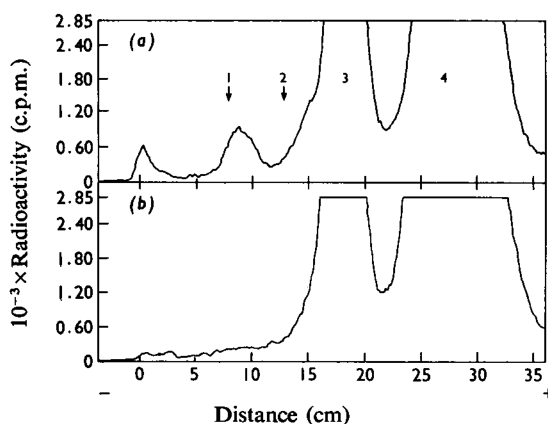


Fig. 1. *ATP sulphurylase-catalysed production of a radioactive selenium peak having the electrophoretic mobility of adenosine sulphatophosphate*

The reaction mixture contained, in μmol: Tris/HCl, pH8.0, 25; ATP, 2.5; $MgCl_2$, 2.5; 3′-AMP, 5; $Na_2{}^{75}SeO_4$, 5 (5μCi/μmol); (*a*) 50μl of ether-treated yeast preparation; in a total volume of 275μl. This amount of enzyme gave maximum yields of adenosine 3′-phosphate 5′-sulphatophosphate under these conditions. (*b*) Enzyme was boiled for 5min before incubation. Both mixtures were incubated for 1h at 37°C and the reaction was stopped by boiling for 90s. After centrifugation to remove protein, 100μl of the supernatant was placed on each strip. Electrophoresis was for 5h with 0.1M-sodium acetate buffer, pH4.5. The numerals indicate the electrophoretic mobility of: 1, adenosine 5′-sulphatophosphate; 2, adenosine 3′-phosphate 5′-sulphatophosphate; 3, SeO_3^{2-}; 4, SeO_4^{2-}.

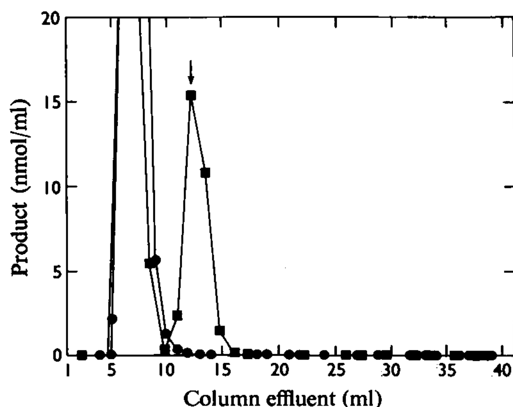


Fig. 2. *Column-chromatographic separation of the products formed by yeast ATP sulphurylase with sulphate and selenate*
The reaction mixture contained, in μmol: Tris/HCl, pH 8.0, 50; ATP, 5; $MgCl_2$, 5; $Na_2{}^{75}SeO_4$ (16.2 μCi/μmol) or $Na_2{}^{35}SO_4$ (1.25 μCi/mmol), 2.5; ATP sulphurylase, 8.2 units; in a volume of 0.5 ml. The mixture was incubated at 37°C for 1 h and stopped by boiling for 90 s. The precipitated protein was removed by centrifugation at 500 g for 5 min. The column was 0.7 cm × 7.0 cm and was eluted with a 40 ml linear gradient of 0.1–1.3 M-NH_4HCO_3. Each fraction contained 1 ml and the flow rate was 2.5 ml/h. The adenosine 5′-sulphatophosphate peak is indicated by the arrow. ■, Elution pattern of the ^{35}S experiment; ●, ^{75}Se experiment. The first peaks of material to emerge are selenate and sulphate.

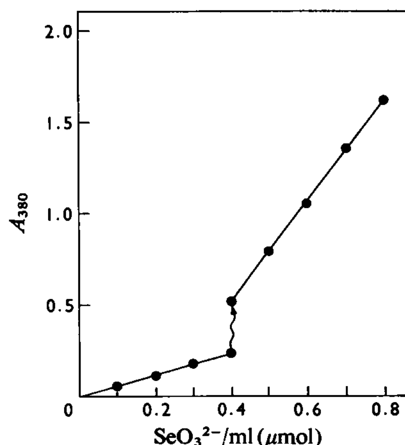
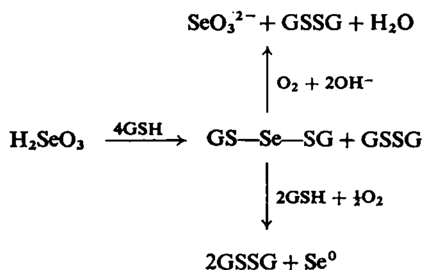


Fig. 3. *Elemental selenium produced by reduction of Na_2SeO_3 by 10 mM-GSH*
The indicated amount of Na_2SeO_3 was added to the reaction mixture described under 'Methods'. A_{380} was determined after mixing and monitored until stable. The A_{380} increase, indicated by the wavy arrow, occurs only at 0.4 μmol of Na_2SeO_3/ml, as discussed in the text, and represents the transition point from catalytic oxidation of GSH by Na_2SeO_3 to reduction of Na_2SeO_3 to elemental selenium by GSH (Scheme 1).

containing buffers. As discussed below, we believe that a selenium anhydride could be catalytically decomposed by DEAE-Sephadex or ammonia buffers.

Since instability precluded chemical characterization of the putative adenosine 5′-selenophosphate, we determined to measure the product by its predicted chemical reactivity by using a reaction noted previously in this laboratory (R. S. Bandurski, unpublished work), in which the addition of cysteine to a sulphurylase incubation mixture containing selenate resulted in deposition of elemental selenium. The elemental selenium was characterized by its pink colour and the formation of piazselenole (2,1,3-benzoselenadiazole) from *o*-phenylenediamine after filtration and oxidation with bromine/hydrogen bromide (Kuder, 1973). Spectrophotometric assay was difficult, since elemental selenium and cystine are insoluble, and we thus devised a new assay. If the reaction solution was made viscous, and GSH substituted for cysteine, enzymically formed elemental selenium formed a clear red solution. The production of elemental selenium was followed by monitoring the increase in A_{380}. This wavelength was selected somewhat arbitrarily, as there was no sharp absorption maximum in the visible range. The absorption spectrum of the enzymically formed product was identical with the spectra obtained when selenite was reduced with GSH, hydrazine or H_2SO_3, three common ways of forming elemental selenium (Nazarenko & Ermakov, 1972).

Fig. 3 shows the relationship between A_{380} and the concentration of selenium produced from Na_2SeO_3. The results were unexpected, in that a linear relationship was not observed. The discontinuity in increase of A_{380} at 0.4 μmol of $SeO_3{}^{2-}$ occurs about 12 min after adding $SeO_3{}^{2-}$ to the GSH-containing reaction mixture and requires about 3 min to reach the stable higher absorptivity. It occurs when the proportions of O_2/GSH/$SeO_3{}^{2-}$ favour the reduction of selenite to selenium as opposed to the catalytic oxidation of GSH (Petersen, 1957; Tsen & Tappel, 1958). These reactions are shown in Scheme 1. The time-course of the ATP sulphurylase-dependent increase in A_{380} in the presence of ATP, Mg^{2+}, Na_2SeO_4 and various amounts of GSH is presented in Fig. 4 and shows the same kinetics and transition to linearity as seen in the standard curve (Fig. 3). The change in the transition point with various GSH concentrations was also like those seen when different GSH concentrations were made to react with increasing amounts of selenite. Data such as those in Figs. 5–7 have been expressed as elemental selenium by using



Scheme 1. *Alternative reactions resulting in either the catalytic oxidation of GSH by SeO_3^{2-} or the reduction of SeO_3^{2-} to elemental selenium by GSH*

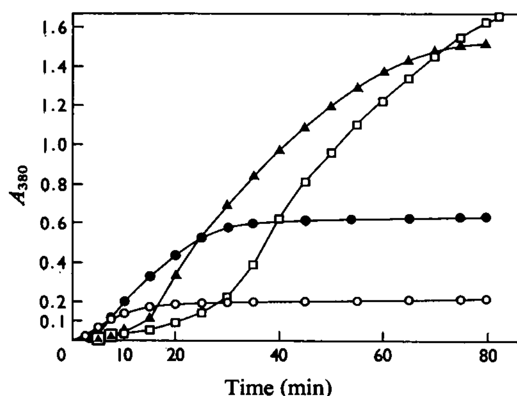


Fig. 4. *ATP sulphurylase-dependent increase in A_{380} with time and at various GSH concentrations*

The reaction conditions were as described under 'Methods'. The following GSH concentrations were used: ○, 2.5mM; ●, 5mM; ▲, 10mM; □, 20mM.

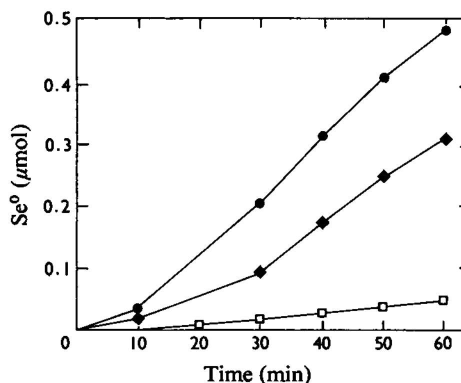


Fig. 5. *Effect of reaction components on the production of elemental selenium by ATP sulphurylase*

The reaction conditions were as described under 'Methods' in a volume of 1.0ml with the indicated omissions: ●, none; ◆, pyrophosphatase; □, Mg^{2+}. No selenium was formed when ATP, GSH, Na_2SeO_3 or ATP sulphurylase was omitted.

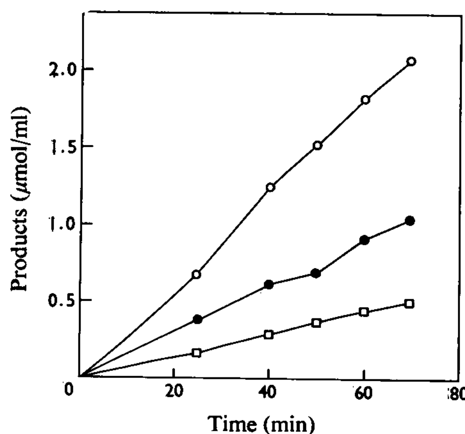


Fig. 6. *Enzymic production of phosphate and elemental selenium as a function of time*

The reaction conditions were as described under 'Methods' and the following symbols are used: ○, total P_i formed; ●, Na_2SeO_4-dependent P_i formed; □, elemental selenium formed. The average ratio of Na_2SeO_4-dependent P_i liberated/selenium formed was 2.07±0.14.

the standard curve of Fig. 3. The dependency of elemental selenium production on time is shown in Fig. 4 and the dependency of the reaction on the components of the reaction mixture is presented in Fig. 5. The addition of inorganic pyrophosphatase stimulated the reaction, but was not essential. The enzyme preparation was not tested for pyrophosphatase activity, and thus an absolute requirement for pyrophosphatase has not been demonstrated.

Fig. 6 shows the ratio of selenate-dependent phosphate release/elemental selenium formation. The average ratio was 2.07±0.14mol of phosphate/mol of elemental selenium. The relationship between phosphate produced and elemental selenium formed having been established, the stoicheiometry of GSH oxidation to selenium and phosphorus production was determined. There was high GSH oxidation during the reaction, much more than could be explained by the reduction of selenate to selenide or elemental selenium. The reaction could not be run anaerobically to prevent the oxidation of GSH by oxygen, since elemental selenium was not formed until air was admitted, as indicated in Scheme 1.

The equilibrium constant for the sulphurylase reaction with sulphate is about 4×10^{-8}, so that product accumulation detectable by colorimetry was

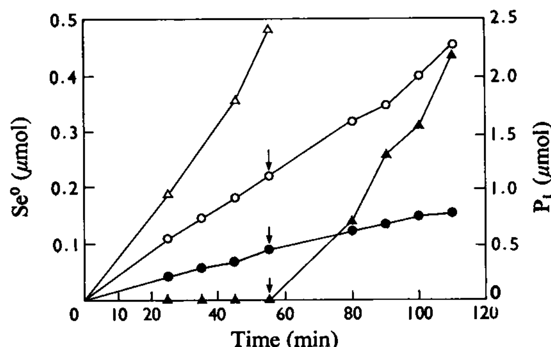


Fig. 7. *Failure to accumulate the enzymically produced, GSH-reducible intermediate*

Reaction conditions were as described under 'Methods', and the volume of the GSH added (at time indicated by the arrow) was 50μl. Elemental selenium formation in the complete system (△) and in a system lacking GSH (▲) is measured on the left-hand ordinate, and P_i liberation in the absence of GSH (○) and in the absence of GSH and Na_2SeO_4 (●) is shown on the right-hand ordinate.

not expected (Wilson & Bandurski, 1958). Decomposition of adenosine 5′-selenophosphate will drive the reaction from left to right (Bandurski *et al.*, 1956) but adenosine 5′-selenophosphate will not be detectable. Nonetheless an attempt was made to detect accumulation of an active selenate. In this experiment GSH was added after prior incubation of the reaction mixture. Selenate-dependent P_i release was only slightly increased and selenate-independent phosphate release slightly inhibited by the presence of GSH, and no GSH-reducible products accumulated (Fig. 7). These data suggest that the rate-limiting reaction in elemental selenium production by ATP sulphurylase and GSH is the rate of dissociation of adenosine 5′-selenophosphate from ATP sulphurylase.

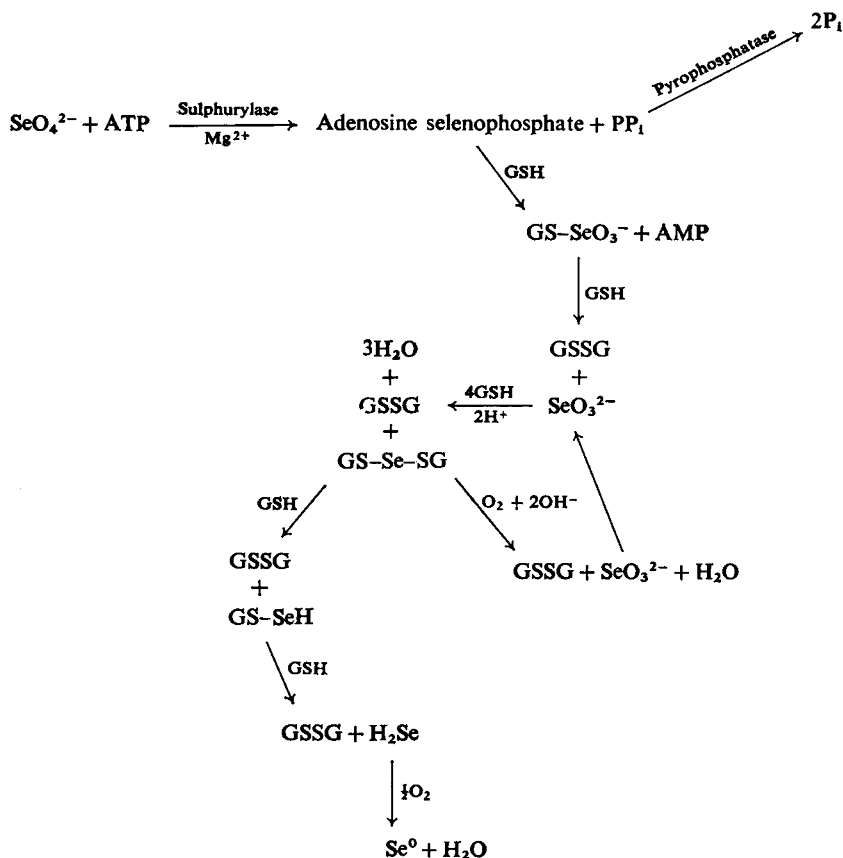
Discussion

Five items of data support the formation of adenosine 5′-selenophosphate from ATP and selenate in the presence of ATP sulphurylase (Wilson & Bandurski, 1956, 1958; Bandurski *et al.*, 1956): (1) the formation of a labelled selenium compound with similar electrophoretic properties to adenosine 5′-sulphatophosphate; (2) the acid-lability of the radioactive compound was similar to that of adenosine 5′-sulphatophosphate; (3) charcoal-adsorbable selenium could be detected after incubation of selenate, ATP and Mg^{2+} with ATP sulphurylase; (4) selenate served as substrate for the ATP sulphurylase-mediated exchange between ATP and radioactive pyrophosphate; (5) selenate behaved as a competitive inhibitor of sulphate activation. To this we now add the demonstration that: (6) sulphurylase plus GSH can reduce selenate to elemental selenium; (7) the stoicheiometry of the reaction is $2P_i$ per selenium formed; (8) the reaction with GSH is characteristic of a selenium anhydride.

Shaw & Anderson (1974), using electrophoresis, were unable to detect the formation of a radioactive selenium compound in the region of adenosine 5′-sulphatophosphate, although they confirmed the pyrophosphate-exchange studies by Wilson & Bandurski (1958). An attempt to settle this apparent disagreement is presented in Fig. 1. This shows that the original results of Wilson & Bandurski (1958) can be duplicated, but that the selenium must have a high specific radioactivity. The specific radioactivity of the labelled selenate used by Shaw & Anderson (1974) was at most one-tenth that of ours and would have prevented their detection of this radioactive compound. However, even when sufficient radioactivity was used, we did not always obtain formation of the putative anhydride with different enzyme preparations. Presumably there must be sufficient thiol for enzyme activity but not enough to degrade the product. It is also possible that the plant and yeast enzymes are different.

Our attempts at column chromatography involved DEAE-Sephadex and ammonia-containing buffers. Although analogous selenate anhydrides are not available, it is known that the tetrameric selenium trioxide reacts easily with nucleophiles (Schmidt & Wilhelm, 1964; Blanka & Toužín, 1967; Toužín & Kratochvíla, 1968; Kurze & Paetzold, 1972) and that potassium diselenate forms an amidoselenate with ammonia (Dostál & Krejčí, 1958). Thus inadvertent aminolysis or ammonolysis may account for inability to isolate the selenophosphate anhydride by DEAE-Sephadex chromatography. Until the properties of selenophosphate anhydrides are studied, care must be used in the interpretation of negative results.

A direct spectrophotometric assay for elemental selenium was developed with GSH as the thiol. The assay was calibrated with selenite and GSH to form elemental selenium non-enzymically. The kinetics of this reaction were unusual (Fig. 3). Studies by Tsen & Tappel (1958) showed that either of two reactions may occur, depending on the ratio of selenite to GSH, namely the catalytic oxidation of GSH or the reduction of selenite. Scheme 1 summarizes the proposed reactions. The intermediate GS–Se–SG (selenodiglutathione) was shown by Petersen (1957), Tsen & Tappel (1958) and Ganther (1968) to be involved in both reactions. Fig. 3 can now be interpreted as follows. At low selenite concentrations the reaction is primarily a catalytic oxidation



Scheme 2. *Proposed pathway for the ATP sulphurylase-catalysed, glutathione-dependent reduction of selenate to selenide or elemental selenium in systems lacking a sulphate reductase*
For details of symbols and discussion of pathways, see the text.

of GSH. Under these conditions, GSH is being consumed but the selenite concentration remains the same. The transition occurs when there is enough selenite compared with GSH to favour the production of elemental selenium. A stoicheiometric production of elemental selenium occurs after the transition point.

The spectrophotometric assay permitted the monitoring of the kinetics of elemental selenium production from selenate by ATP sulphurylase with ATP, Mg^{2+} and GSH. The same kinetics seen with selenite reduction were observed (Fig. 4), and the transition point varied with GSH concentration, as expected from studies with selenite. The observation that excess of GSH was consumed during the reaction relative to P_i or elemental selenium production also indicated that the pathway which leads to the catalytic oxidation of GSH was operating. These results, and the ability to use the standard curve obtained with selenite to show a linear enzymic production of elemental selenium with time, indicated the existence of a common partially reduced intermediate. This intermediate must be between selenate and GS–Se–SG, as it resembled selenite or a product between selenite and GS–Se–SG. GS–Se–SG is the key intermediate giving unusual kinetics to both reactions.

The substrate and cofactor requirements for the formation of elemental selenium are shown in Fig. 5. Except for the GSH requirement, the same components are required as for activation of sulphate. The production of P_i is stoicheiometrically related to the production of elemental selenium. The data in Fig. 6 showed that 2 mol of P_i was released, in the presence of inorganic pyrophosphatase, for each mol of elemental selenium formed. The formation of adenosine 5′-sulphatophosphate from sulphate likewise involve the production of 2 mol of phosphate

per mol of adenosine 5′-sulphatophosphate produced.

The only reductant present in the system is GSH, and thus the conversion of selenate into elemental selenium must involve the participation of GSH. The reaction between adenosine 5′-selenophosphate and GSH cannot be studied directly, as the anhydride has not been isolated in usable amounts from biological systems and has yet to be chemically synthesized. There is, however, a model system, since selenium trioxide is a selenate anhydride.

Selenium trioxide is the tetrameric anhydrous form of selenic acid and is readily converted into selenic acid when exposed to water. Schmidt and co-workers (Schmidt *et al.*, 1963; Schmidt & Wilhelm, 1964) have reported that selenium trioxide reacts explosively with thiols, forming thioselenic acids. A thiolytic cleavage of a phosphoselenate anhydride thus could be predicted and alcoholysis of the selenium anhydride would also be predicted. Nissen & Benson (1964) found ethyl selenate when plants fed on selenate were extracted with hot ethanol, and ethyl selenate is the product expected from an alcoholytic cleavage of a selenium anhydride. The enzymic product, as an analogous anhydride, should, like selenium trioxide, be unstable in water. The enzymic product is unstable, as shown by the difficulty in isolating it and the requirement that it be trapped by GSH as it is formed (Fig. 7). The thioselenic acid formed from thiolytic cleavage can be converted into selenite by the addition of one electron (from one GSH molecule). The conversion of thiosulphuric acids into sulphite and a disulphide by thiols is well documented.

Our proposed mechanism for conversion of selenate into elemental selenium is shown in Scheme 2. This proposal involves the thiolytic cleavage of the selenate anhydride adenosine 5′-selenophosphate. Tissue GSH concentrations of 1–2.5mM (cf. Crook, 1959) are adequate for this reaction. The conversion of thioselenic acid into selenite is analogous to known sulphur chemistry. The mechanism of the catalytic oxidation of glutathione by selenite is that proposed by Tsen & Tappel (1958). The conversion of selenite into elemental selenium is essentially the mechanism proposed by Hsieh & Ganther (1975). There is no direct evidence to distinguish between the two possible paths for the conversion of GS–SeH, glutathione selenopersulphide, into elemental selenium. One possibility is presented in Scheme 2, and the other possibility would be an intramolecular rearrangement forming elemental selenium and GSH (Ganther, 1971). The inclusion of hydrogen selenide production in the proposed pathway is due to the fact that oxygen is required for elemental selenium formation.

Our mechanism for the conversion of selenate into elemental selenium was designed to explain a phenomenon *in vitro*, but it also provides a plausible pathway for the reduction of selenate *in vivo*. The steps requiring oxygen would presumably be side reactions of this reductive pathway. The pathway *in vivo* would include the glutathione reductase (EC 1.6.4.2)-catalysed reduction of GS–Se–SG (Ganther, 1971; Hsieh & Ganther, 1975). This enzyme catalyses the reduction of GS–Se–SG by 2mol of NADPH, forming 2mol of GSH and hydrogen selenide. If enzyme catalysis does occur *in vivo*, the mechanisms of the individual reaction steps would differ from the proposed Scheme 2, but the intermediates would be the same.

This pathway would explain the observed antagonistic effects between selenate and sulphate, since both would be substrates for the same enzyme, ATP sulphurylase. The inability to detect adenosine 3′-phosphate 5′-sulphatophosphate metabolites (selenate esters) would arise from the instability of adenosine 5′-selenophosphate in the presence of tissue thiol. In the proposed Scheme 2 sulphite reductase would not be required to reduce selenite, and thus the low affinity of this enzyme for selenite would not present a problem. The observation that animals can reduce selenate but not sulphate is likewise explained. Animals contain ATP sulphurylase, and, as shown in the present study, would form a product from selenate that would be reduced by GSH in a mechanism different from that of plant and microbial sulphate reduction. In animals there is evidence that the reduction of selenite is mediated by GSH (Ganther, 1966), and the ability of ATP sulphurylase to form an intermediate from selenate that can be converted into selenite would explain the ability of animals to utilize both selenite and selenate as a source of nutritionally required selenium (Schwarz & Foltz, 1958).

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