STUDIES ON E. COLI SULFITE REDUCTASE AND THE ACTIVATION OF SELENATE BY YEAST ATP SULFURYLASE

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ABSTRACT

STUDIES ON E. COLI SULFITE REDUCTASE
AND THE ACTIVATION OF SELENATE BY
YEAST ATP SULFURYLASE

By

Gregory Lee Dilworth

Part I. Studies on E. coli Sulfite Reductase

The six electron reduction of sulfite to sulfide catalyzed by E. coli sulfite reductase was studied. Two approaches were used to elucidate the intermediate forms of sulfur that occur during the reduction. The first series of experiments involved the binding of radioactive sulfur to the purified enzyme under conditions that might lead to partially reduced intermediates. Reduced nicotinamide adenine dinucleotide phosphate-dependent binding of radioactive sulfur from sulfite could be observed under these conditions. This binding was found to be inversely proportional to the specific activity of the homogeneous enzyme preparations. Using published values as the maximum specific activity possible, it could be calculated that there would be no binding if the preparation was completely active. If the preparation was completely inactive, approximately one mole of sulfur would be bound at saturation to one mole of enzyme. The inactivation,

which permitted increased binding, occurred during purification and long term storage of the preparation. The radioactive sulfur appeared to be covalently bound as the radioactivity could not be separated from the enzyme by extensive dialysis, gel filtration, and treatment with sodium dodecyl sulfate or ethanol. The binding was concluded to be associated with an inactivation event as opposed to the formation of a bound intermediate.

The second approach was an attempt to modify the enzyme such that the enzyme catalyzed only a partial reduction. A number of treatments were used, but the reaction catalyzed could not be altered. These studies did, however, result in the finding that sodium formaldehyde sulfoxylate is a good inhibitor of sulfite reductase.

Part II. The Activation of Selenate by Yeast ATP Sulfurylase

Crude yeast extracts containing sulfate activating enzymes, were found to catalyze an adenosine 5'-triphosphate (ATP) and cysteine-dependent reduction of selenate to elemental selenium. The requirement for ATP implied the formation of adenosine phosphoselenate (APSe) analogous to the formation of adenosine phosphosulfate (APS). The formation of a selenium compound with the electrophoretic and stability properties of APS could be detected. However, an ion-exchange chromatographic separation, which required more time than an electrophoretic separation, failed to

resolve an adenine-containing selenium compound. A direct spectrophotometric procedure was developed to assay the ATP, ATP sulfurylase, glutathione, and pyrophosphatase-dependent formation of elemental selenium from selenate. The enzyme-catalyzed formation of elemental selenium showed the same unusual kinetics and glutathione dependency as the chemical reduction of selenite to elemental selenium by glutathione. It was shown that two phosphates were released for each "active" selenium formed. The observed reactivity towards thiols and instability properties of the enzymatic product were found to be those predicted for selenium anhydrides. By analogy to sulfur chemistry, the product of the thiolytic cleavage of a selenium anhydride would be easily converted to selenite. The selenite would then be reduced by the thiol to elemental selenium. Thus it was concluded that ATP sulfurylase can catalyze the formation of APSe, or a similar selenium anhydride. The anhydride can be reduced by thiol compounds in a manner similar to the reduction of selenite by thiols.

The role of ATP sulfurylase in the <u>in vivo</u> reduction of selenate is discussed.

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By

Gregory Lee Dilworth

A DISSERTATION

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To my family

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LIST OF ABBREVIATIONS

ADP = Adenosine 5'-diphosphate
AMP = Adenosine 5'-phosphate
3'-AMP = Adenosine 3'-phosphate
APS = Adenosine 5'-phosphosulfate
APSe = Adenosine 5'-phosphoselenate
ATP = Adenosine 5'-triphosphate
BSA = Bovine serum albumin
DEAE = Diethylaminoethyl-
EDTA = Ethylenediamine tetraacetate
FAD = Flavin adenine dinucleotide
FMN = Flavin mononucleotide
Glucose-6-P = Glucose-6-phosphate
GSSG = Oxidized glutathione
GS-Se-SG = Selenodiglutathione
GS-SeH = Glutathione selenopersulfide
GSH = Reduced glutathione
K-phosphate = Potassium phosphate
MV = Methyl viologen
$NADP^+$ = Nicotinamide adenine dinucleotide phosphate
NADPH = Reduced nicotinamide adenine dinucleotide phosphate
PAPS = 3'-phosphoadenine-5'-phosphosulfate
PAPSe = 3'-phosphoadenine-5'-phosphoselenate
Pi = Inorganic phosphate
PPi = Inorganic pyrophosphate
SDS = Sodium dodecyl sulfate
Tris = 2-amino-2-hydroxymethyl-1,3-propanediole
Vol = Volume
Wt = Weight

INTRODUCTION

This dissertation presents research on enzyme-catalyzed reactions involved in the metabolism of inorganic compounds of sulfur and selenium.

Sulfur Metabolism

Inorganic sulfate can be utilized for the synthesis of organic sulfur compounds by microorganisms and plants, or as the terminal electron acceptor during anaerobic respiration by some bacteria. The first process is called assimilatory sulfate reduction, while the second is termed dissimilatory sulfate reduction. The research presented here was performed on the assimilatory sulfite reductase of Escherichia coli.

The overall assimilatroy sulfate reduction pathway, as it is thought to occur in microorganisms (1), is diagramed in Figure 1. The sulfur passes sequentially through the enzymatic reaction steps catalyzed by ATP sulfurylase (E.C. 2.7.7.4), APS kinase (E.C. 2.7.1.25), the "PAPS reductase system" (which includes enzyme A, enzyme B, and fraction C), sulfite reductase (E.C. 1.8.1.2) and O-acetylserine sulfhydrolase (E.C. 4.2.99.8).

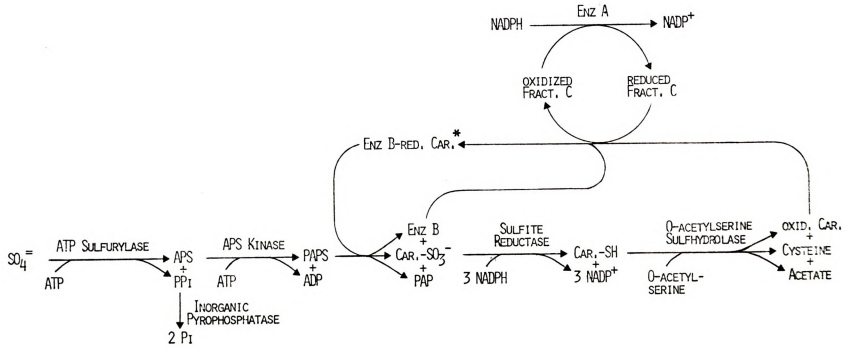


Figure 1. The proposed pathway of assimilatory sulfate reduction in microorganisms.

Adapted from Wilson and Bierer (1).
* Carrier.

Inorganic sulfate is relatively non-reactive and requires activation before other reactions can occur. This activation, which consumes three "high-energy" phosphate bonds, is catalyzed by the enzymes ATP sulfurylase and APS kinase. After being activated the sulfate can be directly incorporated into organic sulfate esters and sulfonic acids, or the activated sulfate can be enzymatically reduced by the "PAPS reductase system" and sulfite reductase to a form that can be incorporated into the sulfur amino acids.

The pathway presented in Figure 1 is a hypothesis that is consistent with the presently available evidence. The mechanism of the two electron transfer to sulfate by the reduced carrier and the six electron reduction catalyzed by sulfite reductase are unknown. The mechanism of the sulfite reductase catalyzed reaction was the area investigated by the studies presented in the first part of this dissertation.

Information about the chemical transformations that occur during the enzyme-catalyzed reduction of sulfite to sulfide was sought by attempting to form and detect intermediates that were only partially reduced. Two different experimental approaches were used. The first approach involved studying the binding of radioactive sulfur, from radioactive sulfite, to the enzyme under incubation conditions that had the potential of trapping partially reduced intermediates. The second approach was an attempt to modify the enzyme such that it would catalyze only a partial reduction. Characterization of the intermediates formed

would have provided information about the chemical conversions taking place during the reaction. Unfortunately, this characterization was not possible as neither approach gave results indicating the formation of intermediate products.

The binding experiments showed that under the proper conditions radioactive sulfur could be bound to the enzyme. However, the binding was concluded to be a phenomenon associated with an inactivation process as opposed to the traping of an intermediate. The attempts to modify the enzyme activity provided information about sulfite reductase inhibitors, but did not change the reaction catalyzed.

Selenium Metabolism

The element selenium was discovered by Berzelius in 1817. It was considered a chemical oddity until the early 1930's, when its role as the toxic agent in certain range plants was described. Further studies have since been conducted on the toxicology of selenium along with nutritional investigations. The nutritional studies were prompted by the recognition that selenium is an essential nutrient for animals and microorganisms.

Early studies on the toxicological properties of selenium compounds revealed the interaction in biological organisms between selenium and another group VIA element, sulfur. Several lines of evidence have evolved since this time that demonstrate that the metabolism of selenium at least parallels the metabolism of sulfur. This conclusion

is based on the following evidence: (a) the antagonism of sulfur compounds towards the toxic effects of selenium compounds, (b) the similarity between naturally occurring selenium compounds and the naturally occurring sulfur compounds, (c) the approximately equal distribution of selenium and sulfur in biological organisms, (d) the ability of several enzymes that normally catalyze reactions involving sulfur compounds to metabolize the analogous selenium compounds.

To date, only one of the enzymes involved in the assimilatory sulfate reduction pathway has been shown to effectively utilize an analogous selenium compound as a substrate. This enzyme, ATP sulfurylase, catalyzes the first reaction of the pathway, the activation of sulfate. Wilson and Bandurski (2) showed that selenate could act as an effective substrate for ATP sulfurylase and provided evidence for the formation of APSe. Recently, another laboratory has reported (3) that the formation of APSe could in fact not be demonstrated using techniques similar to those used by Wilson and Bandurski. The purpose of the second part of this thesis was to investigate the activation of selenate by ATP sulfurylase. It was concluded that APSe, or a similar selenate anhydride was indeed formed during the reaction.

A mechanism for the in vitro reduction of selenate to elemental selenium is proposed and its potential relationship to the in vivo pathway is discussed.

Part I.

STUDIES ON E. COLI SULFITE REDUCTASE

LITERATURE REVIEW

The ability to reduce sulfite to sulfide has been found in most microorganisms and plants. Since the early 1950's, several laboratories have spent considerable effort in studying this reduction and how it relates to the overall biological assimilation of sulfur. Several review articles containing discussions on this reduction have been published (4-10).

Role of Sulfite Reductase in the Assimilatory Sulfate Reduction Pathway

The involvment of a reductive step from sulfite to sulfide in the pathway of biological sulfate reduction was shown by four experimental approaches. Enzyme, mutant, *in vivo* labeling and regulation studies have provided a sound basis for the inclusion of the enzyme, sulfite reductase (E.C. 1.8.1.2. and E.C. 1.8.99.1), which catalyzes the reduction of sulfite to sulfide in the pathway of assimilatory sulfate reduction.

The ability of sulfite to be metabolized has been shown by numerous studies with microorganisms. These studies demonstrated that sulfite can be utilized by these organisms as their sole, nutritionally required, sulfur source (see reference 6). The relationship between the metabolism of sulfite and the metabolism of sulfate can be shown by competition studies. These studies showed that the metabolism of radioactive sulfate was suppressed by adding non-radioactive sulfite. The unlabeled sulfite had mixed with the labeled sulfite intermediate thus diluting the amount of radioactive sulfur appearing as reduced organic compounds. These studies were performed with E. coli (11), Salmonella (12), and mung bean leaves (13). The same kind of study using sulfide has shown that sulfide likewise suppresses the reduction and incorporation of radioactive sulfate (14). The demonstration of the formation of sulfite from sulfate in tomato plants (15), tobacco leaves (16), mung bean leaves (17), and yeast (18), further implicates the role of sulfite as an intermediate in the reduction of sulfate.

Enzymological studies have shown that in vitro the enzyme sulfite reductase can catalyze the reduction of sulfite to sulfide. The occurrence of another enzyme, O-acetylserine sulfhydrolase which forms cysteine from hydrogen sulfide and O-acetylserine provides a potential pathway for the reduction and incorporation of sulfite into amino acids. The formation of free sulfite from an enzymatic system has been shown (19), but further studies have suggested that the product is a "bound" sulfite (20) which can

be liberated by reaction with reduced thiol compounds (21). The existence of enzymes which are capable of catalyzing the proposed reaction, forming the substrate, and utilizing the product also provides evidence for this pathway.

A number of studies have been performed with genetic mutations in the sulfate reduction pathway. Several of these mutations have been shown to be associated with sulfite reductase activity. Mutations which do not permit growth on sulfate or sulfite but do permit growth on sulfide or sulfur amino acids have been shown in Salmonella (22), yeast (23), Neurospora crassa (24), and Aspergillus nidulans (25) to be associated with the lack of detectable sulfite reductase activity. Thus the genetic evidence also supports the concept that sulfite reductase is an essential part of the sulfate assimilatory pathway.

The regulation of sulfite reductase also supports this concept. The level of sulfite reductase has been shown to be regulated by an end-product of the assimilation pathway, cysteine. Cysteine has been shown to repress the level of sulfite reductase activity in E. coli (26), Salmonella (27), Bacillus subtilis (26), Neurospora (28), and yeast (29).

The evidence presented in this discussion strongly support the concept that sulfite reductase is an essential enzyme that catalyzes the biological reduction of sulfur. There is, however, some evidence that suggests that sulfite reductase may not be on the major pathway in all organisms. Schmidt and Schwenn (30) have reported the existence of an enzyme from Chlorella and spinach, which separates from

sulfite reductase during ammonium sulfate fractionation, that will reduce S-sulfoglutathione but not sulfite. S-sulfoglutathione contains the thiosulfuric acid group that has been proposed by Torii and Bandurski (21) to be the product of the PAPS reductase system. The formation of the S-sulfoglutathione from APS has been shown by Schiff and Hodson (10) and Schmidt (31) to be catalyzed by an enzyme they have called APS-sulfotransferase. The sulfotransferase catalyzes the same reaction as enzyme B of the PAPS reductase system except that APS is the preferred substrate.

The exact role of the thiosulfonate reductase in the reduction of sulfate has not been adequately determined, as the in vivo substrate and the enzymatic product have yet to be characterized. It should be noted, however, that Schmidt et al. (32) have reported a Chlorella mutant, incapable of assimilating sulfate, which lacks thiosulfonate reductase activity, but has sulfite reductase activity.

Properties of Sulfite Reductases from Various Organisms

Cell free extracts, capable of catalyzing the six electron reduction of sulfite to sulfide, have been obtained from E. coli (33, 34), Salmonella tryphimurium (35), Saccharomyces cervisiae (23), spinach (36), Allium (37), Aspergillus nidulans (25), Neurospora crassa (24), Porphyra (38, 39), and dissimilatory sulfate reducing bacteria (40, 41). The enzyme responsible for this reduction, sulfite reductase, has been purified from E. coli (42), Aspergillus

(25), Salmonella (43), Saccharomyces (44), spinach (45), and Allium (46). The physical, spectral, and catalytic properties of the enzyme preparations are presented in Table 1. E. coli, Salmonella, and yeast possess sulfite reductases which are similar in molecular weight, electron donors, reductions catalyzed, prosthetic groups and spectral properties. There is some variation in the amount of flavins and iron present in these enzymes but their properties are essentially the same. The enzymes from Aspergillus, Allium and spinach also seem to constitute a separate group. They are lower molecular weight proteins which do not accept electrons from NADPH. They also lack the flavins and the capacity to catalyze the reduction of nitrite. It should be noted that the enzyme from Aspergillus may not belong with this group. Yashimoto et al. (25) have stated that the protein may have broken down during purification, and had lost the NADPH coupling site. Also the ability to use nitrite as a substrate was not examined due to experimental difficulties. From an evolutionary standpoint one would expect that the Aspergillus enzyme would be similar to the yeast enzyme, another fungus, as opposed to the enzymes from higher plants.

The subunit structure of the enzymes from Salmonella and E. coli have been characterized by Siegel et al. (48) and Siegel and Davis (49) respectively. Both of these enzymes are composed of twelve polypeptide chains, eight "α" chains and four "β" chains. The polypeptides have a

Table 1. Properties of the purified sulfite reductase preparations.

Source	Molecular weight	Electron donor	Reductions catalyzed	Prosthetic group	Spectral properties
Yeast (44)	350,000[a]	MV NADPH	$SO_3^= \rightarrow S^=$ $NH_2OH \rightarrow NH_3^+$ $NO_2^- \rightarrow NH_3^+$	1 FMN 1 FAD 5 nonheme iron	5.313:1.0:0.223 (278:386:587)
E. coli (47)	670,000	MV NADPH	$SO_3^= \rightarrow S^=$ $NH_2OH \rightarrow NH_3^+$ $NO_2^- \rightarrow NH_3^+$	4 FMN 4 FAD 20-21 iron 3-4 siroheme 14-15 labile $S^=$	3.56:1.0:0.23 (278:386:587)
Salmonella (48)	674,000	MV NADPH	$SO_3^= \rightarrow S^=$ $NH_2OH \rightarrow NH_3^+$ $NO_2^- \rightarrow NH_3^+$	6 FMN 6.1 FAD 21.4 iron 18.4 acid labile $S^=$ 1.5-3 heme	1.0:0.24 (386:587)
Aspergillus (25)	4.23 S[e]	MV	$SO_3^= \rightarrow S^=$ [b] $NH_2OH \rightarrow NH_3^+$	-[c]	8.26:1.0:0.259 (279:384:585)
Spinach (45)	83,000-85,000	MV	$SO_3^= \rightarrow S^=$ $NH_2OH \rightarrow NH_3^+$	0.76 iron No flavin	2.326:1.0:0.442 (279:404:589)
Allium (46)	4.46 S	MV	$SO_3^= \rightarrow S^=$ $NH_2OH \rightarrow NH_3^+$	-[c]	Peak at 586 nm[d]

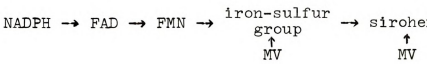
a. By flavin content. b. $NO_2^- \rightarrow NH_3^+$ not determined. c. Not determined.
d. Some contaminating protein present. e. Complete analysis not performed.

molecular weight between 50,000 to 60,000 with the "α" chains being a little heavier than the "β" chains. The enzyme can be dissociated into two subunits with one subunit containing only "α" chains and the other subunit containing only "β" chains (49). The "α subunit" can catalyze the reduction of cytochrome c by NADPH while the "β subunit" catalyzes the reduction of sulfite by reduced methyl viologen. The "α subunit" contains the flavins, while the "β subunit" contains the chromophore, iron, and acid labile sulfide.

All of the sulfite reductases isolated have similar spectral properties (Table 1). The chromophore has been shown by several studies to be closely related to the active site of sulfite reduction (50, 51). The structure of the chromophore was determined by Murphy et al. (52) and shown to be a new type of heme, a siroheme. The only other enzyme that appears to have the same type of heme is nitrite reductase (53) which likewise catalyzes the six electron reduction of an inorganic ion.

The Mechanism of the Enzymatically Catalyzed Reduction of Sulfite

The sequence of electron flow from the donor to the receptor molecules has been studied by Siegel and Kamin's group (48, 51, 54, 55) and Yashimoto and Sato (44, 50, 56). A proposed sequence for the electron flow through the enzyme molecule is shown below.



The yeast, Salmonella, and E. coli enzymes contain the whole sequence while the Aspergillus, Allium, and spinach enzymes lack the flavin containing subunit. The reduced methyl viologen could either donate electrons to the iron-sulfur component or to the siroheme or both. The siroheme passes the electrons to sulfite or other acceptors.

The mechanism of the enzymatic reduction of sulfite to sulfide is very important in biology as there are three, or possibly four, enzymatically catalyzed six electron reductions knwon. These enzymes include nitrogenase, nitrite reductase, sulfite reductase, and possibly thiosulfonate reductase. All biological nitrogen and sulfur have at one time been acted upon by these enzymes, thus the importance of six electron reductions is immense. Yet our knowledge about how the reductions are catalyzed is limited at best. There has been considerable work on the mechanism of nitrogenase, but the basic instability of the enzyme and the lack of radioactive nitrogen compounds have prevented the elucidation of the reduction pathway. The studies on nitrite reductase have also had the same technical problems. Sulfite reductase, on the other hand, is easily isolated and purified. There is a readily available radioactive sulfur isotope and the enzyme is relatively stable. Thus the best experimental system for studying enzyme-catalyzed, six

electron reduction should be sulfite reductase. Yet, only one published report by Kemp et al (34) has directly addressed the mechanism of sulfite reduction. Their hypothesis was based on the belief that serine was involved at the catalytic site of sulfite reduction. It should be noted that many reaction mechanisms may be written for the reduction and the hypothesis presented by Kemp et al (34) was without experimental support.

Siegel and Kamin (51) and Siegel et al (54) have published the only reports that give some potential insight into the mechanism of sulfite reduction. They reported that when radioactive sulfite was incubated with sulfite reductase and limiting amounts of NADPH, a tight enzyme-radioactive sulfur complex was formed. They concluded that the complex represented a covalent bond and hypothesized that this complex may represent a bound intermediate between sulfite and sulfide.

The purpose of the studies presented in the next section was to gather information about the mechanism of sulfite reductase catalysis by attempting to isolate intermediates between sulfite and sulfide. These studies included binding experiments similar to the studies of Siegel and Kamin (51) described above and attempts to inactivate the enzyme such that only a partial reduction could be observed.

MATERIALS AND METHODS

Chemicals

All of the chemicals (unless stated below) were reagent grade from commercial sources. Calcium phosphate gel was obtained from Bio-Rad Laboratories. The Sepharose 6B was purchased from Pharmacia Inc. The methyl viologen was from Mann Research Laboratories. The $Na_2{}^{35}SO_3$ was obtained from New England Nuclear Inc. Dr. G. Kennedy kindly provided the N,N,-dimethyl-p-phenylenediamine. Na_2S standard solutions were prepared according to Asada (36).

E. coli Sulfite Reductase Preparations

The preparation of this enzyme is essentially that used by Siegel and Kamin (42) with modifications as outlined in the text.

Cell Preparation

E. coli B (obtained from Dr. H. Sadoff of the Department of Microbiology at Michigan State University) was grown in the medium given by Siegel and Kamin (42) in two 6 l batches, then transferred to a 100 l fermenter and grown to late log phase. The pH was maintained at 6.5 to 7.0 by periodic additions of concentrated NH_4OH. The cells were harvested in a Sharples centrifuge and the packed cells frozen and stored.

Extraction and Purification

Step 1. E. coli cells (30-100 g) were suspended in two volumes of 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA and disrupted in a Branson Sonifier (model S-125) for 15 minutes in a 180 ml Rosette cell cooled in a salt-ice bath. The suspension was centrifuged for 30 minutes at 15,000 x g, the supernatant was collected and this is referred to as the crude extract.

Step 2. The protein content of the crude extract was determined and the pH of the extract adjusted to approximately 7.5 with 6 % (wt/vol) NaOH. Protamine sulfate (Sigma grade II), in a 5 % solution was adjusted to pH 7.0 using NH_4OH, and was added to the crude extract so that the ratio of protamine sulfate to protein was 0.27. The precipitate formed after 30 minutes of stirring was sedimented at 15,000 x g for 90 minutes and discarded.

Step 3. Ammonium sulfate (250 g) was slowly added to each liter of the supernatant solution. The mixture was stirred for 30 minutes and the pellet resulting from a 20 minute centrifugation at 6,000 x g was dissolved in sufficient 0.05 M K-phosphate buffer, pH 7.75, with 0.5 mM EDTA to yield a solution having a concentration of 20 mg/ml protein.

Step 4. Calcium phosphate gel suspended in water (220 ml of a 30.3 mg dry weight of gel per ml solution) was added to each liter of protein solution and stirred for 30 minutes. The solution was then centrifuged for 20 minutes

at 6,000 x g and the pellet discarded. Twice as much gel solution as added previously was mixed with the supernatant and stirred for 30 minutes. The pellet resulting from a 20 minute centrifugation at 6,000 x g was then resuspended in 167 ml of 0.5 M K-phosphate buffer, pH 7.75, with 0.05 mM EDTA for each liter of original protein solution. The solution was stirred for 20 minutes and centrifuged for 20 minutes at 6,000 x g. The pellet was resuspended in 600 ml of 0.2 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA per liter of original protein solution and stirred for 30 minutes. The solution was then centrifuged at 6,000 x g for 20 minutes to sediment the gel.

Step 5. Ammonium sulfate (186 g) was added to each liter of gel eluate, stirred for 30 minutes and then centrifuged for 20 minutes at 6,000 x g. An additional 45 g of ammonium sulfate was then added and the suspension stirred for 30 minutes. The pellet resulting from 20 minute centrifugation at 6,000 x g was resuspended in a small volume of 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA and dialyzed for 16 hours against the same buffer.

Step 6. The dialyzed material was loaded on a 5 x 70 cm Sepharose 6B column equilibrated with 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA. The column was eluted with the same buffer at a flow rate of 10-15 ml/hour.

Step 7. The active fractions were pooled and precipitated at 50 % ammonium sulfate with 30 minutes of stirring.

The pellet resulting from centrifugation at 6,000 x g for 20 minutes was resuspended in a minimal volume of 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA. FMN (1 mM), 170 µl, was then added and the solution dialyzed for 18 hours against 0.1 M K-phosphate buffer, pH 7.75, with 10 mM EDTA and then 18-24 hours with 0.1 mM K-phosphate buffer, pH 7.75, with 0.1 mM EDTA.

Sulfite Reductase Assays

Sulfite-dependent NADPH Oxidase Assay

The reaction mixture contained in µmoles: K-phosphate buffer, pH 7.75, 100; NADPH, 0.2; Na_2SO_3, 0.5; and enzyme in a total volume of 1.0 ml. The total mixture minus the sulfite was incubated in a spectrophotometer and the endogenous bleaching rate at 340 nm measured. Sulfite was tipped in and the rate of bleaching after tipping measured. The endogenous bleaching rate was subtracted from the rate with sulfite to obtain enzyme activity. One unit of enzyme was defined to be the amount of enzyme that would catalyze the oxidation of 1 µmole NADPH per minute at 24-25°C.

This particular assay may not correctly reflect the level of sulfite reductase activity in crude extracts. As shown in the appendix, this activity may be associated with different reactions.

Siegel's Sulfide Production Assay

This assay was a modification of the assay developed by Siegel (57). The reaction mixture contained in µmoles:

K-phosphate buffer, pH 7.75, 100; glucose-6-P, 5; NADPH, 0.2; Na_2SO_3, 0.5; with 0.06 units glucose-6-P dehydrogenase and enzyme in a total volume of 0.8 ml. The mixture was incubated at 24-25°C for 30 minutes in one-half dram screw cap vials fitted with teflon liners. The reaction was stopped by adding 0.1 ml of 0.03 M $FeCl_3$ in 1.2 N HCl and 0.1 ml of 0.02 M N,N,-dimethyl-p-phenylenediamine in 7.2 N HCl. The cap was replaced immediately after the addition of the reagents. The optical density at 664 nm was measured after 30 minutes. Na_2S was used to prepare a standard curve to relate OD_{664} to nmoles of H_2S produced.

Methyl Viologen Oxidation Assay

The methyl viologen oxidation assay developed by Asada (36) was used in several preliminary studies and in the inhibition studies. The bottom of a glass Thunberg cuvette received 150 µmoles of K-phosphate buffer, pH 7.75, 2 mg BSA, and enzyme in a total volume of 0.95 ml. The side arm received 1.5 µmole Na_2SO_3. The cuvette was evacuated and flushed with argon repeatedly until the cuvette was anaerobic. Reduced methyl viologen (0.6 ml of a 0.145 mg/ml solution) was transferred to the cuvette as previously published (36, 58) and the endogenous bleaching rate at 604 nm was measured. The sulfite was then tipped in and the bleaching rate measured. Activity was defined to be the sulfite dependent bleaching rate. The reduced methyl viologen was made according to Asada (36). The number of m moles oxidized per unit time can be calculated by multiplying the change in optical density by 1.143×10^{-4}.

Protein Assays

Biuret Assay

The method of Zamenhof (59) was used with the optical density of the colored product being read at 310 nm. BSA was used as a standard.

Lowry Assay

The protein was determined by the method of Lowry *et al.* (60), with BSA as a standard.

Gel Electrophoresis

Gel Preparation and Running Procedures

Davis gels. Electrophoresis gels (0.3 x 5 cm) were made and run using the solutions and methods of Davis (61) except that the running buffer was one half as concentrated. After the gels were removed from the glass tubes they were stained according to the procedures described below.

Urea gels. The solutions and methods of Jovin *et al.* (62) were used to make the gels. The enzyme preparation was treated with 45 µl of 0.05 M K-phosphate buffer, pH 7.75, 8 M urea, and 0.3 M mercaptoethanol for 12 hours at 24°C. A small sample of this mixture (20-40 µl) was mixed with 50 µl 4 % sucrose and loaded on the gels. The running buffers were as described by Jovin *et al.* (62). Bromophenol blue was used as a marker.

Staining Techniques

Coomassie Blue staining. The gel was placed in a test tube containing 50 % trichloroacetic acid for 1 hour.

The gel was then placed in 0.01 % Coomassie Blue in 50 % trichloroacetic acid for 1 hour and 20 minutes at 37°C. Acetic acid (7 %) was used to destain the gels.

Diaphorase stain. The gel was placed in a test tube containing 0.25 M K-phosphate buffer, pH 7.75, 2 mM NADPH, and 0.74 mg/ml nitro-blue tetrazolium until bands appeared.

^{35}S Binding Assay

Reaction Conditions

These procedures were those finally adopted to produce saturable binding. The reaction mixture which was in aluminum foil wrapped test tubes was started by adding the enzyme preparation and incubating for 5 minutes at 24°C. The reaction was stopped by placing the test tube in an ice bath. The samples were then loaded into the dialysis cells and the dialysis started. After 18 hours of dialysis the samples were removed and counted in Bray's scintillation fluid in a Beckman LS-133 Scintillation Counter using the ^{14}C settings. A minus NADPH control was run in each experiment and this value was subtracted from the other values to obtain NADPH dependent binding.

Dialysis Equipment and Running Procedures

Two four-cavity 1 ml flow-through microdialysis cells (Bel-Art Products Model 347) were hooked in series so that the effluent of the first chamber of the first cell passed through the first chamber of the second cell before being discarded. The flow through the chambers was regulated by a peristaltic pump. The dialysis was performed with the

cells mounted horizontally on a shaking platform. A bubble left in the solution being dialyzed provided mixing during the operation. Unless otherwise stated in the figure or table legends the buffer used in the dialysis was 0.1 M K-phosphate, pH 7.75, with 0.1 mM EDTA. The flow rate for the first 4-6 hours was 40 ml per hour; the flow rate for the remaining 12-14 hours was 20 ml per hour.

Each cavity of the cell was divided by one layer of dialysis membrane with Parafilm gaskets on both sides of the membrane. The membrane was prepared by the following treatments; the membrane was boiled for 15 minutes in 0.1 M K-phosphate buffer, pH 7.75, with 10 mM EDTA; left in 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA and 10 mM cysteine for 3 hours at 25°C; treated with 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA and 10 mM Na_2SO_3 for 3.5 hours at 25°C; and finally the membrane was thoroughly washed with 0.1 M K-phosphate buffer, pH 7.75, and 0.1 mM EDTA.

Stoichiometry and Inhibition Assays

The reaction mixture and procedures (except when modified as stated in the legend) described for the methyl viologen assay were used. The reaction was stopped by adding 0.15 ml of each of the Siegel reagents (described earlier) which had been placed in the neck of the Thunberg cuvette and sealed with a cap. The vacuum in the cuvette pulled the reagents into the cuvette. After 30 minutes of shaking the reaction mixtures were removed and centrifuged for 5 minutes

to remove the precipitated protein and read at 664 nm in a spectrophotometer. A standard curve using Na_2S was prepared with the cuvettes which did not contain active protein. After reduced methyl viologen was added the tubes were made aerobic and Na_2S and the two Siegel reagents were added. The Na_2S could not be placed under a vacuum because of its volitility. The rest of the procedures described above were then followed.

The ratio of sulfite-dependent methyl viologen oxidized and sulfite-dependent H_2S produced was then calculated.

RESULTS

Enzyme Purification

The purification procedure of *E. coli* sulfite reductase was essentially the procedure developed by Siegel and Kamin (42) with the exception of the concentration step and the FMN activation step which were done at the end of the procedure. The FMN activation step was required to regain activity lost during the Sepharose 6B column and ammonium sulfate concentration step. A detailed description of the purification is presented in the methods section. A summary of a typical purification is presented in Table 2. Certain data points are missing since it was not possible to assay all fractions due to volume constraints. The second ammonium sulfate fraction activity was too concentrated and the protein in the Sepharose 6B column eluent was too dilute.

Gel Electrophoresis of Purified Enzyme

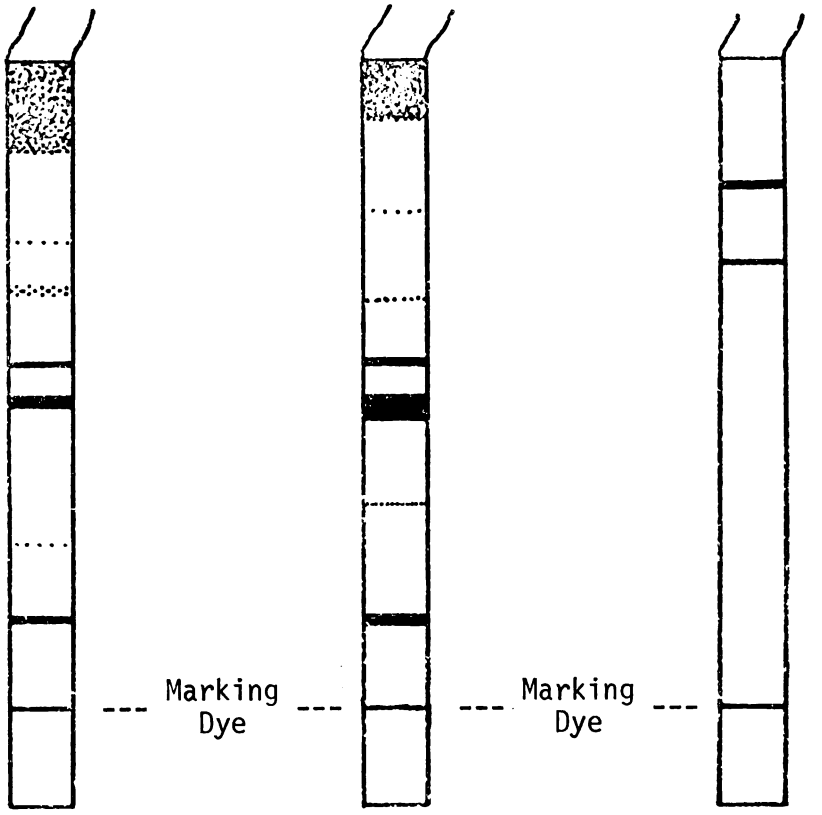
An attempt was made to establish the purity of the preparation using polyacrylamide disc gel electrophoresis. Seven bands were produced when the preparation was run on standard 7 % Ornstein-Davis gels and stained with Coomassie Blue (see Figure 2, number 1). These results were surprising since the preparation was thought to be homogeneous.

Table 2. Purification of sulfite reductase.

Fraction	Volume (ml)	Total protein[a] (mg)	Total activity[b]	Specific activity	Yield (%)
Crude	125	6,625	72.6	0.011	100
Protamine sulfate	142	4,550	57	0.0125	78.5
First $(NH_4)_2SO_4$	82.5	1,650	42.4	0.026	58.5
Ca-phosphate	50	129.5	25.35	0.196	35
Second $(NH_4)_2SO_4$	1.9	43.7	-[c]	-	-
Sepharose 6B	122	-	13.2	-	18.2
$(NH_4)_2SO_4$ + Dialysis[d]	Approx. 1.2	Approx. 6.44	Approx. 17.0	2.64	Approx. 23.4

a. Buiret assay. b. Sulfite-dependent NADPH oxidase activity. c. Could not be determined. d. Accurate volume measurements could not be made.

Packed E. coli cells (60 g) was used for this experiment. The procedures are described in the methods section.



1. Gel: Davis
   Stain: Coomassie Blue

2. Gel: Davis
   Stain: Diaphorase Activity

3. Gel: Urea
   Stain: Coomassie Blue

Figure 2. Gel electrophoresis studies of sulfite reductase.

The procedures used for these experiments are described in the methods section; 26.9 µg of sulfite reductase were used in each experiment.

An experiment was performed to find out which of the protein bands was sulfite reductase. Sulfite reductase has diaphorase activity and an assay for diaphorase is the enzyme dependent reduction of nitro-blue tetrazolium with NADPH. In the reduced form, nitro-blue tetrazolium is insoluble and thus makes a good gel stain. Figure 2 number 2 shows the banding pattern obtained with the activity stain. The five major bands stained with Coomassie Blue also stained with the diaphorase activity stain.

E. coli sulfite reductase has twelve polypeptide chains of two molecular weights with a proposed ratio of 4 chains of one to 8 chains of the other polypeptide (49). A homogeneous sulfite reductase preparation should show only 2 bands when denatured with urea, reduced with mercaptoethanol, and run on urea gels. Number 3 in Figure 2 shows that this was in fact the case. There was a minor third band that was just barely detectable. Its origin is unknown.

The unusual siroheme present in the sulfite reductase (52) permits, by its spectral properties, the estimation of purity by the ratio of the amount of siroheme present to the amount of protein. The absorption at 278 nm reflects the amount of protein present while the optical densities at 386 and 587 nm represent the amount of the prosthetic group. Siegel and Kamin (42, 51) have reported that the 278:386:587 ratio for a homogeneous preparation is 3.6:1.0:0.23. The protein preparation used in the electrophoresis experiments had a ratio of 3.62:1.0:0.22.

^{35}S Binding Studies

Siegel and Kamin (51) reported in 1967 that in preliminary experiments, radioactive sulfur from sulfite was bound to sulfite reductase when the reaction was run to completion with limiting amounts of NADPH. The following series of experiments were performed to verify and hopefully expand these studies. In this experimental design binding was defined as the amount of sulfur not removed from a reaction solution by extensive dialysis.

Non-saturable Binding

Initial experiments were hampered by high blank values which could not be significantly decreased by further dialysis. It was found that in these experiments the dialysis membranes became labeled. This high labeling, and the high blank value could be reduced by pretreating the membranes with EDTA, cysteine and unlabeled Na_2SO_3. Table 3 shows the results from an experiment using the pretreated membranes. The binding was NADPH-dependent.

The difference between the amount of binding, when limiting amounts of NADPH (Table 3, number 1) were used or excess amounts (Table 3, number 2) were used, showed that at least at the limiting concentration of NADPH no "saturation" of the binding had occurred. Figure 3a summarizes further experiments designed to study the saturation of this binding by holding the amount of sulfite used constant and in excess, while varying the amount of NADPH. Figure 3b

Table 3. Dialysis experiments using pretreated membranes.

Experiment number	Reaction conditions	cpm
1	Sulfite reductase $5{,}78 \times 10^{-2}$ nmoles NADPH 0.462 nmoles $^{35}SO_3^{=}$ (59 µc/µmole) 2.6 nmoles K-phosphate buffer (pH 7.75) 100 µmoles EDTA 0.1 µmoles in a total volume of 1.0 ml	2,140
2	Same as 1 with 2 µmoles NADPH	12,780
3	Same as 1 minus NADPH	373
4	Same as 3 minus enzyme	200

The reaction mixtures were incubated for 40 minutes at 25°C and placed in the microdialysis chambers. The dialysis continued for 18 hours with a flow rate of 30 ml/hour for each chamber. The dialysis system and membrane pretreatment procedure are described in detail in the methods section. The specific activity of the enzyme preparation was 2.07 units/mg.

Figure 3. NADPH-dependent binding at various substrate concentrations.

(a) The reaction mixture contained in μmoles: sulfite reductase, 6.57×10^{-5}; $Na_2{}^{35}SO_3$, 60×10^{-3}; K-phosphate buffer, pH 7.75, 100; EDTA, 0.1; and varying amounts of NADPH in 1 ml. The reaction conditions and dialysis procedures are as in Table 3.
(b) The reaction mixture contained in μmoles: sulfite reductase, 6.57×10^{-5}; NADPH, 3; K-phosphate buffer, pH 7.75, 100; EDTA, 0.1; and varying amounts of $Na_2{}^{35}SO_3$ in 1 ml. The reaction conditions and dialysis procedures are as in Table 3.

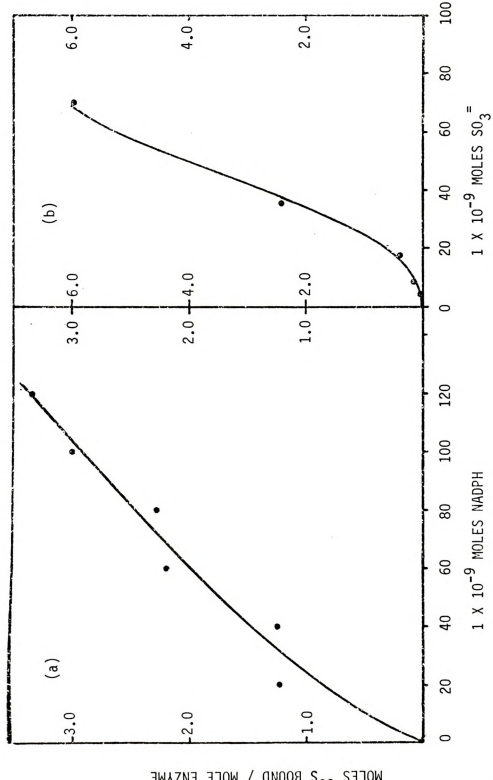

(a)
(b)
3.0
2.0
1.0
6.0
4.0
2.0
0
20
40
60
80
100
120
MOLES ^{35}S BOUND / MOLE ENZYME
1 X 10^{-9} MOLES NADPH
1 X 10^{-9} MOLES $SO_3^=$

Figure 3.

summarizes an experiment where NADPH was held constant and in excess and the amount of sulfite varied. In both cases there was no saturation, and as shown in Figure 3b, excess NADPH did not cause removal of radioactive sulfur from the enzyme. Binding phenomena and intermediate accumulation normally show "saturation", as the catalytic site can only process a limited number of molecules. Inorganic sulfur compounds easily form complex polymers and it is possible that the reaction mechanism of this enzyme involves such polymers.

Experiments to demonstrate a polymeric form of sulfur were somewhat successful. Dialysis of a complete reaction mixture with a sodium sulfide solution removed counts from the dialyzed solution. Sodium sulfide can easily cleave sulfur-sulfur bonds of the type that might be formed in a polymer. Further experimentation, however, showed that the inclusion of Na_2S in the reacting mixture caused a considerable increase in the number of counts bound. Sulfide, when left in solution under proper conditions, can polymerize to form elemental sulfur and polysulfides. These compounds are very insoluble and probably would not be dialyzable, but would be cleaved by excess hydrogen sulfide.

Saturable Binding

The product of the sulfite reduction is hydrogen sulfide. Addition of a small amount of exogenous sulfide to the solution might initiate and accelerate the polymerization. The conditions that catalyze the polymerization of

H_2S are light, heavy metals, and heat. Experiments were designed to minimize the effects of these catalysis, the results of which are shown in Figure 4. The binding was now saturable when NADPH was varied and the amount of sulfite held constant.

The sulfite reductase preparation used in the saturation experiment saturated at 0.42 moles of radioactive sulfur bound (NADPH-dependent) per mole of enzyme. This value was calculated by computing the average for all points obtained from NADPH concentrations of 1 mM and above. This particular preparation was 51.5 % active when compared to the literature value of 2.73 units/mg (42). When a series of experiments was performed with different homogeneous preparations of various specific activities, different molar ratios of binding were obtained. These data are presented in Figure 5. Linear regression analysis of the points showed that if the preparation was completely inactive, the NADPH-dependent binding would saturate at 0.84 moles of radioactive sulfur bound per mole of enzyme. Attempts to increase binding by inactivating the enzyme by flavin removal were unsuccessful.

Characterization of Saturable Binding

Characterization of the bound radioactive sulfur showed that the radioactivity remained bound to the enzyme during column chromatography (Figure 6). The radioactivity could not be released by continued dialysis against saturated SDS or 50 % ethanol. Also, further incubation with

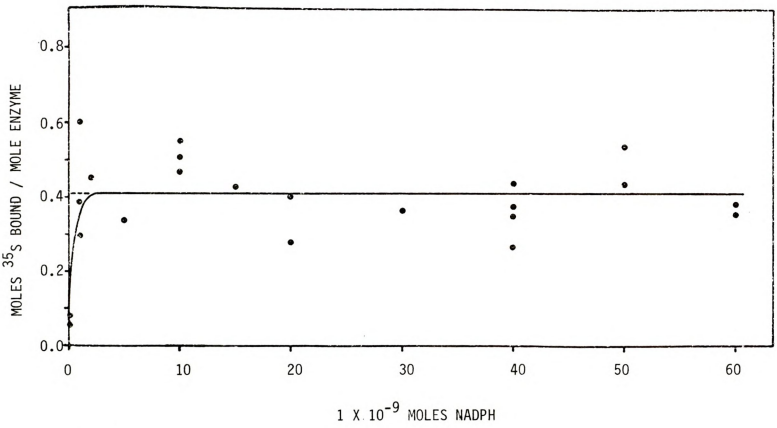


Figure 4. Saturation of NADPH-dependent binding.

The reaction contained in μmoles: K-phosphate, pH 7.75, 100; sulfite reductase, 2.92 x 10^{-5}; $Na_2{}^{35}SO_3$, 11.2 x 10^{-3} (23 mc/mmole); EDTA, 100; NADPH as stated on graph, in a total volume of 1.0 ml. The reaction conditions and dialysis procedures are described in the methods section.

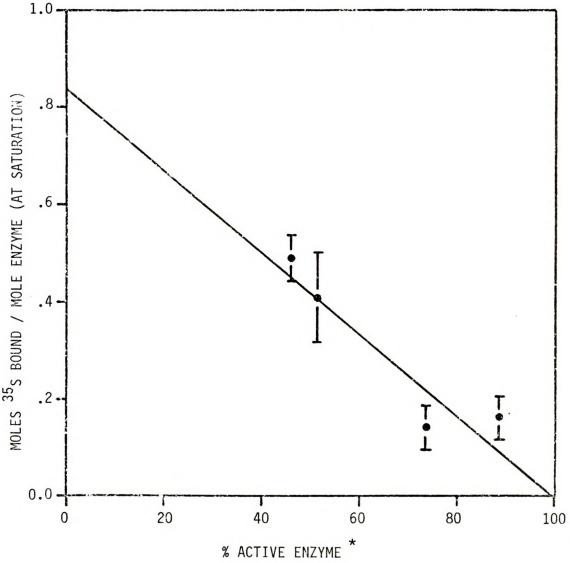


Figure 5. Saturable binding as a function of enzyme inactivation.

The level of saturation for various preparations were measured as in Figure 4.
* Percent activity of the preparation with 2.73 units/mg equaling in 100 %.

Figure 6. Elution of bound radioactive sulfur with protein and sulfite reductase activity during Sepharose 6B column chromatography.

Sulfite reductase (51 % active), 6.57×10^{-5} µmoles, was labeled under conditions that would saturate the binding of ^{35}S. The sample was dialyzed as described in the methods section under saturation binding assay. The sample was then placed on a Sepharose 6B column (1 x 67 cm) and eluted with 0.1 M K-phosphate buffer, pH 7.75, with 0.1 mM EDTA. The flow rate was 4 ml/hour with each fraction receiving 2 ml. The activity was measured by the normal NADPH oxidation assay (see methods section) with 0.02 µmoles FMN included in each reaction mixture.

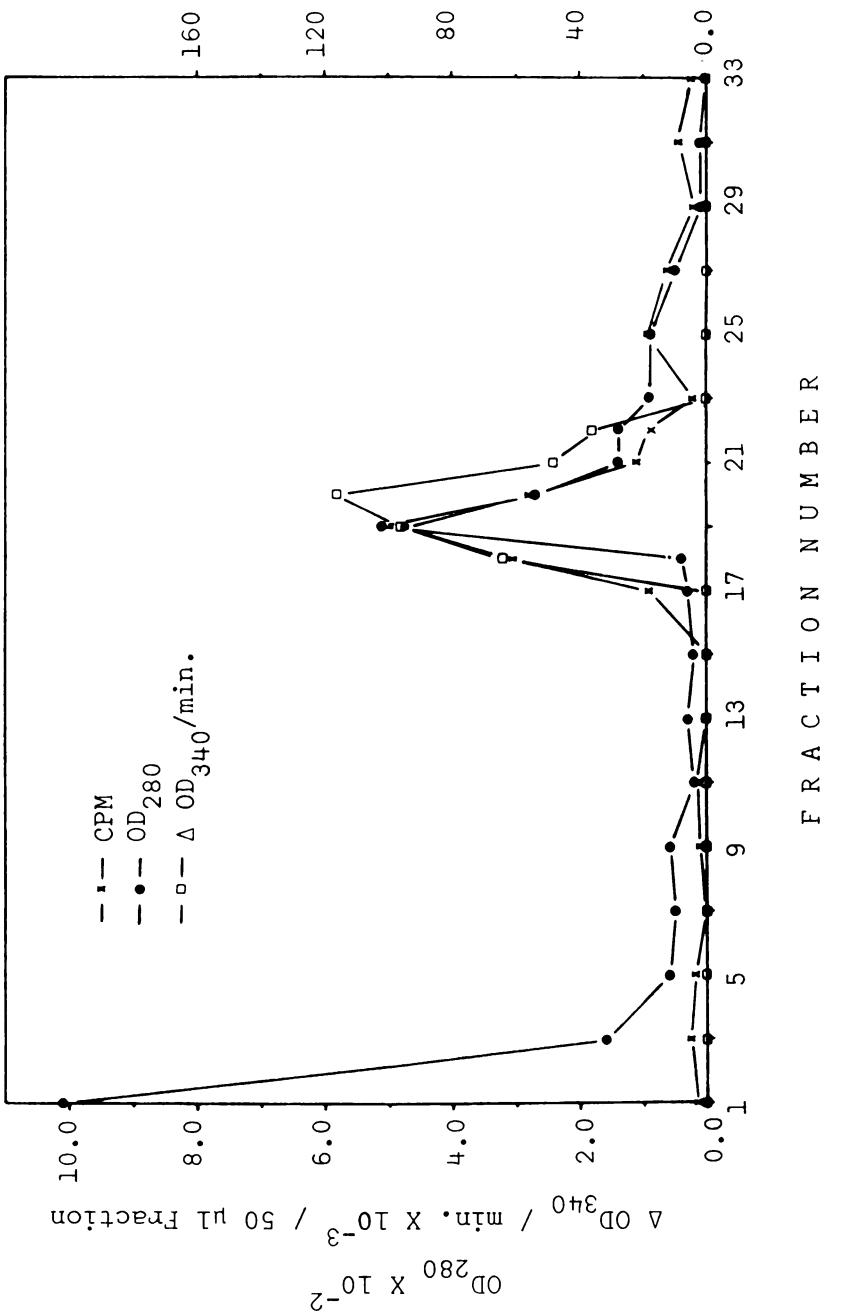

CPM
OD_{280}
Δ OD_{340}/min.
OD_{280} X 10^{-2}
Δ OD_{340} / min. X 10^{-3} / 50 µl Fraction
FRACTION NUMBER
0.0
2.0
4.0
6.0
8.0
10.0
0.0
40
80
120
160
1
5
9
13
17
21
25
29
33

Figure 6.

NADPH did not remove a significant amount of the radioactivity.

Stoichiometry and Inhibition Studies

The following experiments were quite different from the studies presented above. The experiments were designed to modify the sulfite reductase enzyme so that it would not produce hydrogen sulfide, but would still consume reductant (i.e. to create an enzyme that only catalyzed a partial reduction).

As shown in Table 4, the only procedure which dramatically increased the amount of methyl viologen consumed per sulfide produced appeared to be the treatment with sodium formaldehydesulfoxylate. Sodium formaldehydesulfoxylate does inhibit sulfite reductase activity, but the change in stoichiometry was only due to the inhibition of Siegel's sulfide assay. This result was shown as treatment 11, which reflects the inhibition of the sulfide assay, and has the same apparent stoichiometry as treatment 10.

The sequence of sodium formaldehydesulfoxylate addition was important to the amount of inhibition observed. A final concentration of 1.1-1.3 mM sodium formaldehydesulfoxylate was required to produce 50 % inhibition of the methyl viologen oxidation activity when the sodium formaldehydesulfoxylate was added from the side-arm with the sulfite. When the sodium formaldehydesulfoxylate was added to the bottom of the cuvette, prior to evacuation, only

Table 4. Inhibitor studies on sulfite reductase.

Treatment	Inhibition of MV oxidation	Stoichiometry (MV oxid./$S^{=}$ prod.)
1. None	0 %	5.55
2. Hexane	66	6.56
3. 24 hours at 23°C	0	5.51
4. Reduction-oxidation	63	4.9
5. Dichloroethane	20	5.77
6. 50 % ethanol	18.5	5.71
7. 95 % ethanol	0	5.37
8. Pyridine	-[a]	5.59
9. Minus BSA	63	6.91
10. SFSO[b]	38	17.95
11. SFSO added during sulfide assay	0	18.55

a. Percent inhibition varied from 0 to 80%. b. Sodium formaldehydesulfoxylate.

1. The basic assays are described in the methods section.

2. An equal volume of hexane was added to the enzyme preparation and sonicated for 1 minute. The solution was centrifuged and the aqueous layer was used for the enzyme preparation.

3. The enzyme preparation was aged for 24 hours at 23°C before use.

4. Water (0.45 ml) was left out of the assay mixture, then 0.45 ml of reduced methyl viologen was added. The solution was then oxidized and the normal assay procedures followed.

5. Dichloroethane saturated water (0.45 ml) was used.

6. Water (0.45 ml) was left out and replaced with 50 % ethanol.

7. Water (0.45 ml) was left out and replaced with 95 % ethanol.

8. Pyridine (10 µl) was included in the reaction mixture.

9. The BSA solution was left out of the reaction mixture and replaced with water.

10. Sodium formaldehydesulfoxylate (1.5 µmoles) added with Na_2SO_3.

11. Sodium formaldehydesulfoxylate (1.5 µmoles) added with sulfide reagents.

0.15-0.2 mM was required to give 50 % inhibition. These amounts may be higher than actually required as some of the formaldehydesulfoxylate may have been lost during the evacuation process. The varying effects shown by ethanol and pyridine may also be due to losses that occurred during the evacuation and flushing procedures.

DISCUSSION

This series of experiments was an attempt to elucidate the mechanism of the enzymatic conversion of sulfite to sulfide by E. coli sulfite reductase.

The E. coli cells were grown and the enzyme purified using essentially the procedure of Siegel and Kamin (42). The major difference was the requirement for a FMN activation step to obtain maximum activity of the purified preparation. The preparation appeared to lose most of its activity after the Sepharose 6B column step at which point the enzyme was very dilute. The loss of activity upon dilution can be explained by FMN dissociating from the enzyme with a dissociation constant of from 3×10^{-8} M (48) to 1×10^{-8} M (55). The FMN was not lost during a long term dialysis after it had been concentrated and reactivated. Siegel and Kamin (42) used much more starting material and thus did not appear to have this problem.

Studies to determine if the preparation was electrophoretically pure were at first disturbing as seven protein bands were observed. The same gels when stained for diaphorase activity showed that five of the major protein bands also had diaphorase activity, a reaction catalyzed by sulfite reductase. The results obtained from urea gels showing only two polypeptides were the same as reported by

Siegel et al. (48) for Salmonella sulfite reductase and Siegel and Davis (49) for E. coli sulfite reductase, showing that the preparation was pure. It appeared that during electrophoresis on 7 % polyacrylamide gels the protein separated into a number of subunits. Siegel et al. (48) have proposed that the enzyme contains twelve polypeptide chains with eight of them being responsible for the diaphorase activity and four containing the catalytic site itself. Further studies (49) have confirmed this. Thus it would be possible for the protein to break down into various combinations, many of which would retain the diaphorase activity. Another criterion of purity, the ratio of prosthetic group to protein as shown by the height of the absorption maxima, was the same as the published value (42).

In 1967, Siegel and Kamin (51) reported that it was possible to form a stable complex between radioactive sulfur, derived from sulfite, and pufified sulfite reductase. This binding occurred when sulfite reductase was reacted with excess ^{35}S-sulfite and limiting amounts of NADPH. The radioactivity remained bound during gel filtration and showed a maximum binding of 1 to 2 moles radioactive sulfur per mole of enzyme. In their discussion, they speculated that the radioactivity reflected an enzyme-sulfite complex or an intermediate in the reduction of sulfite to sulfide which was trapped by the disappearance of the reducing agent.

The gel filtration step used by Siegel and Kamin (51) did not easily lend itself to a large number of studies and required an appreciable amount of enzyme, thus an assay using dialysis was developed. After technical problems with the dialysis membranes were corrected, NADPH-dependent binding was observed (see Table 3). This binding phenomenon was not saturable, i.e. as either the NADPH or sulfite level was increased so did the binding, as shown in Figure 3. The bound radioactivity could be released by adding H_2S, but the binding could be increased if H_2S was added to the reaction mixture. These results are consistent with the formation of polysulfides or elemental sulfur. Precautions were taken to prevent sulfide polymerization and this "binding" ceased to occur, implying that in fact polysulfides and/or elemental sulfur formation was responsible for the observed "binding". The binding reported by Siegel and Kamin (51) was not exchangable with H_2S and saturated, thus eliminating this explanation for their binding.

The prevention of sulfide polymerization permitted another kind of NADPH-dependent binding to be observed. This binding was saturable (Figure 4). The enzyme preparation used in Figure 4 permitted binding that saturated at 0.41 moles of sulfur bound per mole of enzyme. This particular enzyme preparation had approximately 50 % of the specific acitivity as the preparations described in the literature (42). As different enzyme preparations were purified from the same E. coli, there was some fluctuation

in the total maximum amount of activity restorable by FMN. The ratios of their absorption maxima showed that the preparations were pure. Figure 5 shows that the maximum number of moles of radioactive sulfur bound per mole of enzyme could be related to the specific activity of the various enzyme preparations. The saturated NADPH-dependent binding of radioactive sulfur to the enzyme decreased linearly as the specific activity of the preparations increased. Linear regression analysis indicated that if the preparation was as active as the preparations described in the literature, there would be no observed binding. If the preparation was totally inactive, 0.84 mole of sulfur would have been bound.

The cause of the inactivation could not be determined. The only deliberate way to cause the inactivation (and increased binding) was to keep the preparation in the freezer for long periods of time (6 months to a year). Flavin removal inactivated the enzyme but did not give the enzyme increased binding capabilities. The bond that was formed between the radioactive sulfur and the enzyme appeared to be covalent, as the counts remained bound during gel filtration (Figure 6) and dialysis against SDS and ethanol (preliminary evidence suggested that 8 M urea also did not remove the counts).

Siegel et al. (47) have shown that there are 3 or 4 moles of siroheme per mole of enzyme. The siroheme has been shown to be associated with the catalytic site of

sulfite reduction (50, 51). If there is one heme per active site there would be three to four active sites per molecule of enzyme. If there are two hemes per active site then there would be one and a half to two hemes per enzyme molecule. The binding reported here had a ratio of approximately one mole ^{35}S-sulfur bound per mole of enzyme. The binding was only associated with an inactive enzyme and presumably it was the inactivation that permitted the binding. Accordingly, this would imply that one inactivation event occurred per molecule of enzyme. As there are between one and a half to four active sites per enzyme molecule, the inactivation event and the binding must have occurred at a site other than the site of sulfite reduction. Thus the binding that was observed appeared to be a phenomenon associated with inactivation as opposed to the formation of a sulfite-active site complex or reaction intermediate.

The attempts to modify the reaction catalyzed by sulfite reductase, as shown by a change in the stoichiometry of the reaction, were unsuccessful (Table 4). However, the studies did provide potentially useful inhibitor data. Organic solvents (hexane, dichloroethane, and ethanol) inactivated the enzyme, possibly by altering the configuration of the protein. The siroheme has been shown (51) to be distroyed by heat, acid and urea which likewise alter protein structure. Pyridine, which inhibited as much as 80 % of the activity, complexes with hemes and

presumably the inhibition was caused by this complex being formed.

The inhibition caused by sodium formaldehydesulfoxylate is interesting as it can be a relatively specific inhibitor. The mechanism of the inhibition is probably related to the structural similarity between the formaldehydesulfoxylate and sulfite. Sodium formaldehydesulfoxylate is not very toxic, as 10 g given intravenously can be tolerated by humans (63). Thus it very likely does not cause any general metabolic inhibition, yet can be an effective sulfite reductase inhibitor.

In summary, the purpose of the studies discussed above was to gather information about the mechanism of the enzyme-catalyzed reduction of sulfite by attempting to isolate intermediates between sulfite and sulfide. These studies included binding studies and attempts to modify the enzyme such that only a partial reduction could occur. While the binding studies gave results that were consistent with the formation of a bound intermediate, further studies led to the conclusion that the binding was a phenomenon associated with an inactivation process that was not directly related to the catalytic site of sulfite reduction. The attempts to form an enzyme that would catalyze only a partial reduction were unsuccessful but did provide some potentially helpful information about the inhibition of sulfite reductase.

Part II.

THE ACTIVATION OF SELENATE BY YEAST ATP SULFURYLASE

LITERATURE REVIEW

The Biology of Selenium

Interest in the biological role of the element selenium has peaked several times during the last fifty years. During this time selenium has been shown to cause several animal diseases, with symptoms arising from both too much and too little dietary selenium. Selenium has also been related to such diverse diseases as lung cancer and dental cavities. The discovery that several common livestock diseases were caused by excessive selenium intake first brought attention to the biology of selenium. Another major peak occurred following the demonstration that selenium is an essential micronutrient in animals. Recently the discovery of several enzyme systems that require selenium has brought about renewed interest in the biochemistry of selenium.

During the early thirties, researchers affiliated with the U.S. Department of Agriculture correlated the ability

of certain range plants to cause several cattle diseases with the selenium content of these plants. The common diseases that have been shown to be caused by selenium toxicity include blind staggers and alkali disease. The type of disease symptoms displayed by the animal was related to the chemical form of the ingested selenium (64). These results led to many studies which characterized the selenium compounds found in plants and their relative toxicity in animals. Much of this work is reviewed by Rosenfeld and Beath (64) and Trelease and Beath (65).

In 1957 Schwarz and Foltz (66) discovered that selenium is an essential micronutrient for rats. Diseases which have been shown to be caused by selenium deficiency include exudative diathesis, white muscle disease, liver necrosis and pancreatic fibrosis (see reference 67). Several cases of Kwashirokor, a human disease normally associated with protein deficiency, have been cured by increasing the level of dietary selenium (68, 69). Interest in the nutritional role of selenium declined a little in the late sixties until several enzymes were isolated which required a selenium cofactor for activity. The selenium containing enzymes thus far described in the literatures are glutathione peroxidase (70, 71), formate dehydrogenase (72) and glycine reductase (73). A selenium binding protein has been isolated from muscle (74), but its function is not yet known. Several books have been published which contain extensive sections on the biology of selenium and

are useful references (64, 65, 75, 76, 77). Other useful review articles, which are not included in the books mentioned above, have been published (78, 79, 80).

The Relationship between the Biology of Selenium and Sulfur

Selenium like sulfur, is a group VI element and thus the chemical and physical properties of most selenium compounds are the same or similar to sulfur compounds. The common inorganic forms of selenium; selenate, elemental selenium, and selenide behave much the same as sulfate, elemental sulfur and sulfide. Selenate is more readily reduced than sulfate while selenide is more readily oxidized than sulfide. Both elemental selenium and elemental sulfur are relatively non-reactive. Selenite, and sulfite are the most dissimilar of the selenium-sulfur analogues as selenite is a better oxidizing agent than sulfite. These facts have led, almost since the conception of the biology of selenium, to attempts to relate the biology of selenium to that of sulfur.

There are several basic lines of evidence to support the contention that the biology of selenium is closely related to the biology of sulfur. One strong piece of evidence is the fact that most naturally occurring selenium compounds are analogues of naturally occurring sulfur compounds. Most of the selenium compounds that have been identified in plant material are analogues of cysteine and methionine derivatives. Included in this category would be analogues of cysteine and methionine metabolites,

homocysteine and cystathionine. Many of the derivatives involve methylation, particularly in plants which accumulate large amounts of selenium. The methylation has been proposed to be a detoxification mechanism (81). Even inorganic selenium compounds have been shown to be methylated in plants, microorganisms, and animals. Dimethyl selenide is one of the most commonly found methylated selenium compounds. The chemistry of the naturally occurring selenium containing amino acids has been reviewed by Murti (82).

Hurd-Karrer (83) in 1937 provided evidence of a different type, showing the similarity between the biology of sulfur and selenium. She determined the selenium and sulfur content of different parts of various crop plants. The data showed that the ratio of sulfur to selenium was essentially constant, even though the absolute amounts varied widely. Later studies have shown that this correlation is not always exactly true as some plants grown on seleniferous soil had a different ratio in various plant parts, but the overall trend was still observed.

Hurd-Karrer (84) also demonstrated the ability of sulfate to protect against the toxic effects of selenate in plants. Since this original discovery many studies have confirmed the ability of sulfate to protect against selenate toxicity in many organisms. There are a few exceptions, but the generalization of this phenomenon seems valid. Several studies have shown that this antagonism is competitive in nature, implying that the two

compounds interact directly in some manner. Leggett and Epstein (85) have shown that these two substrates compete for the same uptake process. Other sulfur compounds have been shown to completely inhibit the toxicity of selenium compounds. All of these pairs are strict analogues and in several cases the competition occurs at the absorption or uptake level. These pairs include cysteine-selenocysteine and methionine-selenomethionine.

The toxicity of a selenium compound can often be inhibited by a non-analogous sulfur compound. In these cases the inhibition is non-competitive. For example, the toxic effect of selenate on Desulfovibrio is non-competitively inhibited by sulfite (86); and in Chlorella, selenate toxicity is non-competitively inhibited by methionine (87). This type of antagonism provides strong evidence for the interaction of sulfur and selenium indirectly at a site or process that is metabolically separated.

In these studies the toxicological properties of selenium are being used to assay for the interaction of sulfur and selenium. Thus the results presented above are directly related to the validity and reproducibility of the toxicity itself. The mechanism of selenium toxicity is unknown and does have unusual properties which can cause experimental problems. Some organisms can be adapted to grow on selenium. Schrift and Kelly (88) trained E. coli to utilize selenate instead of sulfate. The cells that were given only selenium, instead of sulfur, were able to grow

and divide. Thus the observed antagonism may, in fact, be an adaptation phenomenon.

Despite some contradictory evidence, the reason for the toxicity of selenium is generally agreed to be that selenium effectively replaces the sulfur in a compound and prevents the normal function of the sulfur compound from occurring. An example of the evidence for this is the data that show that D-selenocystine is much less toxic than L-selenocystine (89), the analogue of the biologically active isomer. Evidence which contradicts this hypothesis has been reported, showing that several common sulfur compounds have been replaced by their selenium analogues yet they function as well or better than the original sulfur compound. Selenium containing ferridoxin can function effectively as an electron transport molecule (90). Methylation reactions using Se-adenosyl methionine proceed as well as with S-adenosyl methionine (91). The important biochemical compound coenzyme A can be effectively replaced by seleno-coenzyme A in vivo (92). The lack of knowledge about the site of selenium toxicity greatly hinders the evaluation of the antagonism studies.

The selenium compound, selenite appears to act differently in that its toxic effects can not be prevented by any sulfur compounds. These results may be explained by the fact that selenite reacts with thiols forming a complex, or oxidizing the thiols. Thus the toxic effect of selenite may be to destroy essential thiols.

Selenium Metabolism

The relationship between sulfur and selenium, as demonstrated by the studies mentioned previously, should also be observed at the biochemical level. The biochemistry of sulfur and selenium may be divided into three main parts; the uptake or absorption process, the activation and reduction of the inorganic anions, and the metabolism of organic compounds. As mentioned earlier the uptake of many selenium compounds appear to use the same enzyme systems that normally function in the uptake of sulfur compounds. The interconversion of the seleno- and sulfur-amino acids, and their incorporation into proteins has recently been reviewed (93). The following discussion will concern itself with the metabolism of inorganic selenium compounds.

Inorganic selenium is commonly found in four oxidation states, + 6, + 4, 0, and - 2. These represent the oxidation states of, respectively, selenate, selenite, elemental selenium, and selenide. The known metabolism of inorganic selenium involves the reduction of one oxidation level to a lower state. There probably are metabolic routes that involve the oxidation of selenium compounds but as of yet they have not been conclusively demonstrated (93).

Selenate Reduction

Selenate, being the most oxidized common selenium compound, logically represents the first possible substrate

for the overall reduction. Potter and Elvehjem (94) first suggested that selenate was metabolized from their studies on succinoxidase. They showed that selenite immediately inactivated the enzyme while selenate toxicity slowly increased with time of incubation in the crude enzyme preparation. From this observation, they speculated that the selenate was slowly being converted to the inhibitory selenite. Selenite has not been shown to be oxidized to selenate by any biological system (95). Thus the equal toxicity of selenate and selenite (95) implies that selenate must be converted to a form resembling selenite or a selenite metabolite. A similar phenomenon was observed with animal nutrition studies. Schwarz and Foltz (96) have shown that selenate and selenite are essentially equally effective in preventing selenium deficiency.

Selenate has been shown to be enzymatically converted to selenite by Rosenfeld and Beath (97) using various bovine tissues. The liver tissue catalyzed the conversion of selenate to selenite while autoclaved tissue did not. Other tissues which could promote this reaction included whole blood (shown to be associated with the plasma fraction) and the spleen. This reduction did not occur in Guinea pig intestine and bovine red blood cells.

The conversion of selenate to elemental selenium (a 6-electron reduction) has been reported to occur in bacteria (98, 99), fungi (99), and beaf spleen (97). The

reduction of selenate to the selenide level has also been reported in many organisms. Rosenfeld and Beath (97) described the formation of "volatile selenium". The volatile selenium could have been hydrogen selenide or more likely dimethyl selenide. The conversion of selenate to dimethyl selenide has been reported in fungi (100, 101), and animals (102). This conversion in animals is believed to be a liver mediated detoxification process (102, 103) as the dimethyl selenide formed is much less toxic than most other selenium compounds (104).

The reduction and incorporation of selenate into more complex organic compounds has also been suggested. Bovine liver, spleen and blood have shown to tightly bind selenate after a 2-hour incubation, but the exact nature of the complex was not determined (97). Hirooka and Galambus (103) noted the conversion of selenate to a non-dialyzable compound by incubating mixtures of liver homogenate, ATP, and methionine. Awwad et al. (105) have shown the incorporation of selenium from selenate into seleno-amino acids in the rat. The selenium-containing cofactor required by several enzymes is thought to be a reduced, organic selenium compound (80) and the ability of selenate to fulfill the nutritional requirement for selenium implies that selenate can be metabolized to this cofactor.

Selenite Reduction

The reduction of selenite has been studied more thoroughly than the reduction of selenate. The probable

reason for this is the fact that selenite is much more rapidly metabolized than selenate. Hirooka and Galambus (103) showed that the conversion of selenate to dimethyl selenide was much slower than the reduction of selenite to dimethyl selenide in rats.

The four electron reduction of selenite to elemental selenium has been shown to occur in bacteria (99, 106, 107), fungi (99, 108), and animal tissues (97). The enzymatic nature of this reduction is hard to establish but this conversion was not observed in autoclaved liver. Ganther and co-workers (109, 110, 111) have studied the chemical reduction of selenite by thiols and have proposed a mechanism for this reduction (see discussion section). They also noted (110, 111, 112) that the enzyme, glutathione reductase, could catalyze several of the reaction steps. The selenium intermediates are the same, but the reduction mechanism is different. The ability of glutathione reductase to catalyze the reduction of selenite has thrown some doubt on the work of Nickerson and Falcone (113) who reported the need for a yeast enzyme preparation, menadione, glutathione, glutathione reductase and NADPH to catalyze this reduction.

The reduction of selenite to selenide has been described in bacteria (114) and rat liver homogenate (115). This reduction, like the reduction to elemental selenium, proceeds easily with reduced thiols. In fact, the formation of elemental selenium may proceed through hydrogen

selenide. Ganther (110) has proposed that the intermediate R-SSeH can be reduced to hydrogen selenide or can rearrange to form elemental selenium. The hydrogen selenide could also be easily oxidized by many compounds to elemental selenium. The oxidation of hydrogen selenide occurs so readily that unless the reaction is performed anaerobically only elemental selenium would be observed. Like the reduction to elemental selenium the reduction to hydrogen selenide is catalyzed by glutathione reductase. The glutathione reductase also catalyzes the last step R-SSeH $\xrightarrow{NADPH}$ R-SH + H_2Se. The formation of dimethyl selenide has been proposed to involve the methylation of hydrogen selenide (111). Thus the production of dimethyl selenide reflects the formation of hydrogen selenide. Dimethyl selenide formation from selenite has been reported in Penicillium (101), liver and other animal tissues (97, 103, 116).

The formation of complex organic compounds, amino acids and cofactors from selenite has also been reported. Falcone and Gianbanco (117) reported the synthesis of seleno-amino acids from selenite in cell free extracts of yeast. The ability of selenite to provide the selenium cofactors required by formate dehydrogenase (118) and glycine reductase (73) has also been demonstrated.

Elemental Selenium Reduction

There has been one report on the biological reduction of elemental selenium to hydrogen selenide. These studies were performed by Woolfolk and Whitely (114) using

anerobic bacteria. The same preparation was able to reduce many inorganic compounds including most inorganic ions and elements.

The Relationship between the Metabolism of Selenium and Sulfur

In the above discussion of the biochemical reduction of inorganic selenium compounds very little reference has been made to the relationship between the metabolism of sulfur and selenium. The evidence for this relationship is contradictory. There is evidence that suggests that different pathways do exist, while other data support the conclusion that at least part of the pathways are the same.

Pathways Where Selenium and Sulfur Metabolism Differ

Several lines of evidence suggest that the pathway of selenium reduction is not the same as the sulfur reduction pathway. A strong argument can be presented from the nutritional toxicity and *in vivo* labeling studies conducted with animals. Non-ruminant animals, with the possible exception of the cat family (95), are incapable of reducing inorganic sulfur. They have the ability to form the activated sulfate compounds, APS and PAPS, but further reduction has not been observed. Selenate and selenite, on the other hand, can be reduced by animals as discussed above. This clearly indicates that the ability to reduce selenium is independent of the ability to reduce sulfur.

There are several biosynthetic pathways which are open to sulfur but not to selenium. Nessen and Benson (119)

noted that all of the pathways closed to selenium involved the intermediate, PAPS. The major pathways involved are the formation of the sulfate esters and sulfolipids (see the review by Schrift (93) for a complete summary of these closed pathways) Wilson and Bandurski (2) were not able to detect radioactive PAPSe formation. Thus the synthesis of this key intermediate seems doubtful. The reduction of sulfate in microorganisms proceeds through PAPS, hence the inability to form PAPSe would require a different pathway that avoids this step. The reduction of sulfite is catalyzed by the enzyme sulfite reductase, yet the enzyme has such a low affinity for selenite that the *in vivo* reduction of selenite by this enzyme would be effectively prevented. The existence of a viable pathway for the reduction of selenite to the selenide level, as discussed in the selenite reduction section above, provides a mechanism for this reduction, that is different from the reduction of sulfite. However, the nutritional and toxicity studies have shown that selenate and selenite have equal biological activity, yet selenate can not be reduced by thiol compounds. Thus it would appear that the reduction of selenate to selenite would be the most likely place for the reduction of selenium to be catalyzed by the sulfur reducing system. The formation of PAPS, as discussed above, can not be involved, but as shown below, the evidence for the participation of the sulfur pathway always seem to involve the uptake mechanism and the first enzyme of

sulfate activation, ATP sulfurylase.

Pathways Where Selenium and Sulfur Metabolism Appear to be the Same

Studies which implicate the use of the sulfur pathway by selenium include the studies mentioned previously on selenate uptake. The demonstration that both selenate and sulfate use the same binding site (85) shows that for at least this step, selenium follows sulfur.

The reduction of selenate in animals occurs in distinct tissues, reportedly being primarily a hepatic process (102, 103). The liver is also thought to be the site of sulfate activation. The metabolism of selenite appears to be much less specific. Erythrocytes have been shown to metabolize selenite while leaving selenate unchanged (120). Thus even though the overall process must be different, the first steps occur in the same location and could possibly use the same enzyme.

In bacteria and fungi, several experiments have been performed that indirectly provide evidence for the influence of the sulfate reduction pathway on the reduction of selenate. These studies used genetic mutants blocked at various enzymatic steps of sulfate activation and reduction. Cuppoletti and Segel (100), while doing studies on sulfate and selenate transport, used ATP sulfurylase-less Penicillium mutants and stated that the wild type produced a volatile odoriferous compound (presumably dimethyl selenide) while the mutants did not. McKillen and Spencer (121) showed that ATP sulfurylase was required for

molybdate to be toxic. Molybdate is an alternative substrate for the enzyme, thus this study might reflect on a potential site of selenate toxicity. Arst (122) and Hulanicka (123) used selenate toxicity to characterize sulfate activation and reduction mutants. Unfortunately these studies involve some cyclic reasoning as they assumed that the selenate requires the sulfur enzymes for toxicity. The fact that the system worked for them provides some evidence for this hypothesis.

The ability of the first enzyme of the sulfate activation system, ATP sulfurylase, to utilize selenate as a substrate has been shown by several different methods by Wilson and Bandurski (2, 124- 127). They showed that selenate caused pyrophosphate liberation from ATP with ATP sulfurylase preparations. Selenate also catalyzed the ATP sulfurylase-dependent exchange between ATP and radioactive pyrophosphate. Selenate and sulfate were the only compounds which catalyzed this exchange. The pyrophosphate liberation caused by other group VIA and VIB anions was much greater than the liberation by selenate and sulfate, also implying the similarity between selenate and sulfate. Wilson and Bandurski (2) reported the appearance of an unstable compound resembling APS in its electrophoretic and charcoal absorption properties. The pyrophosphate liberation and exchange data have been repeated by several laboratories (3, 128), but Shaw and Anderson (3) have reported that they were unable to repeat the electrophoresis data.

Anderson and Shaw (129) have stated that the affinity for selenate is higher than the affinity for sulfate using an ATP-^{32}P-pyrophosphate exchange assay with purified plant ATP sulfurylase. Reuveny (130), using an assay for the formation of APS, has shown that the K_I for the competitive inhibition by selenate is also lower than the Km for sulfate. Thus the ability of ATP sulfurylase to utilize selenate has been shown. Data demonstrating the formation and existence of APSe are suggestive but not complete. It was thus the objective of this study to determine whether APSe, or something functionally equivalent to APSe, is formed by ATP sulfurylase. It was concluded that sulfurylase does indeed form APSe or, at least, something with the properties of a selenate anhydride. The product formed was shown to be reducable by thiol compounds, in a manner similar to selenite. The reduction of selenate can be connected to the proposed mechanism of selenite reduction (111) to provide a potential pathway for the biological reduction of selenate.

MATERIALS AND METHODS

Chemicals

The chemicals used unless otherwise stated were of reagent grade. Glass distilled water was used throughout. The o-phenylenediamine was recrystalized from an aqeous 1 % sodium thiosulfate solution following filtration through charcoal. The crystals formed in the cold, were collected on a Buchner funnel, washed with cold water and dried in a vacuum (131).

$Na_2{}^{35}SO_4$ and $Na_2{}^{75}SeO_4$ were obtained from New England Nuclear Corporation. $Na_2{}^{35}SO_4$ was used without prior treatment. $Na_2{}^{75}SeO_4$ was treated as follows. $Na_2{}^{75}SeO_4$ was mixed with unlabeled carrier to the desired activity in a total volume of 50 to 150 µl, and 10 µl of 30 % H_2O_2 added. The mixture was placed in a boiling water bath for 10 minutes, little if any evaporation occurred. The solution was neutralized with 2 N Tris (free base) (0-5 µl) and 10 µl of catalase was added. After the foaming subsided, the mixture was placed in a boiling water bath for 5 minutes. The precipitated catalase was not removed as it was not present in significant amounts.

Enzyme Preparations

Yeast Sulfate Activating Enzymes

Wilson and Bandurski's yeast sulfate activating enzymes. The enzyme preparation is essentially that described by Wilson and Bandurski (2). Budweiser starch-free yeast cakes were crumbled and air dried. The dried yeast (5 g) was ground with 5 g sea sand for 15 minutes with a motorized mortar and pestle. The grinding continued for another 70 minutes after the addition of 15 ml of water. Another 15 ml of water was added and the grinding continued for 20 minutes. The ground mixture was then centrifuged at 19,500 x g for 15 minutes, filtered through cheesecloth, and the filtrate was frozen. The thawed supernatant was dialyzed for 36 hours against 5 mM Tris-HCl, pH 7.5, and centrifuged for 15 minutes at 19,500 x g. The filtrated supernatant was frozen, thawed, and centrifuged at 78,000 x g for 1 hour. The supernatant was then used in the various experiments and is referred to as Wilson and Bandurski's yeast sulfate activating enzymes.

Robbins and Lipmann's ether-treated sulfate activating enzymes. The ether-treated Robbins and Lipmann preparation of yeast sulfate activating enzymes was prepared according to Robbins (132) as adapted by Dr. L. Wilson's laboratory.

The enzyme was obtained by the following procedure. One-half to 1.0 kg of Budweiser starch free yeast cakes

were crumbled into an equal weight of diethyl ether which contained 1.5 times its weight of dry ice. The ether was decanted off 30 minutes later and the yeast and dry ice mixture spread on a flat tray until the smell of ether was gone. During this evaporation step, dry ice was added to keep the yeast frozen. The frozen yeast was then placed in a descicator and a vacuum was applied until the yeast began to melt. The yeast was stored in a freezer until used.

A portion of the ether-treated yeast (150-200 g) was thawed in an equal amount (wt/vol), of 0.05 M K_2HPO_4. The mixture was stirred at 4°C for 16 hours and then centrifuged at 2,000 x g for 30 minutes. The pH of the supernatant was lowered to 5.0 using 2 N acetic acid. The acid-treated solution was stirred for 10 minutes at 4°C and then centrifuged at 4,000 x g for 5 minutes. Sodium chloride was slowly added to the supernatant in the amount of 250 g of NaCl per liter of supernatant fluid. The mixture was stirred for 30 minutes at 4°C and allowed to sit for 4 hours. The solution was centrifuged for 15 minutes at 13,200 x g. The pellet was resuspended in a minimum volume of 0.02 M Tris (free base) and the pH was adjusted to neutrality with 2 N Tris (free base). The preparation was stored frozen.

Dry yeast enzyme. A four to one mixture (wt/wt) of Red Star dry yeast and sea sand was placed in a blender and ground for 10 minutes with intermittent stops to

clean the sides of the blender. The yeast-sand mixture (50 g) was suspended in 200 ml of 0.05 M K_2HPO_4 and left stirring in a cold room for 16 hours. This mixture was then centrifuged at 2,000 x g for 30 minutes as described for the ether-treated enzyme. The rest of the procedure was identical to the ether-treated enzyme procedure.

Yeast ATP Sulfurylase

The dry yeast enzyme preparation was analogous to Fraction III of Robbins (132) and his procedure was carried through to Fraction IV. This procedure involved dialyzing Fraction III for 1 day against 0.01 M Tris-HCl, pH 7.5, with 1 mM EDTA. The pH was adjusted to 5.8 with 2 N acetic acid and centrifuged at 4,000 x g for 15 minutes. The pellet was resuspended in 3 ml of 10 mM Tris-HCl, pH 8.0, containing 1 mM ATP. The volume was adjusted to 5 ml with water and 3.3 ml of saturated ammonium sulfate (enzyme grade), pH 8.0, was added. The solution was centrifuged at 4,000 x g for 10 minutes. The supernatant received 1.6 ml of the ammonium sulfate solution and allowed to stand for 1 hour. The solution was then centrifuged at 19,200 x g for 15 minutes and the pellet resuspended in a minimum volume of 0.02 M Tris-HCl, pH 7.5. After dialyzing for 12 hours against 0.01 M Tris-HCl, pH 7.5, with 1 mM EDTA the preparation was stored in a refrigirator.

The preparation was assayed by the molybdolysis reaction described by Robbins (132), using the same

definition of activity units. The amount of protein was determined by OD_{280} using the methods described by Layne (133).

Cultured Tobacco Cell Extract

The cultured tobacco cell extract was provided by Dr. Z. Reuveny of the AEC/MSU Plant Research Laboratory. The preparation of the extract and the growth conditions of the cells have been published elsewhere (130).

Elemental Selenium Filtration Assay

The incubation mixture was vacuum filtered through a scintered glass filter (fine) fitted with a Millipore filter (45 μ size) and washed with 0.5 ml of water. A test tube was placed under the funnel and 0.1 ml of 1.5 % Br_2 in 46 % HBr was layered on top. After 5 minutes the mixture was drawn into the test tube followed by 0.9 ml of water. The millipore filter was then discarded and the glass filter washed sequentially with 1 N HCl, chloroform, concentrated HNO_3, 1 N HCl and water.

When all of the samples had been filtered 5 μl of liquified phenol (88 %) was added with shaking to each tube. This solution was mixed with 0.1 ml of 0.1 M o-phenylenediamine (freshly prepared) and the mixture heated at 85°C for 15 minutes. The solutions were then read at 332 nm while still hot.

Electrophoresis

The electrophoresis studies were performed on a E-C Apparatus Electrophoresis machine in the laboratory of Dr. L. Wilson which has a bed length of 45 cm. Whatman 3 MM chromatography paper, 6 cm wide, was used and it extended at least 3 cm into each bath. The origin was placed 3 cm from the anode side of the bed. The running buffers were either 0.1 M sodium acetate, pH 4.5 or 0.1 M Tris-HCl, pH 7.6. The voltage was 10 V/cm bed length, and the temperature during the runs was 7-9°C. The running time was either 5 or 5.5 hours. The strips were air dried and counted on a Packard Model 7200 strip counter using dual gas-flow counting heads.

Column Chromatography

Column Procedure

DEAE Sephadex-A 25 was allowed to swell in 0.5 M ammonium formate, pH 7.0, for several days. The gel was then equilibrated with the loading buffer, 0.05 M ammonium formate, pH 7.0. The column was packed in the loading buffer and the sample layered on top of the gel. The column was then eluted with a linear ammonium bicarbonate gradient. The other column parameters are presented in the figure legends.

Assays for Sulfur and Selenium in the Column Eluent

The sulfur and selenium concentrations in the column

eluent were determined using ^{35}S and ^{75}Se. The ^{35}S was counted in Bray's scintillation fluid on a Packard Tri-Carb scintillation counter model 3003 using ^{14}C settings. The ^{75}Se was counted using the same method, except ^{3}H settings were used.

Assay for Phosphate in the Column Eluent

The phosphate assay presented here is a micro-adaptation of the methods presented by Ames (134). The sample and 10 µl 4 % $Mg(NO_3)_2 \cdot 6H_2O$ in 95 % ethanol were placed in ignition tubes that had been sonicated for 45 minutes in 50 % concentrated HNO_3. The tubes were then heated on a Bunsen burner for approximately 10 seconds until the yellow fumes had subsided. The tubes were allowed to cool and 30 µl 0.5 N HCl was added. The tubes were placed in a boiling water bath for 15 minutes. The loss of liquid due to evaporation was prevented by placing marbles on top of the tubes and blowing cold air over the upper part of the tubes. The solution in the tubes was mixed with 80 µl of molybdate reagent (1 part 10 % ascorbic acid to 6 parts 0.42 % ammonium molybdate$\cdot 4H_2O$ in 1 N H_2SO_4) and incubated for 20 minutes at 45°C. The resulting color was measured on a Beckman DK-2 spectrophotometer with masked cell holders at 820 nm using a lead sulfide detector. The percent transmittance was read on a 100-90 % T slide wire and converted to optical density units. The system was calibrated with AMP using a molar extinction coefficient of 15,400 at 260 nm.

Adenine Assay for Column Eluent

The adenine was measured by triangulating the optical density at 245 nm, 260 nm and 275 nm using the formula $OD_T = OD_{260} - \frac{1}{2}(OD_{245} + OD_{275})$. The OD_T can be converted to adenine using a molar extinction coefficient of 7,470.

Direct Spectrophotometric Assay of Elemental Selenium

The reaction mixture contained in μmoles: Tris-HCl, pH 8.0, 100; $MgCl_2$, 10; ATP, 10; Na_2SeO_4, 10; GSH, 10; and inorganic pyrophosphatase, 0.2 unit; 0.2 ml of 4 % (wt/vol) carboxymethyl cellulose; ATP sulfurylase, 27 units, in a total volume of 1.0 ml. The reaction was started by the addition of enzyme while monitoring the optical density at 380 nm. When a standard curve was made both the enzyme and Na_2SeO_4 were left out and various amounts of Na_2SeO_3 were added. When the OD_{380} was converted to elemental selenium (selenite equivalents) the standard curve was used directly. Beer's law was obeyed except that there was a break in the curve and care was used to avoid concentrations in the region of the break.

Selenate-dependent Phosphate Release from ATP

The assay used was that of Sumner (135). Part of the solution in the cuvette was removed (0.1 ml) and added to 0.8 ml of 6.6 % $(NH_4)_2MoO_4 \cdot 4H_2O$ in 2.5 N H_2SO_4 that had been diluted 1 to 7 with water. The solution was

mixed with 0.1 ml of a solution that contained 2 g $FeSO_4$ in 20 ml water plus 0.5 ml 20 % concentrated H_2SO_4. After 15 seconds of mixing the sample was read at 660 nm. The assay was not completely linear but the amount of color formed versus the amount of phosphate present could be matched to a curve generated by the formula $y = 3.35x^2 + 2.705x$. The amount of phosphate in 0.1 ml was equal to $(0.163 + 0.29851 \cdot OD_{660})^{\frac{1}{2}} - 0.40373$.

The amount of phosphate formed in the presence of selenate (total phosphate) minus the amount of phosphate formed without selenate equaled the selenate-dependent phosphate released.

RESULTS

Elemental Selenium Formation from Selenate

It was previously noted in this laboratory (136) that when exogenous cysteine was added as a thiol protecting agent to assay mixtures containing yeast sulfate activating enzymes using selenate as a substrate, the incubated mixture turned red or pink. These experiments were repeated and qualitatively verified.

Elemental selenium appears red when formed in an aqueous solution, thus it was considered as a possibility of being the red compound. An assay for elemental selenium has been developed (64) using its colloidal properties and its ability to be converted to selenous acid, and a modification of this assay was used in these studies. The selenous acid can then be assayed spectrophotometrically after complexing with ortho-phenylenediamine to form "piazselenole" (2,1,3-benzoselenadiazole) (137). The complex showed an absorption maximum at 332 nm, and this wavelength was used to measure its formation. Figure 7 shows the optical density at 332 nm with increasing amounts of Na_2SeO_3. The insert in Figure 7 gives the structure of "piazselenole".

The red product produced enzymatically reacted in this

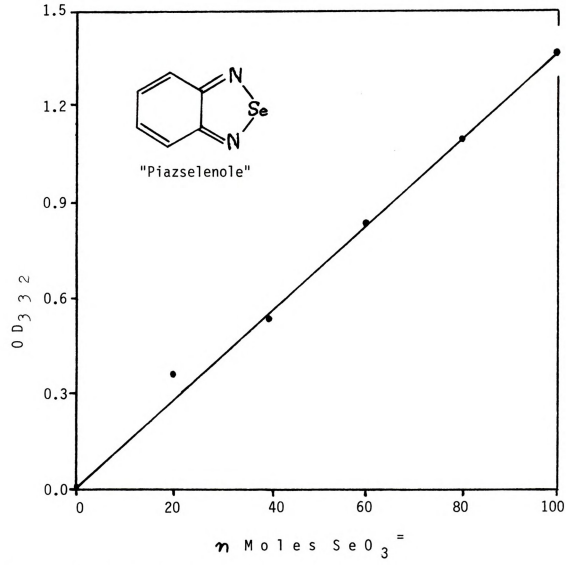


Figure 7. Standard curve for selenite assay.

Assay mixture contained 0.1 ml of the Br_2-HBr solution (described in the methods section) and 0.9 ml of H_2O containing the appropriate amount of Na_2SeO_3. The reaction conditions are described in the methods section.

procedure as predicted showing that the red component was in fact elemental selenium. Table 5 shows the effect of ATP and cysteine on the enzyme catalyzed production of elemental selenium while Table 6 shows the interaction of NADPH and the reaction. The enzyme preparation used in Table 5 was less active than the enzyme used in Table 6 thus explaining the descrepancy between the experiments that were duplicated.

Studies on APSe and PAPSe Formation

The positive influence of ATP on the reaction and the previous reports from this laboratory on the formation of APSe (2) made the formation of APSe a logical place to initiate studies to determine the mechanism by which this relatively crude yeast enzyme preparation catalyzed the reduction of selenate to elemental selenium. The identity of APSe had previously been determined by its charcoal absorptivity and its electrophoretic mobility properties which were similar to APS (2). Another laboratory using a different enzyme source reported that no compound with the electrophoretic properties ascribed to APSe could be detected (3). It therefore appeared necessary to repeat the experiments of Wilson and Bandurski (2) and to attempt to chemically characterize the product formed. The experiment could also check for the possible existence of PAPSe by including 3'-AMP in all of the reaction mixtures to protect any PAPSe formed from the

Table 5. The effect of cysteine, ATP, and enzyme on elemental selenium formation.

Treatment	Average OD_{332}	Average nmoles Se^{o} produced
Complete	1.866 ± 0.050	135.0 ± 3.7
-ATP	0.532 ± 0.016	36.4 ± 1.2
-CySH	0.322 ± 0.057	20.8 ± 4.2
-Enzyme	0.469 ± 0.106	31.7 ± 7.9
-ATP-Enzyme	0.463 ± 0.036	31.2 ± 2.7
-CySH-enzyme	0.247 ± 0.026	15.3 ± 1.9
-ATP-CySH	0.326 ± 0.076	21.1 ± 5.6
-ATP-CySH-Enzyme	0.249 ± 0.115	15.4 ± 8.5

Reaction mixture contained in μmoles: ATP, 10; Tris-HCl, pH 7.5, 15; CySH, 10; $MgCl_2$, 2; Na_2SeO_4, 10; glucose-6-P, 10; NADPH. 1: and 0.2 ml of enzyme(Wilson and Bandurski's yeast sulfate activating enzymes) in a total volume of 1.0 ml. The assay procedures are described in the methods section. The incubation was performed at 23° C for 100 minutes. The reaction was stopped by boiling for 5 minutes.

Table 6. Influence of NADPH on elemental selenium formation.

Treatment	Average OD_{332}	Average nmoles Se^{o} produced
Complete	3.430 ± 0.24	251.0 ± 17.8
-ATP	0.420 ± 0.095	28.1 ± 7.0
-CySH	0.754 ± 0.124	52.8 ± 9.2
-Enzyme	0.325 ± 0.035	21.0 ± 2.8
$-NADP^{+}$	3.235 ± 0.035	236.6 ± 2.6
$-ATP-NADP^{+}$	0.210 ± 0.025	12.5 ± 1.9
$-CySH-NADP^{+}$	0.268 ± 0.053	16.8 ± 3.9

The reaction conditions were as in Table 5 except $NADP^{+}$ was substituted for NADPH and 1 unit of glucose-6-P dehydrogenase was added to the mixture.

action of 3' nucleotide phosphatases.

Electrophoretic Analysis of Enzymatic Products

The electrophoretograms presented in Figure 8 show the enzyme-dependent production of a radioactive selenium peak at the location of APS. There was no discernable peak in the PAPS region. Figure 9 shows the acid lability of this peak. The amount of enzyme used in these experiments was determined by maximizing the amount of PAPS they could produce under the conditions of the assay. The electrophoretograms presented in Figures 8 and 9 could not be obtained reproducibly and the radioactive peaks could not be eluted and rerun to give peaks of the same mobility. Thus a system was used which had high resolution capabilities and provided the resolved compounds in a form suitable for further characterization.

Column Chromatographic Analysis of Enzymatic Products

The system used was column chromatography on DEAE Sephadex-A 25. This column had been shown by Wilson and Bierer (1) to be an effective way of separating APS and PAPS from various nucleotides and sulfur compounds. The eluted compounds could then be characterized by measuring the phosphorous, adenine, and sulfur or selenium in the various fractions.

Radioactive sulfur or selenium was used to quantitate

Figure 8. The enzymatic production of a radioactive selenium peak with the same Rf as APS during paper electrophoresis.

The reaction mixture contained in μmoles: Tris-HCl, pH 8.0, 25; ATP, 2.5; $MgCl_2$, 2.5; 3'-AMP, 5; $Na_2{}^{75}SeO_4$, 5 (5 μc/μmole); and 50 μl enzyme (ether-treated yeast preparation) in a total volume of 0.275 ml. The enzyme was boiled for 5 minutes prior to incubation as a minus enzyme control. The mixture was incubated for 1 hour at 37°C and the reaction stopped by boiling for 90 seconds. The precipitated protein was removed by centrifugation. Each strip received 100 μl of the supernatant. The electrophoresis was run for 5 hours with 0.1 M Na-acetate buffer, pH 4.5.

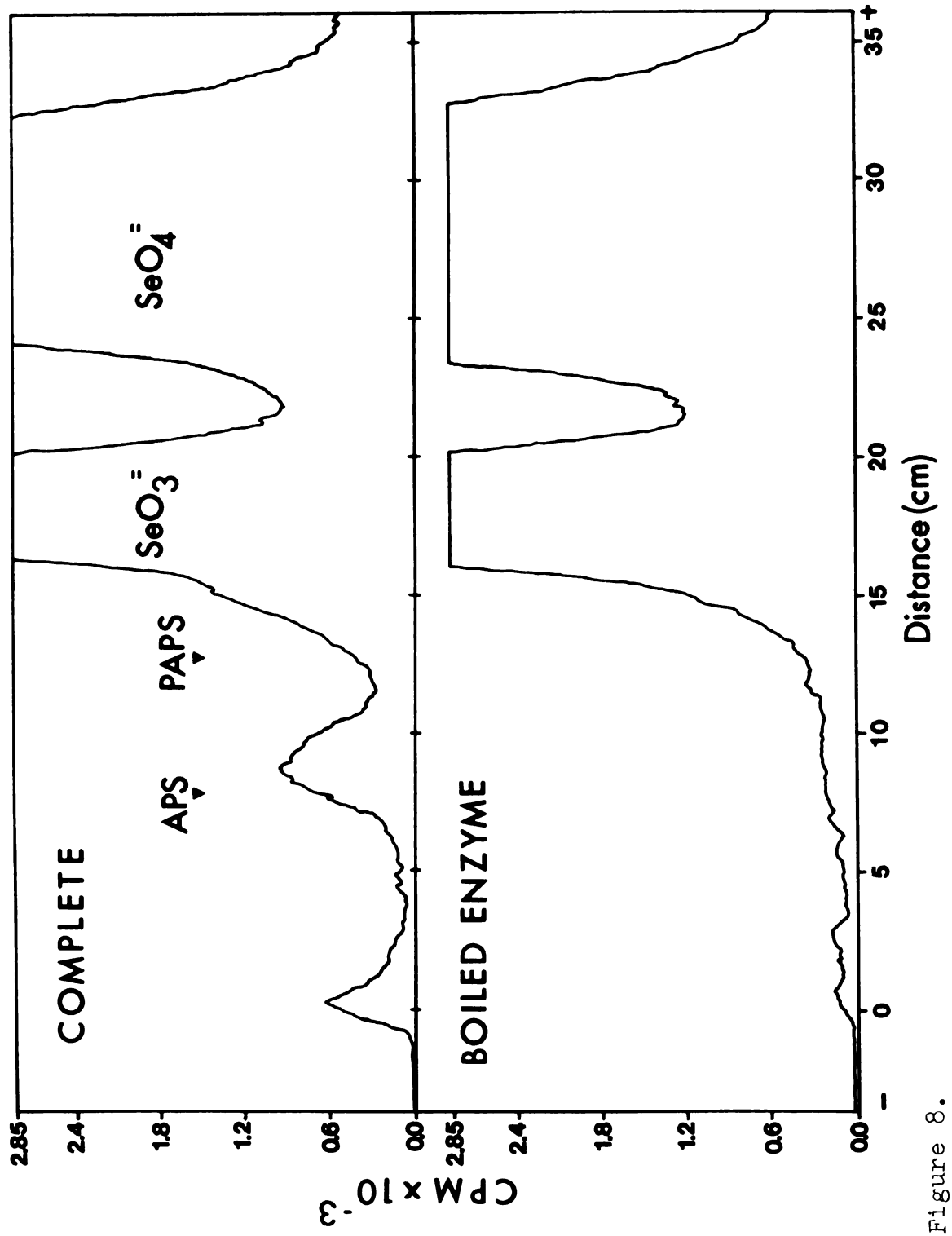

COMPLETE
APS
PAPS
$SeO_3^=$
$SeO_4^=$
BOILED ENZYME
CPM x 10^{-3}
2.85
2.4
1.8
1.2
0.6
0.0
— 0 5 10 15 20 25 30 35 +
Distance (cm)

Figure 8.

Figure 9. The acid lability of a radioactive selenium peak with the same Rf as APS during paper electrophoresis.

The reaction mixture contained in μmoles: Tris-HCl, pH 8.0, 25; $MgCl_2$, 2.5; ATP, 2.5; 3'-AMP, 5; $Na_2{}^{75}SeO_4$, 5 (50 μc/μmole); and 30 μl of enzyme (dry yeast preparation) in a total volume of 0.275 ml. The incubation lasted for 1 hour at 37°C and stopped by boiling for 90 seconds. The precipitated protein was removed by centrifugation, the supernatant, 100 μl, was placed directly on the paper. Another 100 μl was adjusted to pH 0.8-1.0 with HCl using methyl green as an indicator and incubated for 20 minutes at 37°C. The pH was then raised to 7.5 with NaOH and the total treated sample was placed on the paper. The electrophoresis was run for 5.5 hours at pH 7.6. The procedure discribed in the methods section was then followed.

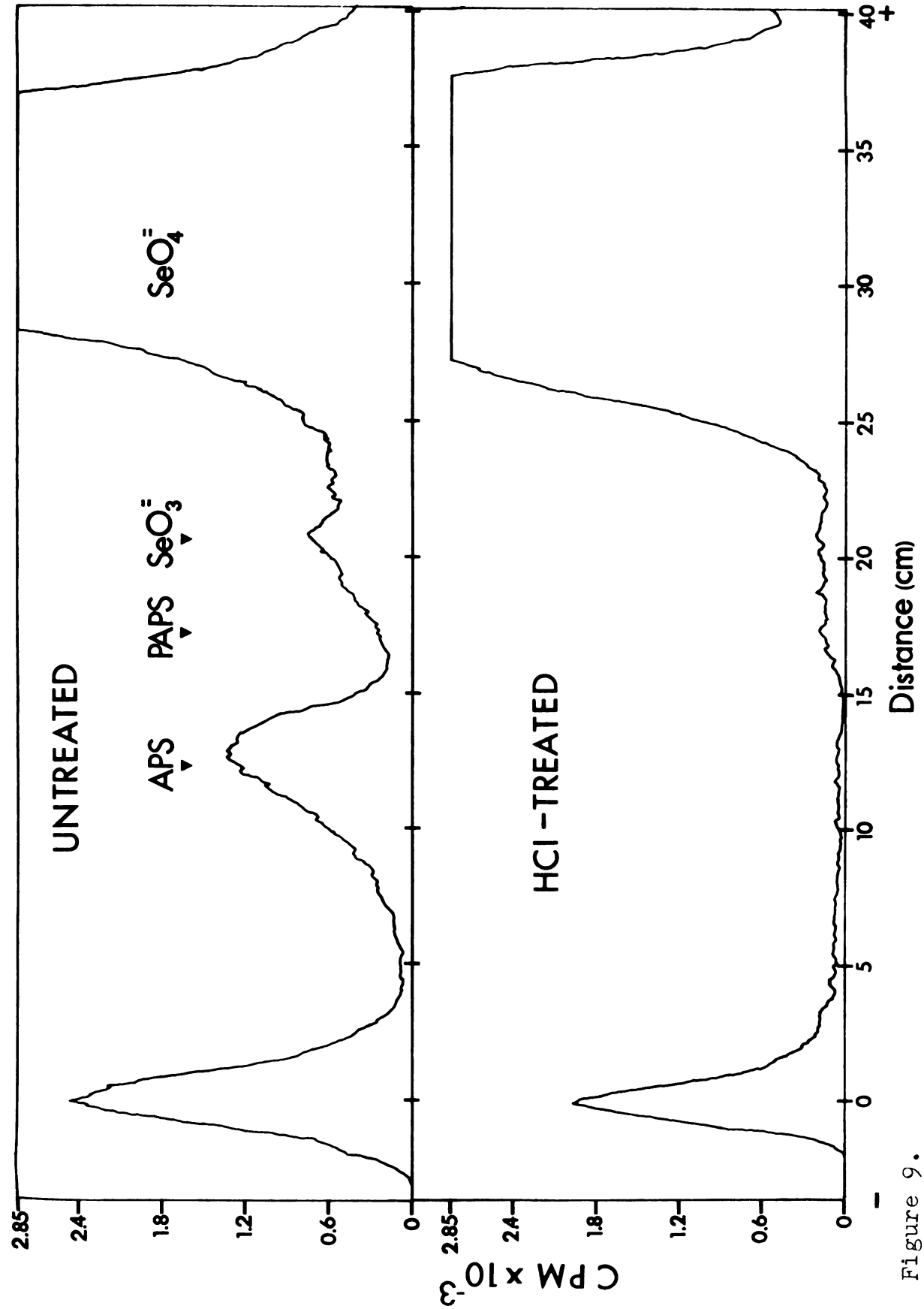

UNTREATED
APS
PAPS
$SeO_3^=$
$SeO_4^=$
HCl-TREATED
C PM x 10^{-3}
2.85
2.4
1.8
1.2
0.6
0
5
10
15
20
25
30
35
40
Distance (cm)

Figure 9.

these values on a scintillation counter. The adenine was measured using its spectral properties. Phosphate was assayed by forming a colored complex with molybdic acid. The normal assay procedures as described by Ames (134) require a relatively large amount of material so procedures were developed that permitted much smaller amounts to be assayed. Figure 10 presents a standard curve for the assay showing that pmoles of organic phosphate can be quantitated.

This system allowed the separation and characterization of the enzymatic products when sulfate was used as a substrate and subsequent determination of the same parameters when selenate was used. Figure 11A shows the separation and characterization of enzymatically derived PAPS. The average ratio of adenine to sulfate to phosphate for the four peak tubes was 1:1:2.3.

When selenate was exchanged for sulfate with all other parameters remaining constant the data presented in Figure 11B were obtained. None of the radioactive selenium peaks contain adenine although the peak at Fraction number 24 had to be run on a longer column with a shallower gradient to get complete separation between adenine and radioactive selenium. It must be noted here that the resolution into this large number of peaks was only possible on DEAE Sephadex-A 25 from one bottle. It was not possible to get other bottles or gel batches to give this kind of resolution. All other batches tried

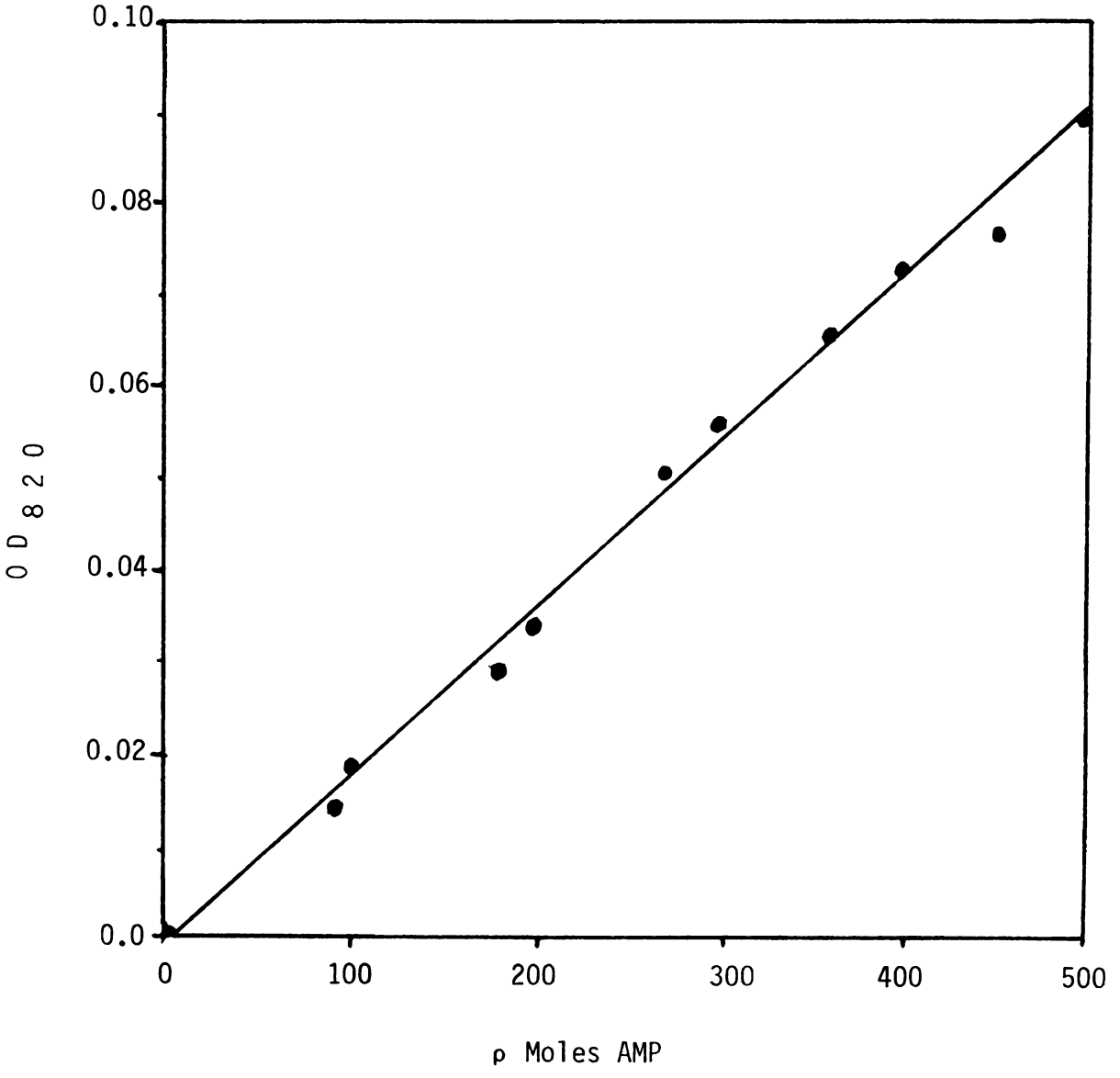


Figure 10. Standard curve for organic phosphate determination.

The assay conditions were as described in the methods section.

Figure 11 A. Enzymatic PAPS production as resolved by DEAE Sephadex-A 25 column chromatography.

The reaction mixture contained in μmoles: Tris-HCl, pH 8.0, 25; ATP, 2.5; $MgCl_2$, 2.5; 3'-AMP, 5; $Na_2{}^{35}SO_4$, 5 (0.38 μc/μmole); and 50 μl enzyme (dry yeast preparation) in 0.275 ml. The mixture was incubated for 1 hour at 37°C and stopped by boiling for 90 seconds. The precipitated protein was removed by centrifugation. Column dimensions were 0.7 x 7.0 cm. The column was eluted with a linear gradient of 0.1 M-1.3 M ammonium bicarbonate. The total gradient was 40 ml with each fraction receiving 1 ml. The flow rate was 2.5 ml/hour.

B. Enzymatic production of selenate metabolites by the sulfate activating enzymes as resolved by DEAE Sephadex-A 25 column chromatography.

The conditions used were exactly the same as in Figure 11A except that the $Na_2{}^{35}SO_4$ was replaced by 5 μmoles of $Na_2{}^{75}SeO_4$ (3.53 μc/μmole).

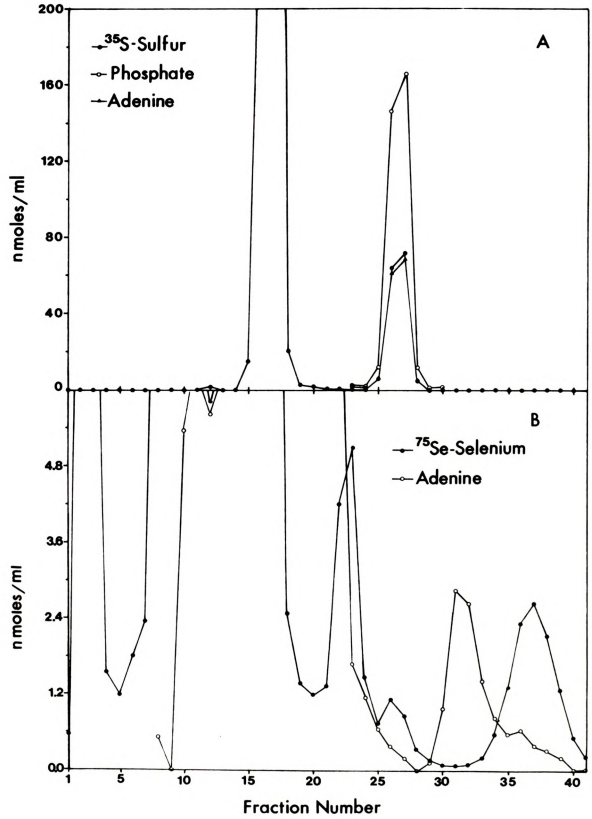

35S-Sulfur
Phosphate
Adenine
A
nmoles/ml
200
160
120
80
40
0
B
75Se-Selenium
Adenine
4.8
3.6
2.4
1.2
0.0
1
5
10
15
20
25
30
35
40
Fraction Number

Figure 11.

would resolve sulfate, selenate and other nucleotides from APS and PAPS but it was not possible to see the number of ^{75}Se peaks presented in Figure 11B. Whether these peaks are real or were artifacts of one gel batch could not be determined.

The elution profile obtained on "normal" gel with an enzyme extract provided by Dr. Z. Reuveny of cultured tobacco cells which did not accumulate PAPS is shown in Figure 12. The average adenine to sulfur to phosphate ratios for the tubes containing the product were 0.96: 1:1.05 showing that this peak was in fact APS. The figure also presents data from a similar column run when selenate was exchanged for sulfate. No discernible radioactive selenium peak in the region of APS can be seen. The peak near the end of the column (tubes 31 and 32) does not contain adenine although this is near the place where PAPS would elute. Figure 13 shows the same data accumulated for yeast ATP sulfurylase. The absence of a radioactive selenium peak in the area of either APS or PAPS is again clear.

The evidence for the formation of APSe from selenate is contradictory - that is - the apparent formation of APSe could be observed electrophoretically but no APSe could be observed following a (more time consuming) column assay. Thus it was decided that reinvestigation of the original phenomena might resolve the mechanism involved. The filtration assay used in the original

Figure 12. Column chromatography of the products formed from cultured tobacco cell extracts with sulfate and selenate.

The reaction mixture contained in μ moles: glycine buffer, pH 9.0, 25; ATP, 5; $MgCl_2$, 5; $Na_2{}^{75}SeO_4$ (11.26 μc/μmole), 5 or $Na_2{}^{35}SO_4$ (5.14 μc/μmole), 10; and inorganic pyrophosphatase, 7.3 units, plus 0.2 ml of tobacco extract in a total volume of 0.5 ml. The mixture was incubated for 1 hour at 30°C and stopped by boiling for 90 seconds. The precipitated protein was removed by centrifugation. The column dimentions were 0.7 x 7 cm. The gradient was linear from 0.5-1.1 M ammonium bicarbonate in a total volume of 40 ml. Each fraction received 1 ml. The flow rate was 2.5 ml/hour.

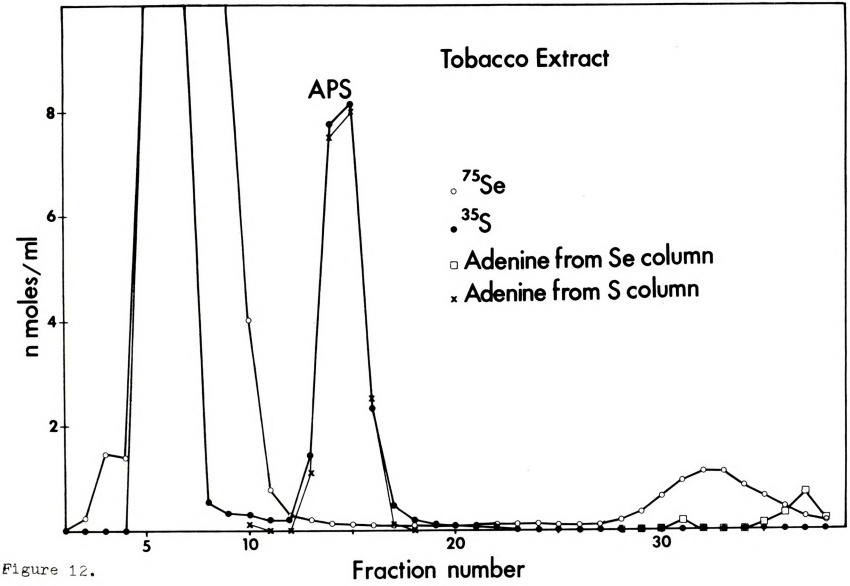

Tobacco Extract
APS
^{75}Se
^{35}S
Adenine from Se column
Adenine from S column
n moles/ml
8
6
4
2
5
10
20
30
Fraction number

Figure 12.

Figure 13. Column chromatography of the products formed by yeast ATP sulfurylase with sulfate and selenate.

The reaction mixture contained in μmoles: Tris-HCl, pH 8.0, 50; ATP, 5; $MgCl_2$, 5; $Na_2{}^{75}SeO_4$ (16.2 μc/μmole) or $Na_2{}^{35}SO_4$ (1.25 μc/μmole), 2.5; and 10 μl enzyme (8.16 units) plus 0.1 unit inorganic pyrophosphatase in a total volume of 0.5 ml. The mixture was incubated for 1 hour at 37 °C and stopped by boiling for 90 seconds. The precipitated protein was removed by centrifugation. The column was 0.7 x 7.0 cm and was eluted with a linear 40 ml gradient from 0.1 M to 1.3 M ammonium bicarbonate. Each fraction received 1 ml. The flow rate was 2.5 ml/hour.

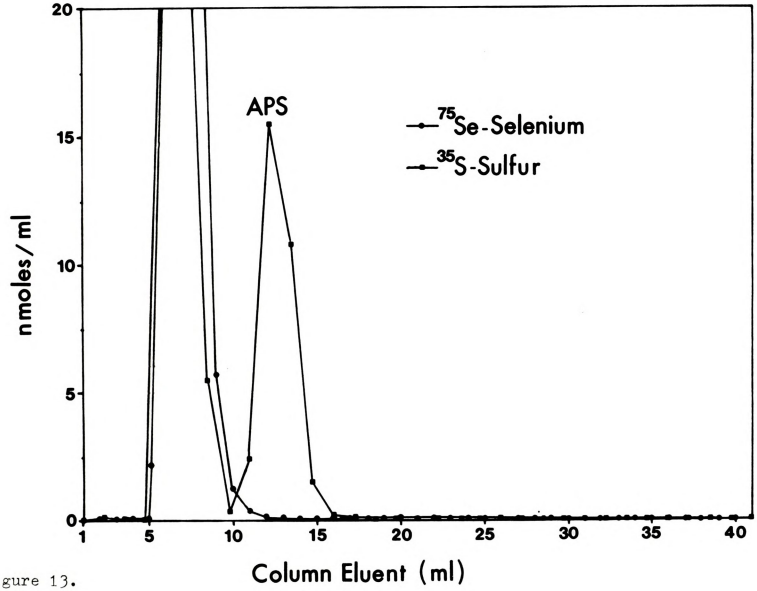

APS
^{75}Se-Selenium
^{35}S-Sulfur
nmoles/ml
Column Eluent (ml)
0
5
10
15
20
1
5
10
15
20
25
30
35
40

Figure 13.

studies was cumbersome and time consuming so another assay system was developed. The appearance of a colored product (in this case elemental selenium) can normally be readily adapted to a direct spectrophotometric assay.

Direct Spectrophotometric Assay

Unfortunately two of the products of the reaction, elemental selenium and presumably cystine, are insoluble and the turbidity that is produced greatly interferes with the quantitation of the red color. It was found that if the solution was made sufficiently viscous, elemental selenium could be made to form a relatively stable and non-turbid solution. The problem of cystine precipitation was solved by using glutathione which likewise remained non-turbid when oxidized in the viscous solution. Thus it was possible to follow the production of elemental selenium by monitoring the increase in optical density at 380 nm. This wavelength was selected somewhat arbitrarily as there was no absorption maximum in the visible range. The absorption spectrum of the enzymatically formed red color was identical with the spectra obtained when selenite was reacted with glutathione, hydrazine or sulfurous acid which are three common ways of forming elemental selenium.

The convenient way to form elemental selenium is to react glutathione with selenite (64). This reaction was used to make a standard curve to hopefully quantitate

the amount of elemental selenium formed. Figure 14 shows the amount of absorption at 380 nm versus various concentrations of sodium selenite. The results were unusual in that the expected linear response was not observed. The insert labeled "Jump" Kinetics shows the time course of the transition from the first line to the second, the entire transition process occurred in approximately 3 minutes. The time course of the production of OD_{380} by an enzyme in the presence of ATP, Mg^{+}, $SeO_4^{=}$ and varying amounts of reduced glutathione is presented in Figure 15. The data show the same kind of kinetics and transition as were presented in the selenite standard curve (Figure 14). The change in the transition point with varying glutathione concentrations was analogous to those shown when different glutathione concentrations were reacted with increasing amounts of selenite. The data presented in Figure 15 can be related to elemental selenium formation (selenite equivalents) using a standard curve of the type presented in Figure 14 to form a graph showing an approximate linear production of elemental selenium with time.

Requirements and Stoichiometry of the ATP Sulfurylase-catalyzed Reaction

The dependence of the reaction on the various components of the reaction mixture is presented in Figure 16. The addition of inorganic pyrophosphatase stimulates the

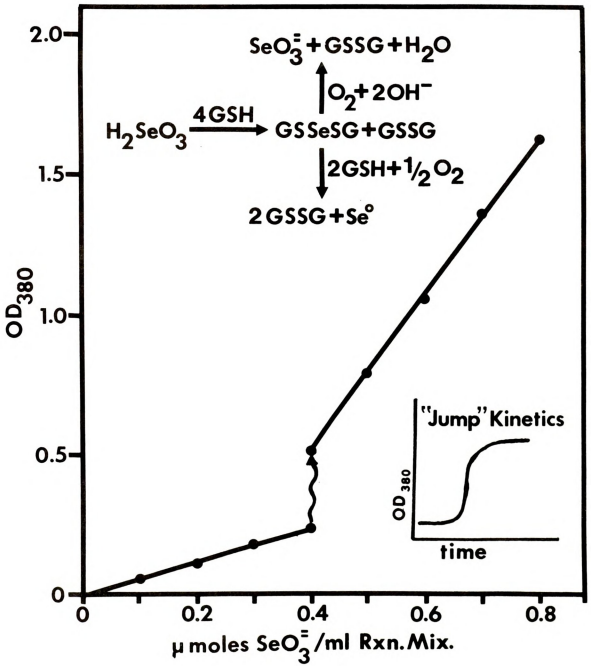


Figure 14. Optical density at 380 nm of various concentrations of sodium selenite in 10 mM reduced glutathione.

The reaction mixture and conditions are described in the methods section.

Figure 15. The ATP sulfurylase-dependent increase in the optical density at 380 nm with time at various reduced glutathione concentrations.

The reaction conditions were as described in the methods section.

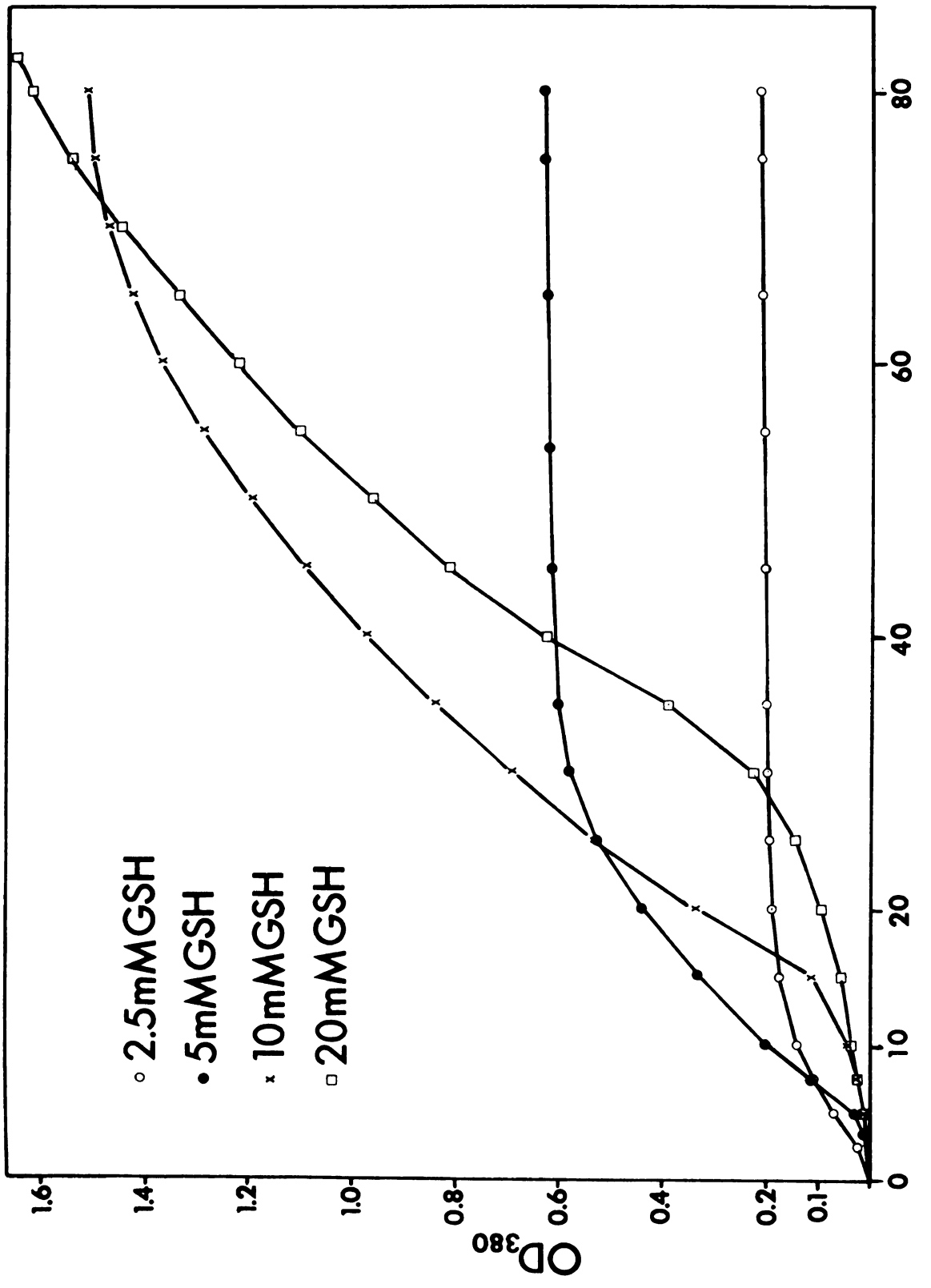

2.5mMGSH
5mMGSH
10mMGSH
20mMGSH
OD380
Time (minutes)
0
10
20
40
60
80
0.1
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Figure 15.

Figure 16. The effect of various reaction components on the production of elemental selenium (selenite equivalents) by ATP sulfurylase.

The reaction conditions were as described in the methods section. The volume was 1.0 ml in all cases.

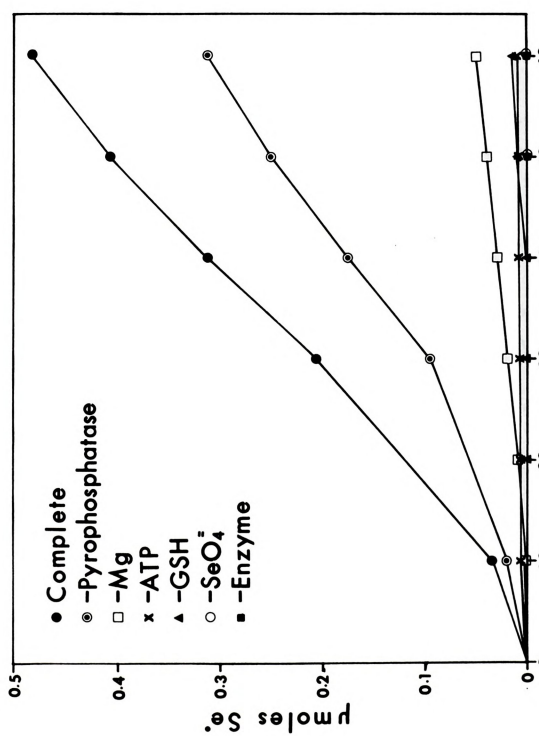

● Complete
◉ -Pyrophosphatase
□ -Mg
x -ATP
▲ -GSH
○ -$SeO_4^=$
■ -Enzyme
μmoles Se°
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
Time (minutes)

Figure 16.

reaction but is not absolutely required. The enzyme preparation was not tested for pyrophosphatase activity, thus the absolute requirement for pyrophosphatase was not determined.

Figure 17 presents graphically the ratio of selenate-dependent phosphate release to the formation of elemental selenium (selenite equivalents). The average ratio in these experiments was 2.07 $\pm$ 0.14. Elemental selenium (selenite equivalents) was quantitated by the measurement of the optical density at 380 nm and converted to elemental selenium using selenite as a reference. Once the relationship between phosphate produced and elemental selenium produced had been established, the stoichiometry of glutathione consumed to either of the other known parameters became of interest. Unfortunately, there was a high level of glutathione oxidation during the reaction, much more than could be explained by the reduction of selenate to selenide or elemental selenium. When the reaction was run anerobically to prevent oxidation of glutathione by oxygen there was no red color produced until air was readmitted. It should be noted that neither catalase nor superoxide dismutase had any effect on the enzymatic production of OD_{380}.

Stability of the Enzymatic Product

An attempt was made to explain the inability of the analytical methods to detect reproducibly APSe or other

Figure 17. Ratio of phosphate and elemental selenium (selenite equivalents) formation with time.

The reaction conditions were as described in the methods section. The phosphate assay used is described under the heading of "selenate-dependent phosphate release from ATP sulfurylase".

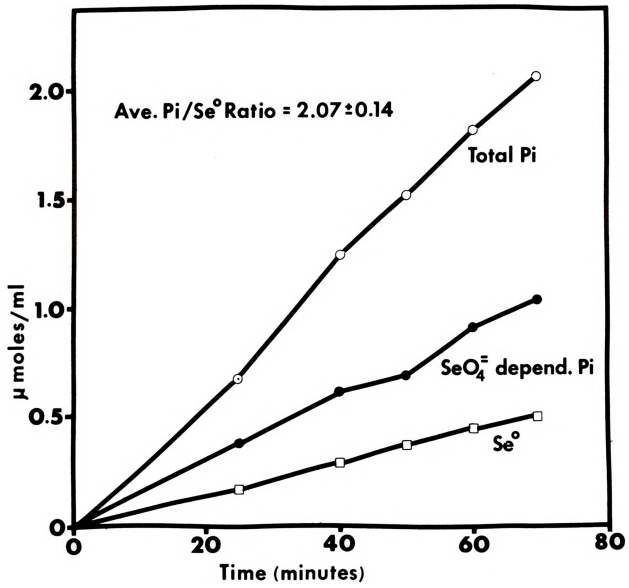

Ave. Pi/Se° Ratio = 2.07±0.14
Total Pi
SeO4= depend. Pi
Se°
μ moles/ml
2.0
1.5
1.0
0.5
0
0
20
40
60
80
Time (minutes)

Figure 17.

intermediate. In this experiment glutathione was added after the reaction had been running for a time and in order to see whether there was an accumulation of the reactive intermediate. The selenate-dependent phosphate release and selenate-independent phosphate release were not affected by the presence or absence of glutathione. Thus the active intermediate should have been produced at a level of one-half of the level of selenate dependent phosphate release, yet when glutathione was added there was no detectable amount of accumulated intermediate. This last study is presented graphically in Figure 18.

Figure 18. Stability of the enzymatic product.

The reaction conditions were as described in the methods section. The volume of the glutathione addition was 50 µl.

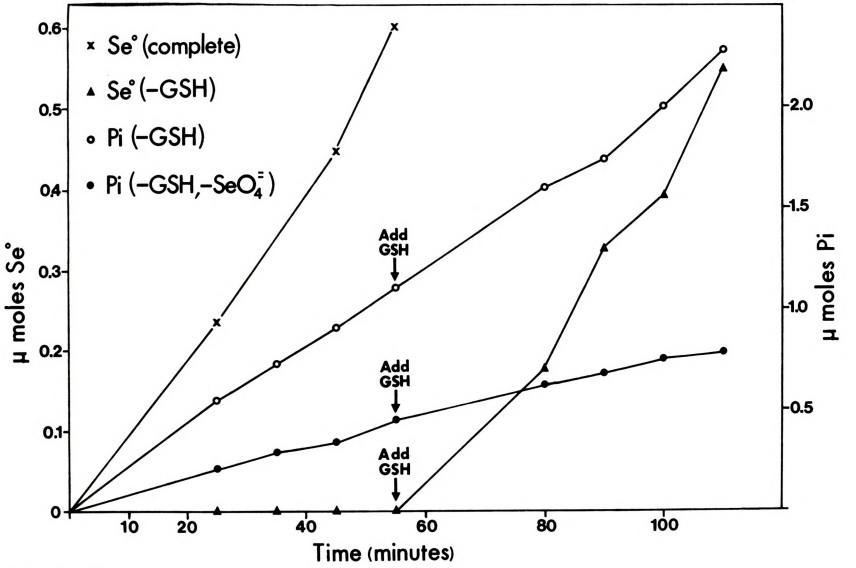

Se° (complete)
Se° (-GSH)
Pi (-GSH)
Pi (-GSH,-SeO4=)
Add GSH
Add GSH
Add GSH
μ moles Se°
μ moles Pi
Time (minutes)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.5
1.0
1.5
2.0
10
20
40
60
80
100

Figure 18.

DISCUSSION

It was observed over 15 years ago that an enzyme extract had the potential to convert selenate to a compound resembling elemental selenium. The phenomenon was reinvestigated because of the renewed interest in the metabolism of selenium. The studies presented here were an attempt to elucidate the mechanism of this conversion.

The first series of experiments demonstrated that the phenomenon was repeatable and that the compound formed was elemental selenium. The reaction definitely required the crude preparation of sulfate activation enzymes, ATP, and cysteine, while the requirement for NADPH or a NADPH-generating system was somewhat unclear. The experimental results presented in Table 6 showed that if NADPH was left out of the reaction mixture there was little, if any, difference in the amount of elemental selenium formed. There was a difference, however, between the reaction mixture which lacked cysteine and a mixture which lacked cysteine and NADPH. The presence of cysteine was sufficient to perform the reduction, but NADPH could replace part of the reducing power of cysteine.

The requirement for cysteine was mechanistically hard to comprehend while the requirement for the enzyme

extract and ATP was very familiar, since ATP is required for the enzymatic activation and reduction of sulfate. Previous reports from this laboratory had reported the formation of the activated selenate, APSe. Thus a detailed study of the ATP required reaction seemed in order.

There were four lines of evidence to support the formation of APSe from ATP and ATP sulfurylase (2, 125). (1) The formation of a labeled selenium compounds which electrophoresed with APS. (2) The radioactive compound was acid-labile as was APS. (3) Charcoal absorbable Se could be detected after incubation with enzyme and ATP. (4) Selenate was able to catalyze the ATP sulfurylase-mediated exchange between ATP and radioactive pyrophosphate. The evidence was strong but not definitive. Also, Shaw and Anderson (3) reported that they were unable to detect any radioactive compound in the region of APS upon electrophoresis. Thus it was necessary to settle this apparent disagreement. The results presented in Figure 2 and Figure 3 show that the original data can be duplicated, but large amounts of radioactivity must be used. The amount of radioactivity used by Shaw and Anderson itself would probably have prevented the detection of this radioactive compound. Even when large amounts of radioactivity were used, the results were not repeatably obtained from experiment to experiment. The reason for the inability to repeatedly obtain the radioactive peaks could not

be determined. Presumably, a component of the enzyme preparations had the capacity to alter or degrade the product.

A column chromatographic separation which provided better resolution and more sensitivity, but required longer running time than electrophoresis was developed. The DEAE Sephadex column permitted separation and the characterization of both APS and PAPS by the ratios of sulfur to adenine to phosphate (Figures 11 and 12). A sensitive phosphate assay permitted the characterization of very small amounts of these compounds (Figure 10). Even though several different enzyme preparations were used, at no time was an adenine-containing selenium compound detected (see Figures 11-13).

The inability to further characterize the ATP sulfurylase product and the problems associated with its detection, led back to studies using a thiol for reduction. During the chromatographic studies a more purified ATP sulfurylase had been prepared and this enzyme preparation was also capable of catalyzing the conversion of selenate to elemental selenium in the presence of ATP and cysteine. A refined direct spectrophotometric assay for elemental selenium was developed using reduced glutathione instead of cysteine. This assay was calibrated using selenite and glutathione to form elemental selenium. The kinetics of this reaction were very unusual (see Figure 14) with a definite transition stage plainly visible.

Studies by Tsen and Tappel (138) have shown that

depending on the ratio of selenite to glutathione, either one of two reactions could occur; the catalytic oxidation of glutathione or the reduction of selenite. The insert presented in Figure 14 summarizes the proposed reactions. The intermediate GS-Se-SG was shown by Peterson (139), Ganther (109) and Tsen and Tappel (138) to be involved in both reactions. The graph can be interpreted as follows. At low selenite concentrations the reaction goes primarily toward the catalytic oxidation of glutathione. Under these conditions reduced glutathione is being consumed while the selenite concentration remains the same. The transition occurs at that point where there is enough selenite compared to reduced glutathione to favor the production of elemental selenium. The "second line" would then represent an approximately stoichiometric production of elemental selenium.

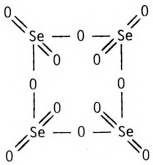
The direct spectrophotometric assay permitted the monitoring of the kinetics of elemental selenium production from selenate by ATP sulfurylase, ATP, and glutathione. The same kind of unusual kinetics that were seen with selenite were observed (Figure 15), and the transition point varied with glutathione concentration as expected from studies with selenite. These results and the ability to use the standard curve obtained with selenite to show a linear enzymatic production of elemental selenium with time, indicated the existence of a common intermediate. This intermediate must be

between selenate and GS-Se-SG, as it resembled selenite or a product between selenite and GS-Se-SG. The GS-Se-SG is the key intermediate in both of the reactions, which gives rise to the unusual kinetics.

The requirement for the various components of the reaction mixture for the formation of elemental selenium (selenite equivalents) is shown in Figure 16. Except for the obvious requirement for glutathione, the reaction shows the same requirements as does the activation of sulfate, the reaction catalyzed by ATP sulfurylase. The production of inorganic phosphate could also be related to the production of elemental selenium. The data presented in Figure 17 showed that 2 moles of inorganic phosphate were released for each mole of elemental selenium (selenite equivalents) formed. The formation of APS from sulfate likewise involves the production of 2 moles of phosphate per mole of APS produced.

The only reductant present in the system is reduced glutathione, thus the conversion of selenate to a compound resembling selenite must involve the participation of glutathione. The reaction between APSe and glutathione cannot be studied directly as APSe cannot be isolated from biological systems and has yet to be chemically synthesized. There is only one commercially available selenium anhydride, selenium trioxide. The structure of selenium trioxide has been determined by X-ray crystalography (140) and can be represented as

follows:



Selenium trioxide

Selenium trioxide is the anhydrous form of selenic acid and is readily converted to selenic acid when exposed to water. Schmidt and co-workers (141, 142) have reported that selenium trioxide reacts explosively with thiols forming thioselenic acids. The concept of a thiolytic cleavage of phosphoselenate anhydride thus seems very plausible if not probable. Alcoholysis of the selenium anhydride would also be predicted. Nissen and Benson (119) found ethyl selenate when plants fed selenate were extracted with hot ethanol. Ethyl selenate is the product expected of an alcoholytic cleavage of a selenium anhydride. As mentioned above, selenium trioxide is unstable in water. The enzymatic product should likewise be unstable. The enzymatic product is in fact unstable as shown by the great difficulty in detecting its formation by either electrophoresis or column chromatography and the inability of glutathione to show any significant accumulation of this product (Figure 18).

The thioselenic acid formed from the thiolytic cleavage could be easily converted to selenite (or

selenite equivalent) by the addition of one electron (one reduced glutathione). The conversion of organic thiosulfuric acids to sulfite and a disulfide by thiols is well documented.

The evidence presented above can be summarized as follows: (1) Selenate could be converted in the presence of ATP, Mg^{++}, ATP sulfurylase, reduced glutathione, and inorganic pyrophosphatase to elemental selenium through a compound that was functionally equivalent to selenite. (2) The stoichiometry of this conversion was the same as the stoichiometry observed for APS production from sulfate by ATP sulfurylase. Two moles of inorganic phosphate were released for each mole of product formed. (3) The properties associated with the enzymatic product were the same as the predicted properties of a phosphoselenate anhydride. These properties included the reactivity with thiol compounds and its basic instability in aqeous solutions. (4) It was possible, using known sulfur and selenium chemistry, to propose the reaction steps from a selenate anhydride to selenite (whose role as an intermediate in the overall reduction to elemental selenium was implied). (5) A compound can be detected which has the electrophoretic and acid lability properties of APS. This evidence, plus the evidence described in the literature showing the ability of selenate to be an effective substrate for ATP sulfurylase catalyzed reactions, led to the conclusion that APSe, or a compound

functionally equivalent to APSe was formed from selenate by ATP sulfurylase.

The proposed mechanisms of the conversion from selenate to elemental selenium as described in these studies can be diagramed as shown in Figure 19. This proposal includes the thiolytic cleavage of a selenate anhydride, which due to the participation of ATP sulfurylase (Figure 16) and the consumption of one ATP per one molecule of active selenium formed (Figure 17), was presumed to be APSe (either free or enzyme-bound). The conversion of the thioselenic acid to selenite is analogous to known sulfur chemistry.

The mechanism of the catalytic oxidation of glutathione by selenite is the proposal made by Tsen and Tappel (138). The conversion of selenite to elemental selenium is essentially the mechanism proposed by Hsieh and Ganther (111). There is no direct evidence to distinguish between two possible paths for the conversion of GS-SeH to elemental selenium. One possibility is presented in the diagram and the other possibility would be an intramolecular rearrangement forming elemental selenium and reduced glutathione (110). The rationale for including the production of hydrogen selenide in the proposed pathway was the fact that oxygen was required for elemental selenium formation.

The proposed mechanism for the conversion of selenate to elemental selenium was designed to explain an <u>in vitro</u>

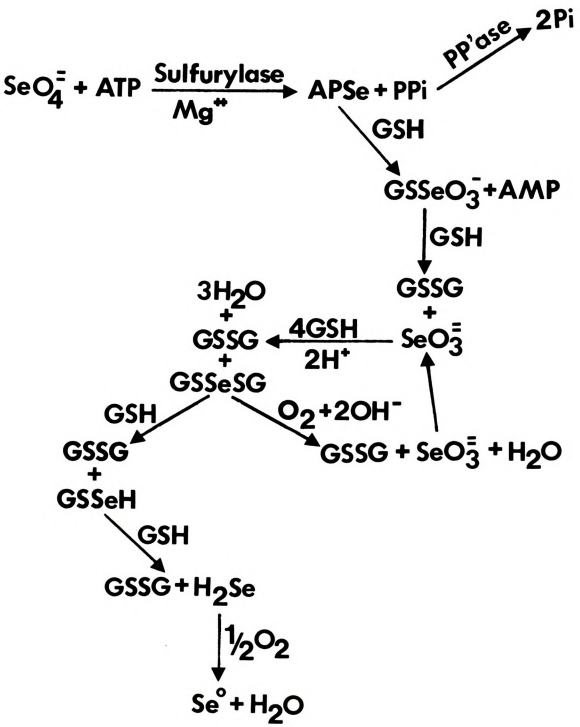


Figure 19. Proposed pathway for the ATP sulfurylase-catalyzed reduction of selenate.

phenomenon, but part of it has the potential of being a viable pathway for the reduction of selenate *in vivo*. The steps requiring oxygen would be side reactions of the reductive pathway. Although the reactions described can easily produce reduced selenium chemically, several of the reaction steps have been shown to be catalyzed by the enzyme glutathione reductase (110, 111). The catalyzed reactions couple the reducing power of NADPH to GS-Se-SG forming 2 GSH and H_2Se. This catalysis reduces the amount of reduced glutathione required for the reaction. Several other enzymes which normally metabolize sulfur compounds may catalyze other reaction steps; *i.e.* the S-sulfoglutathione transhydrogenase described by Winell and Mannervick (143) could catalyze the conversion of $GS\text{-}SeO_3^-$ to $SeO_3^=$; APS sulfotransferase (10, 31) might catalyze the thiolytic cleavage, *etc.* If enzyme catalysis does occur *in vivo* the mechanisms of the individual reaction steps would be different but the intermediates formed would be the same.

This pathway would predict the observed antagonistic effects between selenate and sulfate as both would be substrate for ATP sulfurylase and presumably for the enzymes which synthesize and metabolize the sulfur amino acids. The inability to detect PAPSe or predicted PAPSe metabolite (selenate esters) would be explained as selenate would not form this intermediate. Also explained would be the inability of selenite to serve as an effective

substrate for sulfite reductase (the Km for sulfite and nitrite in E. coli is 10^4 times lower than the Km for selenite (34)). The observation that animals can reduce selenate, but not sulfate is likewise explained. In animals there is considerable evidence that the reduction of selenite is mediated by reduced glutathione (116) and the ability of ATP sulfurylase to form a product which can easily be converted to selenite would explain the ability of animals to utilize both selenite and selenate equally well (96).

The potential for such a pathway to be operable in biological organisms exists, but further in vivo studies must be performed to decide whether this is the pathway that evolution has selected.

APPENDIX

APPENDIX

SULFITE-DEPENDENT NADPH OXIDASE ACTIVITY IN CULTURED TOBACCO CELL EXTRACTS

INTRODUCTION

This study presents data from a series of experiments on the sulfite-dependent NADPH oxidase activity present in crude extracts of cultured tobacco cells. Sulfite-dependent NADPH oxidase activity is often used to assay NADPH-linked sulfite reductase (42, 144), the enzyme which catalyzes the reduction of sulfite to hydrogen sulfide with the oxidation of three NADPH. The data presented in the following report show that sulfite reductase is not the only enzyme that can catalyze this oxidation. It was concluded that an enzyme system existed that was capable of initiating the self-perpetuating oxidation of sulfite. The sulfite oxidation chain reaction involved the intermediate production of superoxide radicals. NADPH, like many chemicals, can be oxidized by the radicals giving an enzyme-dependent and sulfite-dependent oxidation of NADPH.

MATERIALS AND METHODS

Chemicals

The materials used were laboratory reagent grade chemicals.

Tobacco Extract Preparations

Three to five day old tobacco cells were grown according to Filner (145), collected on a Buchner filter with Whatman No. 1 paper and rinsed with water. The cells were weighed and homogenized with fifteen strokes of a teflon plunger in a glass homogenizer. The homogenizing medium was 1.5 x the weight of the cells and contained 0.1 M Tris-HCl, pH 7.5, with 1 mM cysteine. The homogenate was centrifuged for 20 minutes at 10,000 x g. The supernatant was made 45 % saturated with solid enzyme grade ammonium sulfate and the solution stirred for 30 minutes. The solution was centrifuged at 10,000 x g for 10 minutes. The supernatant solution, if boiled for 5 minutes is referred to as the boiled enzyme preparation. If the supernatant was dialyzed against 0.1 M Tris-HCl, pH 7.5, with 1 mM cysteine and 45 % saturation ammonium sulfate for 14 hours, it is referred to as dialyzed enzyme preparation. The boiled enzyme preparation used in these

experiments was derived from 5.2 g of cells, while the dialyzed enzyme preparation was obtained from 4.9 g of cells.

Sulfite-dependent NADPH oxidase Activity

The complete reaction mixture contained, in μmoles: K-phosphate buffer, pH 7.4, 100; NADPH, 0.2; Na_2SO_3, 1.5; and varying amounts of boiled and dialyzed enzyme preparations in a total volume of 1.0 ml. The change in optical density at 340 nm was measured before the addition of sulfite and this value was subtracted from the rate obtained after sulfite was added.

Grant's Sulfite Assay

The procedure used was that described by Bandurski (146) except that no protein precipitating agents were used. The 1.0 ml reaction mixture was added to 4 ml of color reagent and the optical density at 585 nm read after 10 minutes.

The color reagent was freshly prepared before use by adding 2 ml of Fuchsin, 3 % (wt/vol) in 95 % ethanol, and 0.5 ml 40% formaldehyde to 120 ml of 0.8 M sulfuric acid. After 1 hour the mixture was filtered through Whatman No. 1 paper and used.

Oxygen Uptake Studies

These experiments were performed using a Clark-type oxygen electrode (Yellow Springs Instrument Company)

covered with a Teflon membrane, that was kindly made available by Dr. Norman Good. The liquid capacity of the chamber was 2 ml. The signal generated was amplified and recorded. The mixture was kept at 19°C for the duration of these experiments.

RESULTS

Preliminary studies (147) had indicated that cultured tobacco cell extracts contained a large amount of sulfite-dependent NADPH oxidase activity, an activity often used to monitor the enzyme NADPH sulfite reductase. This observation was confirmed and extended showing that the activity was heat labile and that dialysis removed a compound required for activity. Table 7 shows the activation of the dialyzed preparation by a heat-treated preparation which presumably still contains the dialyzable cofactor.

Table 7. The activation of dialyzed tobacco extract by boiled tobacco extract.

Preparation used	Sulfite-dependent NADPH oxidase activity ΔOD_{340}/minute
50 µl dialyzed prep.	0.0
50 µl boiled prep.	0.00338
50 µl boiled prep. + 50 µl dialyzed prep.	0.0358

The reaction conditions and enzyme preparations were as described in the methods section. The pH of the final mixture was 7.25.

The reaction had a rather precise pH optimum so care had to be taken that there was no change in the pH upon addition of other reactants. Figure 20 graphically presents the pH optimum studies.

The kinetic data obtained when the dialyzed preparation and sulfite concentrations were held constant and the concentration of the boiled preparation was varied, showed saturation. These data could be handled in the normal Michaelis-Menten sense. When the sulfite was varied and the other parameters held constant, there was no saturation and the shape of the curve showed that doubling the sulfite concentration would more than double the rate of NADPH oxidation.

This last result was unexpected as all known sulfite reductases show normal Michaelis-Menten kinetics with respect to sulfite. The production of H_2S is also required to define sulfite reductase activity. In this system there was no detectable production of H_2S using Siegel's sulfide assay (57). The system itself did not prevent H_2S from being assayed as a small amount of E. coli sulfite reductase added to the system produced the expected amount of H_2S. Thus the activity being measured was not sulfite reductase.

The sulfite could be acting as a catalyst or could be consumed or metabolized during the reaction. This question was answered by the experiment presented in Table 8. The data show that sulfite disappearance is dependent

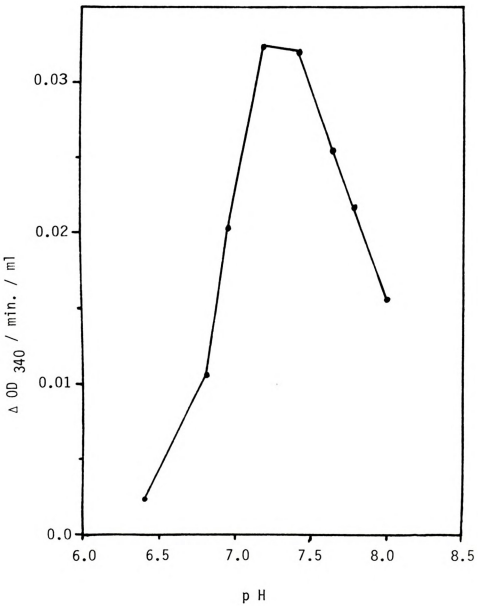


Figure 20. pH optima for tobacco sulfite-dependent NADPH oxidase activity.

The assay conditions were described in the methods section. The pH was measured at the conclusion of the assay. The enzyme preparation used were 50 µl boiled extract from 5.2 g of cells and 50 µl dialyzed enzyme preparation from 4.9 g of cells.

Table 8. The disappearance of sulfite in the reaction mixtures.

Treatment	OD_{585}
Complete system with 50 µl dialyzed enzyme and 20 µl boiled enzyme	1.73 ± 0.12
Complete system with 50 µl dialyzed enzyme and 50 µl boiled enzyme	0.97 ± 0.03
50 µl dialyzed enzyme and 50 µl boiled enzyme with NADPH and glucose-6-P being added after Grant's reagents	1.21 ± 0.04
NADPH plus glucose-6-P with 50 µl dialyzed enzyme and 50 µl boiled enzyme being added after Grant's reagents	2.97 ± 0.05
Complete system with Na_2SO_3 being added after Grant's reagents	3.01 ± 0.08

The conditions of the assay were the same as described in the methods section except that only 0.1 mole of Na_2SO_3 was used. The reaction was run for 30 minutes and was stopped by the addition of Grant's reagents. Glucose-6-P, NADPH, and Na_2SO_3 were in a volume of 50 µl. Grant's sulfite assay is described in the methods section.

on the presence of the enzyme preparations. The presence or absence of reducing agent has no effect when the enzyme preparations are absent, and only a marginal effect when the enzymes are present. Table 9 demonstrates the consumption of the boiled enzyme preparation cofactor. The sulfite could be combining with a compound present in the boiled preparation or undergoing a reduction or oxidation reaction. The combination with an organic compound is unlikely as only a few such derivatives are known.

There are three common inorganic compounds into which the sulfite could be converted; sulfide, elemental sulfur, and sulfate. Hydrogen sulfide was eliminated by previous studies and elemental sulfur was also eliminated as the reaction mixture did not become turbid during the reaction. The conversion of sulfite to sulfate is an oxidative process and a source of oxidizing potential could be molecular oxygen. This guess was substantiated by running the reaction anerobically. There was no change in the optical density at 340 nm until air was readmitted into the system.

The oxygen dependency suggested the use of an oxygen electrode to study the consumption of oxygen during the reaction. The curves presented in Figure 21 show the oxygen consumption with various components of the complete reaction mixture. In all cases the oxygen consumption is sulfite-dependent and proceeds without the addition of reductant as does sulfite consumption. The

Table 9. Boiled enzyme factor consumption during the reaction.

Treatment	Δ OD_{340}/min/ml
Complete reaction mixture with 50 µl dialyzed enzyme minus sulfite and boiled enzyme	0.003 ± 0.0004
+ 1 µmole Na_2SO_3	0.004 ± 0.0009
+ 5 µl boiled enzyme	0.011 ± 0.0015
+ 10 minutes and mixing	0.005 ± 0.0008
+ 5 µl boiled enzyme	0.011 ± 0.0006
+ 10 minutes and mixing	0.006 ± 0.0006

The additions were made serially to the basic reaction mixture which was described in the methods section, with the modification as stated in the table. The activity was corrected for the dilution caused by the volume of the additions.

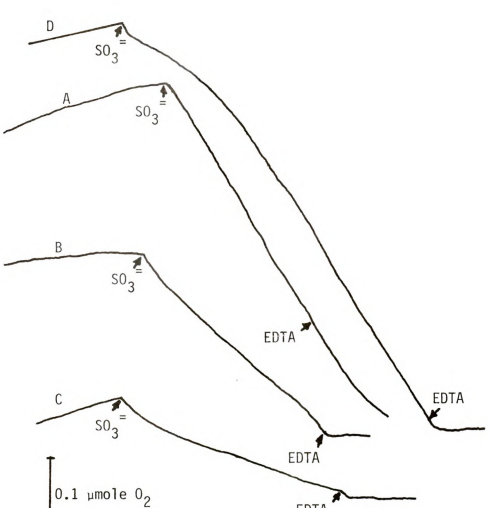


Figure 21. Oxygen consumption with various reaction mixtures.

Reaction A contained 1.8 ml of 0.1 M K-phosphate buffer, pH 7.4, 50 µl dialyzed enzyme, and 50 µl of boiled enzyme preparation. Reaction B contained 1.85 ml of 0.1 M K-phosphate buffer, pH 7.4, and 50 µl dialyzed enzyme. Reaction C contained 1.85 ml of 0.1 M K-phosphate buffer, pH 7.4, and 50 µl boiled enzyme. Reaction D contained only 1.9 ml of 0.1 M K-phosphate buffer, pH 7.4. Na_2SO_3, 50 µl (2 µmoles) were added. EDTA, 50 µl (2 µmoles) were added. The methods section includes information about the oxygen electrode.

chelating reagent, EDTA, was added to these experiments because heavy metals, especially Mn^{++} are known to catalyze the oxidation of sulfite. Figure 21 shows that the mixture represented by curve A is the only system in which EDTA does not have an immediate effect. The mechanism of metal catalyzed sulfite oxidation is believed to include a superoxide radical. Other experiments tested the effect of superoxide dismutase on the complete reaction and showed that it inhibited the oxygen consumption. Superoxide dismutase catalyzes the destruction of superoxide radicals.

Table 10 shows the effect of various additions on the sulfite-dependent NADPH oxidase activity. The data show that sulfite can be replaced by hydrogen peroxide at low concentrations but it becomes inhibitory at higher concentrations. The enzymes superoxide dismutase and catalase also inhibited the reaction along with the reductant and free radical scavenger, ascorbic acid.

Table 10. Effect of various additions on NADPH oxidase activity.

Enzyme	Additions (serial)	Δ OD_{340}/min/ml
50 µl BE[a] + 50 µl DE[b] - Na_2SO_3	none	0.006
	+ 0.5 µmoles $SO_3^=$	0.053
	+ 10 µl SOD[c]	0.017
50 µl BE + 50 µl DE - Na_2SO_3	none	0.006
	+ 0.5 µmoles $SO_3^=$	0.055
	+ 0.5 µmoles H_2O_2	0.044
	+ 1.0 µmoles H_2O_2	0.018
50 µl BE + 50 µl DE - Na_2SO_3	none	0.004
	+ 1.5 µmoles $SO_3^=$	0.055
	+ 10 µl catalase	0.004
50 µl BE + 50 µl DE - Na_2SO_3	none	0.004
	+ 0.2 µmoles H_2O_2	0.052
	+ 0.2 µmoles H_2O_2	0.039
	+ 0.5 µmoles H_2O_2	0.024
50 µl BE + 50 µl DE - Na_2SO_3	none	0.038
	+ 1.5 µmoles $SO_3^=$	0.045
	+ few grains ascorbate	0.002

a. Boiled enzyme. b. Dialyzed enzyme. c. Superoxide dismutase.

See the legend of Table 9 for conditions.

DISCUSSION

Extracts of cultured tobacco cells have relatively large amounts of sulfite-dependent NADPH oxidase activity. The activity was heat labile, sulfite-dependent, and required a heat stable and dialyzable cofactor (see Table 7). Another characteristic of the reaction was its sharp pH optimum at 7.4. This activity is a common assay for NADPH sulfite reductase, however, several experiments eliminated the possibility that sulfite reductase was responsible for the activity. The definitive result was that during the reaction no H_2S was produced. It was possible, however, to show that sulfite was consumed during the reaction, along with the boiled enzyme cofactor (Table 8 and 9).

Studies to determine the fate of the sulfite demonstrated that molecular oxygen was required for the reaction. The consumption of oxygen during the reaction was monitored with an oxygen electrode. The oxidation of sulfite by oxygen is often a problem encountered when working with aqueous sulfite solutions. Heavy metals are effective catalysts for this oxidation and EDTA is routinely added to sulfite solutions to prevent this oxidation. The data presented in Figure 21 show that EDTA stops the consumption of oxygen in all the reaction

mixtures except the reaction mixture containing both active enzyme (dialyzed enzyme) and cofactor (boiled enzyme). These results can be interpreted to mean that heavy metal-catalyzed sulfite oxidation is occurring in all cases except the complete reaction mixture which has a different mechanism for promoting the oxidation of sulfite.

The oxidation of sulfite is a chain reaction (i.e. the oxidation of one sulfite promotes the oxidation of another sulfite, etc.) (148). The process has been reported to involve the intermediate formation of a superoxide radical (149). The removal of the superoxide radical would break the chain reaction and stop the oxidation process. Superoxide dismutase, which catalyzes the reaction $2\ O_2^{\cdot -} + 2\ H^+ \rightarrow\ O_2 + H_2O_2$ (150) inhibits both the oxygen uptake in the complete reaction mixture and the sulfite-dependent NADPH oxidation (Table 10). The free radical scavenger, ascorbate (151), likewise inhibits the NADPH oxidase activity. Hydrogen peroxide (as shown in Table 10) can replace sulfite in oxidizing NADPH when used at low concentrations, but at higher concentrations becomes inhibitory. Catalase also inhibits the sulfite-dependent NADPH oxidation, indicating a requirement for H_2O_2.

The ability of the free radicals involved in sulfite oxidation to oxidize reduced NADPH to $NADP^+$ was shown by Klebanoff (152) in 1961. There are presently several

enzyme systems known which in the presence of their substrate initiate sulfite oxidation (150, 153). The systems include xanthine oxidase, aldehyde oxidase, cytochrome oxidase, lipoxygenase and peroxidase. While the role of H_2O_2 in the xanthine oxidase system can be eliminated, it has been shown that the peroxidase system absolutely requires the presence of H_2O_2 (152, 153).

The original study on the ability of free radicals, formed during the sulfite oxidation, to oxidize NADPH (152) were performed using a peroxidase-peroxidizable substrate initiating system and the results presented here parallel exactly the results obtained with that system.

It was concluded that the dialyzed enzyme preparation contained an enzyme whose substrate was contained in the boiled enzyme preparation. The requirement for H_2O_2, as seen by catalase inhibition, suggested that the enzyme-substrate system observed here was a peroxidase-peroxidizable substrate system. The enzyme-substrate interaction initiated the self-perpetuating oxidation of sulfite. The oxidation of sulfite involves the intermediate participation of superoxide radicals. The free radicals generated were thus responsible for the oxidation of NADPH.

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