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Enzymic Synthesis of Dimethyl Selenide from Sodium Selenite in Mouse Liver Extracts*

Howard E. Ganther†

ABSTRACT: The enzymic synthesis of dimethyl selenide from sodium selenite was studied with mouse liver extracts as a model system for the reductive utilization of selenium. Dimethyl selenide was identified as the major volatile product in doubly labeled studies with carbon-14 and selenium-75. Both the 165,000 × *g* supernatant fraction of liver and washed microsomes synthesize dimethyl selenide, but neither fraction is as active as a 9000 × *g* supernatant fraction. Activity is proportional to protein concentration, and heated extracts are inactive. Optimal conditions were determined for the over-all reaction. The crude system has a specific requirement for glutathione that cannot be eliminated by various thiols or by dithiothreitol. *S*-Adenosyl-L-methionine is the probable methyl donor. Reduced triphosphopyridine nucleotide, coenzyme A, adenosine 5′-triphosphate, and magnesium are also required for optimal activity. Incubation under nitrogen increases the yield of dimethyl selenide approximately tenfold compared to incubation in air, apparently by preventing the oxidation of labile reduced forms of selenium. The crude system is inhibited 50% by 10^{-6} M arsenite in the presence of a large excess of thiols and is also inhibited by cadmium. The possible role of glutathione derivatives of selenium in the synthesis of organoselenium compounds is discussed.

The trace element selenium prevents a number of nutritional diseases at dietary concentrations of 0.1 ppm or less, including liver necrosis in rats, exudative diathesis in chicks, and white muscle disease in ruminants. This subject is treated in detail in a recent monograph by Rosenfeld and Beath (1964). Although the specific biological role of selenium is not established, selenium is known to be most active in the form of organoselenium compounds such as Factor 3, an incompletely characterized substance isolated from pig kidneys by Schwarz and Foltz (1957). The forms of selenium used in most nutritional studies and veterinary applications have been inorganic salts such as sodium selenite and sodium selenate. These compounds are less active per mole of selenium but are convenient to use and give full protection against the deficiency symptoms, indicating that animals are able to synthesize the biologically active selenium compounds.

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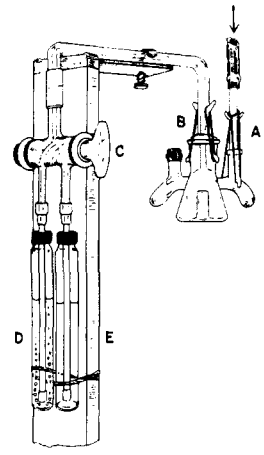

FIGURE 1: Apparatus used for interval collecting of volatile selenium.

The biosynthesis of organoselenium compounds has previously been studied indirectly by injecting animals with radioactive selenite and isolating from their tissues small quantities of labeled metabolites such as selenomethionine, selenocystine, and selenocoenzyme A (McConnell and Wabnitz, 1957; Lam *et al.*, 1961). Such studies are hampered by the difficulty in isolating trace amounts of labile, highly reactive selenium compounds from complex biological materials. A more convenient pathway to study is the synthesis of dimethyl selenide from sodium selenite, a major metabolic route for detoxifying subacute doses of selenite (Schultz and Lewis, 1940; McConnell, 1942). This volatile selenide is readily isolated and its formation involves a six-electron reduction in the oxidation state of selenium, making this system a useful model for other reductive pathways of selenium utilization. In this paper the biosynthesis of dimethyl selenide from sodium selenite in cell-free systems derived from mouse liver is described.

Experimental Section

Preparation of Extracts. Young male mice of the CBA/J strain maintained on a commercial diet[1] and tap water were used in all studies. The animals were killed by cervical dislocation and bled from the carotid arteries. The liver was chilled in ice-cold 0.25 M sucrose containing 10^{-4} M EDTA, pH 7.0, and homogenized at 2° in nine volumes of the same medium in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 × *g* for 10 min to remove cellular debris and nuclei, and this supernatant was centrifuged at 9000 × *g* for 10 min to remove mitochondria. The 9000 × *g* supernatant was centrifuged for 1 hr at 100,000 × *g* in a Beckman Model L-2 preparative ultracentrifuge to obtain the microsomal and the soluble fractions. The microsomes were resuspended by homogenization in sucrose–EDTA medium and centrifuged for another hour at 100,000 × *g*. All centrifugations were at 0–5°.

Incubation Procedures. Incubations were carried out at 37° in Warburg flasks equipped with two side arms. The complete incubation medium contained, in micromoles: sodium phosphate buffer (pH 6.25) 100, glutathione 60, $MgCl_2$ 40, ATP[2] 12, *S*-adenosyl-L-methionine 2, $Na_2{}^{75}SeO_3$ 0.15, coenzyme A 0.6, EDTA 3, TPN^+ 0.4, glucose 6-phosphate 6, plus 5 μg of glucose 6-phosphate dehydrogenase, in a total volume of 3 ml. Solutions of all thiol compounds were prepared immediately before use. Aliquots of freshly prepared liver extract were added to the medium minus selenium in the main compartment, and the flasks were flushed with prepurified nitrogen at 37° for 5 min. Radioactive selenium as $Na_2{}^{75}SeO_3$ was then tipped into the flasks at 15-sec intervals to start the reaction. The volatile radioactive products were trapped in 8 N nitric acid (Challenger, 1935) by means of the apparatus shown in Figure 1, mounted on standard manometer holders of a Warburg apparatus. During the incubation, carrier gas was passed from a manifold through the venting stopper (A) in the side arm of the flask; volatile selenium was swept out the top of the flask through a delivery tube (B) and stopcock (C) to a micro bubbling stick (D) immersed in a 14 × 140 mm counting vial containing 5 ml of 8 N nitric acid. The flow rate was approximately 200 ml/min per flask. At suitable intervals the volatile selenium was diverted to a second bubbler (E) connected in parallel through the three-way stopcock. The first vial was then removed and capped for direct counting in a well-type scintillation counter, and a fresh vial of nitric acid was attached for the next collection interval. At the end of the incubation period (usually 20 min) 0.3 ml of 5 N sodium hydroxide was injected through a serum stopper in the second side arm to stop the reaction, and gassing was continued for 10 min. The flask contents were transferred to 25-ml volumetric flasks, and duplicate 2-ml aliquots were assayed for radioactivity. Between 90 and 95% of the radioactive selenium was recovered in the volatile fractions and flask contents. Recovery was quantitative when no volatile selenium was produced, indicating that incomplete recoveries resulted from small losses of volatile products. For this reason the amounts of volatile selenium produced were calculated by difference from the values for residual radioactivity in the flask contents unless otherwise indicated. Rate curves for selenium volatilization were plotted for each flask from the amount of radioactivity trapped during successive 5-min periods. These curves were useful in following the kinetics of the reaction as well as for verifying the volatilization data obtained by difference.

Other Methods. Selenite was determined colori-

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

[2] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; TPN^+ and TPNH, oxidized and reduced forms, respectively, of triphosphopyridine nucleotide; DMSe, dimethyl selenide; GSH and GSSG, reduced and oxidized forms, respectively, of glutathione.

metrically by the diaminobenzidine method (Cheng, 1956). Selenium dioxide (99.9%) was used as a standard. The concentration of *S*-adenosylmethionine was determined spectrophotometrically at 256 mμ using an extinction coefficient of 15,000 l. mole^{-1} cm^{-1} (Schlenk and DePalma, 1957). Protein was determined by the biuret method (Gornall *et al.*, 1949), with bovine serum albumin as a standard. After color development the crude protein samples were extracted with petroleum ether and centrifuged to eliminate turbidity due to lipid prior to the measurement of optical density.

Materials. Radioactive selenium was obtained from the Oak Ridge National Laboratory as $H_2{}^{75}SeO_3$. Selenium dioxide was obtained from Alfa Inorganics. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine was purchased from the New England Nuclear Corp. *S*-Adenosyl-L-methionine, biosynthesized by the procedure of Schlenk and DePalma (1957), was a generous gift from Dr. Douglas Coleman of this laboratory. Prepurified nitrogen was obtained from Matheson. Coenzyme A, TPN$^+$, Tris, Tris–maleate, flavin mononucleotide, tetrahydrofolic acid, and cyanocobalamin were obtained from Sigma. Coenzyme Q_{10}, menadione, and *N*-ethylmaleimide were obtained from Mann. Hexahydrocoenzyme Q_4 and Q_4-chromanol were the gift of Dr. Karl Folkers, Stanford Research Institute. Mercaptoethanol and *dl*-α-tocopheryl acetate were obtained from Eastman, and thioglycolic acid from Fisher. All other biochemicals were purchased from Calbiochem or from C. F. Boehringer, Mannheim, Germany.

Results

Formation of Dimethyl Selenide. Figure 2 shows the formation of dimethyl selenide by liver extracts incubated with *S*-adenosyl-L-[*methyl*-^{14}C]methionine and sodium [^{75}Se]selenite. Both isotopes were incorporated at nearly identical rates into a volatile product having an average carbon/selenium ratio of 1.86, in reasonable agreement with the expected ratio of 2.0 for dimethyl selenide. Of the 148 mμmoles of selenium added to the flask, 87 mμmoles was recovered in the volatile fractions and 52 mμmoles in the flask contents after incubation, leaving only 9 mμmoles or 6.1% unaccounted for. These data and the requirement for a methyl donor (Table III) indicate that dimethyl selenide is the major volatile product and support the previous evidence for the formation of this compound in animals injected with inorganic selenium (McConnell and Portman, 1952). Challenger and North (1934) have identified dimethyl selenide as the volatile selenide produced by certain molds grown in media containing selenite or selenate.

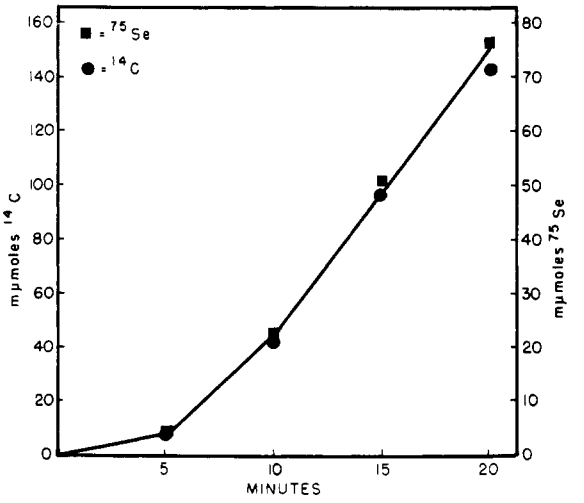

FIGURE 2: Formation of dimethyl selenide doubly labeled with ^{14}C (●) and ^{75}Se (■). The complete medium (see Experimental Section) containing [^{14}C]methyl-labeled *S*-adenosyl-L-methionine and [^{75}Se]labeled sodium selenite was incubated with the 9000 × *g* supernatant (6.03 mg of protein) from a liver homogenate. Duplicate 1-ml aliquots of the nitric acid trapping solutions containing the doubly labeled products were taken for well-type scintillation counting and for Geiger–Müller counting. For Geiger–Müller counting the samples were dried on stainless steel planchets in a hood at room temperature. Nitric acid solutions of volatile selenium labeled only with ^{75}Se were counted by both methods to determine the relative counting efficiency for ^{75}Se so that the observed Geiger–Müller count for the doubly labeled samples could be corrected for the contribution from ^{75}Se to yield the ^{14}C count for the doubly labeled product. The amount of carbon and selenium in each aliquot was then calculated from the specific activities of the labeled precursors. The values are the average of two flasks.

TABLE I: Synthesis of Dimethyl Selenide by Liver Fractions.[a]

Fraction	Protein (mg)	Specific Activity[c]
Experiment 1		
0.3 ml, 10% homogenate	7.78	58
0.4 ml, 600 × *g* supernatant	8.80	62
0.5 ml, 9000 × *g* supernatant	8.22	73
1.0 ml, 100,000 × *g* supernatant	7.98	41
0.5 ml, microsome suspension	8.28	61
0.5 ml, washed microsomes	7.28	66
0.25 ml, washed microsomes plus	3.64	
0.5 ml, 100,000 × *g* supernatant	3.99	78
Experiment 2[b]		
0.40 ml, 9000 × *g* supernatant	5.38	92
1.0 ml, 100,000 × *g* supernatant	7.62	42
1.0 ml, 165,000 × *g* supernatant	7.20	43

[a] Mouse liver homogenates were fractionated by differential centrifugation and assayed in the complete system as described in the Experimental Section. [b] The results in expt 2 are the average of duplicate flasks. [c] mμmoles of Se/min per mg of protein × 10^{-2}.

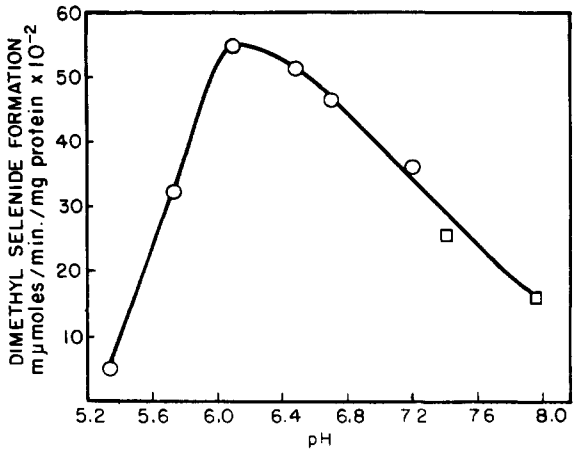

FIGURE 3: Optimum pH for synthesis of dimethyl selenide. Incubation conditions were those described in the Experimental Section except 250 μmoles of sodium phosphate (○) or Tris–HCl (□) buffer was used instead of 100 μmoles of sodium phosphate buffer. The final pH was measured at 37°. Specific activities were calculated from the amount of dimethyl selenide retained in the nitric acid trapping solutions during a 20-min incubation.

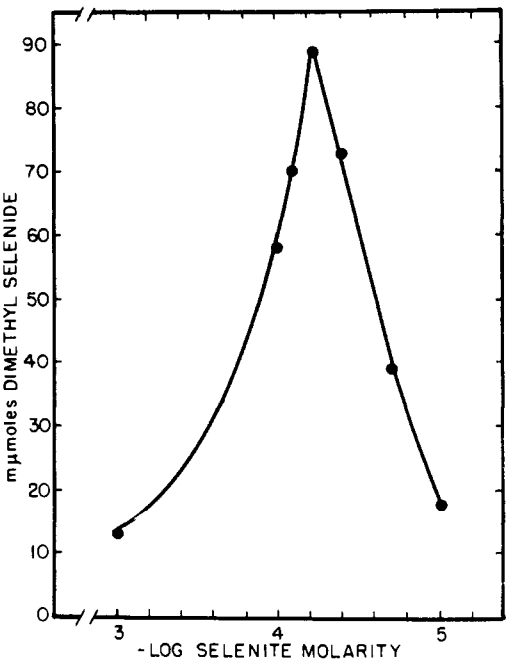

FIGURE 4: Optimum selenite concentration. The 9000 × *g* supernatant from 10% liver homogenate (5.26 mg of protein) was incubated for 20 min in complete medium (see Experimental Section) containing the indicated concentrations of sodium selenite. The data are based on DMSe retained in the trapping solution.

Distribution of Activity. Table I compares the activity of various cell fractions for the synthesis of DMSe. The volume of each fraction added was varied so that the concentrations of protein were approximately equal. Specific activity of homogenates increased as the nuclei, cellular debris, and mitochondria were removed. Further centrifugation to separate the microsomal and soluble fractions resulted in a separation of activity. Washed microsomes were nearly as active as the 9000 × *g* supernatant; the soluble fraction had a considerably lower specific activity but produced an apparent stimulation of microsomal activity when the fractions were recombined. In view of these results 9000 × *g* supernatant was used for the studies in this paper. Possible interactions between soluble fraction and microsomes will be investigated in connection with subsequent purification of the system. To determine if the presence of lighter particulate matter contributed to the activity of the 100,000 × *g* supernatant, this fraction was centrifuged for 1 hr at 165,000 × *g* in the No. 50 rotor of the Spinco Model L-2 ultracentrifuge (expt 2). This procedure removed a small amount of particulate material but did not alter the specific activity of the supernatant fraction.

TABLE II: Preliminary Survey of Tissues for Ability to Synthesize Dimethyl Selenide.[a]

Tissue	Protein (mg)	Specific Activity (mμmoles) Se/min/mg protein × 10^{-2}
Liver	6.98	89
Kidney	3.86	74
Lung	4.18	30
Leg muscle	1.91	11
Spleen	3.52	10
Heart	4.04	5

[a] The 9000 × *g* supernatant fractions from 10% homogenates of mouse tissues were assayed in the complete system as described in the Experimental Section.

A preliminary survey (Table II) of various tissues for their ability to synthesize DMSe demonstrated that liver and kidney had the highest activity of the tissues studied, with intermediate activity in the lungs. Leg muscle, spleen, and heart were the least active.

Optimum Conditions. To determine the optimum pH for the reaction, incubations were carried out in mixtures containing 250 μmoles of sodium phosphate or Tris–HCl buffer, and the final pH was measured at 37° without terminating the reactions. Figure 3 shows that activity was maximal at pH 6.1 to 6.3, falling off sharply on the acid side but less rapidly on the alkaline side. The activity of the system was nearly as good in Tris–HCl as in phosphate buffer, but approximately 50% inhibition was observed with Tris–maleate buffer. Falcone and Nickerson (1963) have also reported that Tris–maleate inhibits selenite reduction.

The optimum selenite concentration range for DMSe synthesis is rather low and narrow. In a typical experiment (Figure 4) maximum activity is obtained at selenite concentrations of 4 to 6 $\times$ 10^{-5} M and activity decreases rapidly on either side of the optimum. The rate curves for volatilization (not shown) reveal that volatilization is rapid at first and then falls off with time when the initial substrate concentration is below the optimum, while at higher concentrations volatilization lags for a longer time at the start but then increases with time. The maximum rates of volatilization attained are approximately the same between 2 $\times$ 10^{-5} and 1 $\times$ 10^{-4} M selenite.

The synthesis of DMSe is in general proportional to the amount of 9000 $\times$ *g* liver supernatant added up to approximately 6 mg of protein (Figure 5). Liver extract (5.65 mg of protein) heated at 95° for 5 min lost 96% of its activity and represents zero protein in the graph of protein *vs.* activity. The extensive binding of selenite by protein (McConnell and Cooper, 1950; McConnell and Roth, 1962) requires that relative concentrations of selenite and protein must be controlled rather carefully in studies of DMSe synthesis. Selenite itself is an inhibitor of DMSe synthesis at higher concentrations (Figure 4) and is known to inhibit other enzymes (Tsen and Collier, 1959). Substrate inhibition at low protein concentrations therefore is one possible explanation for the slightly sigmoid character of the activity curve in Figure 5. Similarly, the observed activity may decrease at high concentrations of protein due to lowering of the selenite concentration through binding to protein, apart from the kinetic effects due to lowering of selenite concentration through volatilization.

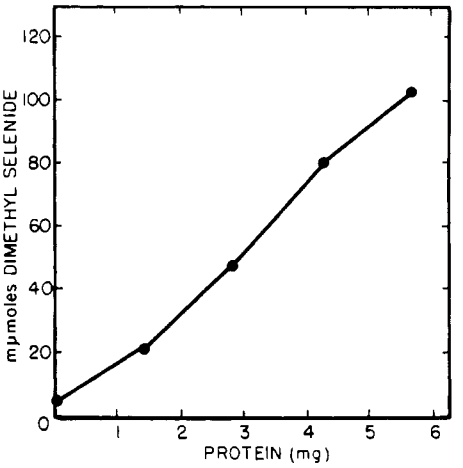

FIGURE 5: Proportionality of dimethyl selenide synthesis to protein concentration. The protein source was a 9000 $\times$ *g* supernatant from 10% liver homogenate, incubated for 20 min with the complete medium (see Experimental Section). Supernatant (5.65 mg of protein) heated at 95° for 5 min represents zero protein.

TABLE III: Cofactor Requirements for Synthesis of Dimethyl Selenide by Liver Fractions.

Incubation Medium	Relative Activity: 9000 $\times$ *g* Supernatant	Relative Activity: Washed Microsomes
Complete[a]	100[b]	100[b]
Minus GSH, TPNH-generating system[c]	6	0
GSH	19	0
TPNH-generating system[c]	64	94
S-Adenosyl-L-methionine	16	2
Magnesium	78	84
ATP	90	64
Coenzyme A	97	59

[a] The complete medium is described in the Experimental Section. [b] Specific activity of complete system with 9000 $\times$ *g* supernatant was 92 $\times$ 10^{-2} mμmole of Se/min per mg of protein, with washed microsomes 81 $\times$ 10^{-2} mμmole of Se/min per mg of protein. [c] The TPNH-generating system consisted of 0.4 μmole of TPN, 6 μmoles of glucose 6-phosphate, and 5 μg of glucose 6-phosphate dehydrogenase.

The synthesis of DMSe requires a number of substances for optimal activity, and the optimal concentration of each required substance was determined in preliminary studies. Table III shows the relative importance of the components in the incubation medium at optimal or near optimal concentrations for each substance. The 9000 $\times$ *g* supernatant of mouse liver homogenates has a high requirement for glutathione and is nearly devoid of activity when both glutathione and the TPNH-generating system are omitted. Presumably endogenous glutathione can be regenerated by TPNH and glutathione reductase present in the crude extract. The other major requirement is for *S*-adenosylmethionine, which stimulates the reaction sixfold. A moderate stimulation results from the addition of a TPNH-generating system. Magnesium is required for optimal activity, and ATP also stimulates the system significantly, even in the presence of optimal amounts of *S*-adenosylmethionine. Coenzyme A did not stimulate the reaction in this experiment, but under very similar conditions in three other studies the activity of the system minus coenzyme A was only 67–85% of that for the complete system containing 1 $\times$ 10^{-4} to 4 $\times$ 10^{-4} M coenzyme A, and this cofactor was routinely added at a concentration of 2 $\times$ 10^{-4} M. Table IV also demonstrates the stimulating effect of coenzyme A.

Washed microsomes show more stringent cofactor requirements, notably for ATP and coenzyme A, and have an absolute requirement for glutathione and *S*-adenosylmethionine but are stimulated very little by a TPNH-generating system.

A number of additional cofactors were tested for their ability to stimulate the crude liver extract. At concentrations ranging from 10^{-4} to 10^{-6} M, the follow-

TABLE IV: Specificity of Requirement for Glutathione and Coenzyme A in Dimethyl Selenide Synthesis.

Additions to Basal Medium	Molarity	Basal Medium −GSH +CoA	Basal Medium +GSH −CoA
None		20[a]	87[b]
Glutathione	10^{-4}	33	
	10^{-3}	73	
	10^{-2}	87	
Dithiothreitol	10^{-3}	34	33
Mercaptoethanol	10^{-3}	8	55
Thioglycoate	10^{-3}	11	78
L-Cysteine	10^{-3}	13	86
L-Cystine	10^{-3}		30
Coenzyme A	4×10^{-4}		100

[a] The values are mμmoles of dimethyl selenide synthesized/20 min per 5.48 mg of protein from the 9000 × *g* liver supernatant incubated with the complete system (see Experimental Section) minus glutathione. [b] The values are mμmoles of dimethyl selenide synthesized/20 min per 5.16 mg of protein from the 9000 × *g* supernatant of liver homogenate incubated with the complete system (see Experimental Section) minus coenzyme A.

ing substances either inhibited the synthesis of DMSe or had no effect: pyridoxal phosphate, flavin mononucleotide, flavin–adenine dinucleotide, *dl*-L-tetrahydrofolic acid, cyanocobalamin, thiamine pyrophosphate, menadione, coenzyme Q_{10}, hexahydrocoenzyme Q_4, hexahydrocoenzyme Q_4-chromanol, and *dl*-α-tocopheryl acetate.

Table IV demonstrates the effect of various concentrations of glutathione on DMSe synthesis and compares the ability of other thiols to substitute for glutathione and coenzyme A. A small amount of DMSe is formed in the absence of supplemental glutathione, presumably because traces of endogenous glutathione are present in the 9000 × *g* supernatant. The addition of 10^{-4} M glutathione causes a partial response, and the effect is nearly maximal at 10^{-3} M, although 10^{-2} M is the approximate amount required for optimal activity. Dithiothreitol is the only compound tested which increases the synthesis of DMSe under these conditions and it is much less active than glutathione at the same concentration. Dithiothreitol effectively maintains monothiols and certain dithiols in the reduced state (Cleland, 1964) and may have stimulated by regenerating traces of endogenous glutathione. All other thiols inhibited the system to some extent. These results indicate that glutathione has a specific role in selenium metabolism as opposed to a "euphoristic" action.

In the presence of an optimal concentration of glutathione, coenzyme A is the only sulfhydryl compound that stimulates the synthesis of DMSe, and all except cysteine inhibit at 10^{-3} M. In view of the well-known reactivity of selenite with sulfhydryl compounds it is not surprising that many thiols inhibit the conversion of selenite to DMSe. Cystine inhibits strongly at 10^{-3} M, however, and the inhibition by thiol reagents might be due in part to the presence of their disulfides. The inhibition by dithiothreitol in the presence of glutathione may result from complex formation with selenite, since Nickerson and Falcone (1963) have reported the formation of a yellow precipitate when selenite was added to another dithiol, British Anti-Lewisite.

The activity of *S*-adenosylmethionine as a methyl donor was compared to that of methionine (Table V).

TABLE V: Comparison of Methionine and *S*-Adenosylmethionine as Methyl Donors for Dimethyl Selenide Synthesis.[a]

Additions (μmoles)	Dimethyl Selenide Produced (mμmoles)
L-Methionine (30), ATP (30)	33
S-Adenosyl-L-methionine (4)	78
S-Adenosyl-L-methionine (4), ATP (30)	99
S-Adenosyl-L-methionine (4), ATP (30), L-methionine (30)	94

[a] All flasks contained the complete medium (see Experimental Section) minus *S*-adenosylmethionine and ATP. The source of enzymes was the 9000 × *g* supernatant (5.42 mg of protein) from 10% liver homogenate. The incubations were for 20 min.

In this experiment the concentration of ATP (when present) was increased to 10^{-2} M so that conditions would be more nearly optimal for endogenous synthesis of *S*-adenosylmethionine. The enzymic synthesis of this compound also requires unusually high concentrations of magnesium for optimal activity, but magnesium could not be increased above the usual level (1.33×10^{-2} M) because higher concentrations inhibited the synthesis of DMSe, possibly through the formation of magnesium selenide.

Under these conditions, the amount of DMSe formed in the presence of *S*-adenosylmethionine is more than double that formed in the presence of a combination of methionine and ATP, indicating that *S*-adenosylmethionine is the probable methyl donor and that the synthesis of this compound is a rate-limiting step in the synthesis of DMSe when methionine is added as the source of methyl groups. Methionine does not stimulate when added to the complete system. Even when *S*-adenosylmethionine is present in excess of the amount required for optimal activity, ATP stimulates the syn-

thesis of DMSe and may therefore be involved in some other reaction of the over-all pathway.

Incubation under nitrogen is an important requirement for optimal activity (Table VI). Synthesis of DMSe in the presence of air is only 10–15% of that obtained when a nitrogen atmosphere is used. Volatilization under either atmosphere is increased by glutathione. If an anaerobic atmosphere is maintained without removing volatile selenium as it is formed, the system is only 50% as active as when the flasks are swept continuously with nitrogen, possibly because dimethyl selenide is inhibitory to the system. The characteristic lability of organoselenium compounds, which is amply documented in a recent report describing the synthesis of selenocoenzyme A (Gunther and Mautner, 1965), would indicate that anaerobic conditions favor the reduction of selenium.

TABLE VI: Importance of Anaerobic Conditions for Synthesis of Dimethyl Selenide.[a]

Gas Phase	Dimethyl Selenide (mμmoles) GSH present	GSH absent
Experiment 1		
Nitrogen (flowing)[b]	112	30
Air (flowing)[b]	13	8
Experiment 2		
Nitrogen (flowing)[b]	96	
Air (flowing)[b]	15	
Nitrogen (closed system)[c]	52	

[a] The complete incubation medium (see Experimental Section) with or without 60 μmoles of GSH was incubated for 20 min under the indicated atmosphere. The enzyme source was 8.76 mg (expt 1) or 5.54 mg (expt 2) of protein from the 9000 × *g* supernatant of a liver homogenate. [b] Flow rates were approximately 200 ml/min. [c] The flask was flushed with nitrogen for 5 min, then sealed.

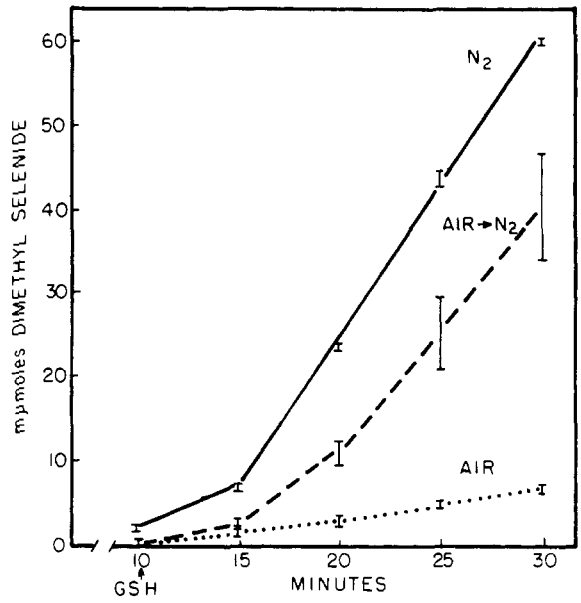

FIGURE 6: Reversal of air inhibition. Three pairs of flasks were preincubated under air or nitrogen in the complete medium (see Experimental Section) minus glutathione; the reaction was started by tipping in selenite (zero time). Glutathione (60 μmoles) was added at 10 min. Gas phase: ———, N_2 throughout; . . . ., air throughout; – – – – air 0–10 min, N_2 10–30 min. Bars indicate the range of duplicates. The protein source was 6.19 mg of 9000 × *g* supernatant from a liver homogenate.

The ability of nitrogen to reverse the effects of air was studied to determine if air caused an irreversible loss of activity. Three pairs of flasks were preincubated at 37° under nitrogen or air for 5 min, then radioactive selenite was tipped into the flasks at zero time. At 10 min, glutathione was tipped into all flasks and one pair of flasks was switched from air to nitrogen while incubation of the control flasks was continued under air or nitrogen (Figure 6). During the first 10 min of incubation dimethyl selenide synthesis was minimal because glutathione had not been added to the medium, but the amount produced in the presence of air was only 35% of that synthesized under nitrogen. After the addition of glutathione, volatilization of selenium in the control flasks incubated under nitrogen increased to a rapid rate in less than 5 min. Flasks incubated under air prior to the addition of glutathione and then changed to nitrogen attained nearly this same rate of selenium volatilization after a slightly longer lag period, while those continued under air showed only a slightly increased rate of dimethyl selenide synthesis. These results show that incubation of homogenates under air in the presence of selenite does not result in an irreversible loss of activity for DMSe synthesis, suggesting that air does not inhibit the system by oxidizing a labile protein involved in the catalytic process. Presumably air inhibits because it oxidizes labile products of selenium reduction, and synthesis of DMSe therefore does not occur at an appreciable rate until anaerobiosis is attained. In agreement with this interpretation, a slightly longer lag period is present when air is not previously removed by flushing with nitrogen (Figure 6). The rapidity with which activity is restored makes it less likely that reversal of inhibition is due to regeneration by glutathione of a labile sulfhydryl moiety. Inhibition by air is only partially reversed by nitrogen if glutathione is present during the prior incubation under air, even if additional glutathione is tipped in after anaerobiosis is attained.

Inhibition Studies. Sodium arsenite has a pronounced inhibitory effect on selenium volatilization, as shown

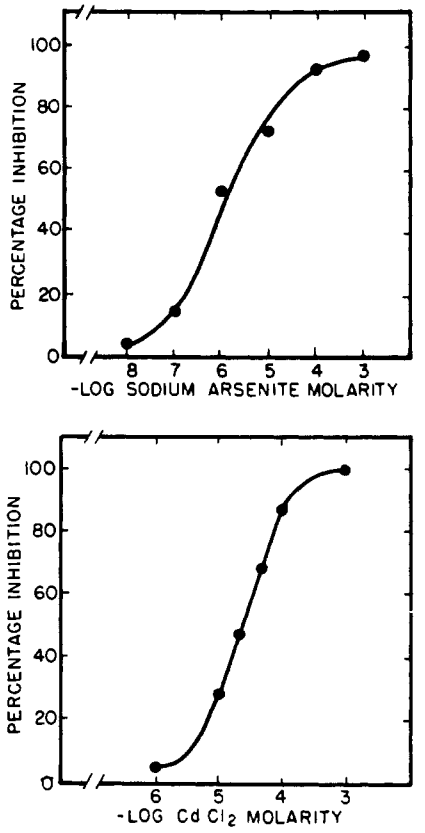

FIGURE 7: Inhibition by arsenite (A) or cadmium (B). Sodium arsenite or cadmium chloride at the concentrations indicated was preincubated with the complete medium (minus selenite) for 5 min at 37° before selenite was added. The medium contained 2×10^{-2} M GSH, 2×10^{-4} M coenzyme A, 1×10^{-3} M EDTA, and the other components described in the Experimental Section. The 9000 × *g* supernatant (5.48 mg of protein) from a liver homogenate was the enzyme source.

in Figure 7a. Inhibition is detectable with 10^{-6} M arsenite and 50% inhibition is obtained with 10^{-6} M arsenite. Since these effects of arsenite are observed in the presence of 2×10^{-2} M glutathione and 2×10^{-4} M coenzyme A, the effect of arsenite is specific and not effectively reversed by monothiols. Table VII presents further evidence of the unusual sensitivity to arsenite. At concentrations of 10^{-5} and 10^{-6} M, *N*-ethylmaleimide has almost no inhibitory effect while *p*-mercuribenzoate, another commonly used inhibitor of sulfhydryl enzymes, inhibits only 10% at 10^{-6} M compared to 51% for arsenite at the same concentration.

The inability of monothiols to prevent the inhibitory action of arsenite suggests that arsenite might react with a vicinal dithiol required for DMSe synthesis. Cadmium is another effective inhibitor of dithiol proteins (Sanadi *et al.*, 1959) and was therefore tested under the same conditions (Figure 7b). Cadmium proved to be a less effective inhibitor than arsenite, but inhibited 47% at a concentration of 2×10^{-5} M. This concentration

TABLE VII: Inhibition of Dimethyl Selenide Formation by Arsenite Compared to Other Sulfhydryl Reagents.[a]

Compound	Molarity	Inhibition (%)
N-Ethylmaleimide	1×10^{-6}	4
	1×10^{-5}	6
p-Mercuribenzoate	1×10^{-6}	10
	1×10^{-5}	29
Sodium arsenite	1×10^{-6}	51
	1×10^{-5}	75

[a] Inhibitors at the indicated concentrations were preincubated with complete medium (minus selenite) for 5 min at 37° before selenite was added. The 9000 × *g* supernatant (5.70 mg of protein) from a liver homogenate was the enzyme source.

is in the same range as the substrate concentration and suggests that cadmium may inhibit by reacting with selenide intermediates or products to form insoluble cadmium selenide. The effects of cadmium are difficult to interpret quantitatively, however, because of the unknown extent to which mercaptide formation with glutathione or protein fractions and chelation by EDTA compete for added cadmium.

Reaction of Glutathione with Selenite. Painter (1941) proposed a general reaction between selenous acid and sulfhydryl compounds to form unstable compounds of the type RS–Se–SR

$$4RSH + H_2SeO_3 \longrightarrow RS\text{–}Se\text{–}SR + RSSR + 3H_2O$$

Compounds of this type known to be formed from simple thiols include selenodiglutathione (Petersen, 1951) and selenodicysteine (Klug and Petersen, 1949). Figure 8 shows the formation of a chromophoric substance in the reaction of 4 μmoles of glutathione with 1 μmole of selenous acid. The substance, presumably selenodiglutathione, has an ultraviolet absorption band extending to approximately 360 mμ with a maximum at 258 mμ. Glutathione and selenous acid do not absorb in this range and oxidized glutathione (1 μmole) has only a weak absorption band beginning at 320 mμ with no maximum at 258 mμ. The reaction is complete within 1–3 min at 25° and the compound formed is stable for some time before it decomposes to liberate elemental selenium. Oxidized glutathione and selenous acid did not react under these conditions. The nonenzymic reaction between glutathione and selenite suggests that the over-all conversion of selenite to dimethyl selenide is a complex pathway involving both enzymic and nonenzymic reactions.

Discussion

The enzymic synthesis of dimethyl selenide from sodium selenite by mouse liver homogenates confirms

that the reductive utilization of inorganic selenium occurs directly in mammalian tissues without the involvement of intestinal flora. Although most animals do not synthesize significant amounts of reduced organosulfur compounds from sulfate, it is interesting that they apparently utilize an organosulfur compound, glutathione, in the synthesis of the organoselenium compound dimethyl selenide.

The requirement for glutathione is specific and indicates a unique role for this substance in selenium metabolism. Selenite is known to react nonenzymically with glutathione to form selenodiglutathione. This compound is believed to be an intermediate in the oxidation of glutathione catalyzed by selenite, reacting with oxygen to yield selenite and oxidized glutathione (Tsen and Tappel, 1958). Under anaerobic conditions, selenodiglutathione or a similar derivative may be an intermediate in the further reduction of selenium to the selenide level. Selenite also reacts with other thiols such as cysteine to form compounds analogous to selenodiglutathione, but the inability of these thiols to substitute for glutathione may reflect the special suitability of glutathione derivatives of selenium as substrates for enzymes that metabolize this very reactive element. Research is in progress to determine if glutathione is involved in the synthesis of other organoselenium compounds in addition to dimethyl selenide.

The requirement for *S*-adenosylmethionine indicates a direct role for this substance in the methylation of selenium. Bremer and Natori (1960) have described a microsomal system from rat liver that catalyzed the methylation of hydrogen selenide and methylselenol by *S*-adenosylmethionine. Whether hydrogen selenide is an intermediate in the synthesis of dimethyl selenide or other organoselenium compounds, however, is not known.

The crude liver system described in this paper requires a number of substances for optimal activity, including ATP, coenzyme A, and a TPNH-generating system. The assignment of a role for these substances in the reaction is an interesting possibility that must await purification of the system. It should be noted that ATP is required in nitrogen fixation (Hardy and D'Eustachio, 1964) and in the reduction of ribonucleotides (Reichard, 1962).

There are a number of interesting biological relationships between arsenite and selenium. Arsenite antagonizes both the chronic and the acute toxicity of selenium (Moxon, 1938; Kamstra and Bonhorst, 1953). Arsenite also prevents the formation of volatile selenium in animals injected with selenite (Kamstra and Bonhorst, 1953; Ganther and Baumann, 1962). In the present study arsenite has been shown to be a very effective inhibitor of dimethyl selenide synthesis *in vitro*, indicating that the action of arsenite on selenium volatilization *in vivo* is a direct inhibition of volatile selenium formation. Cadmium also inhibits dimethyl selenide formation *in vitro* and was previously shown to inhibit *in vivo* (Ganther and Baumann, 1962). Since these effects of arsenite and cadmium are not prevented by a large excess of glutathione, the possibility exists

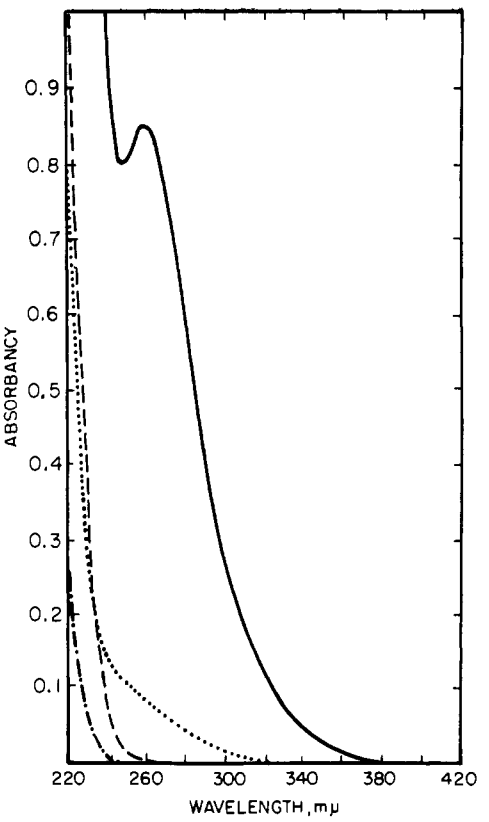

FIGURE 8: Absorption spectrum of chromaphore formed in nonenzymic reaction between glutathione and selenite. (———), 4 μmoles of GSH plus 1 μmole of H_2SeO_3; (– – – –), 4 μmoles of GSH; (—·—·—), 1 μmole of H_2SeO_3; (. . . . .), 1 μmole of GSSG. Conditions for all spectra: temperature 25°, pH 4–4.4, gas phase air, volume 3.0 ml, path length 1 cm, reference cuvet filled with distilled water.

that a dithiol protein is involved in the reduction of selenium. A dithiol protein of low molecular weight participates in the reduction of sulfate by yeast extracts (Wilson *et al.*, 1961), and functionally similar dithiols have been described in other enzyme reactions (Massey and Veeger, 1960; Black *et al.*, 1960; Reichard, 1962). Arsenite is known to inhibit the oxidation of glutathione catalyzed by selenite (Tsen and Tappel, 1958), and presumably could inhibit dimethyl selenide synthesis by preventing a reaction of selenite with glutathione, although it must be emphasized that arsenite inhibits 50% at a concentration of 10^{-6} M, or $^1/_{50}$ the concentration of selenite, whereas arsenite/selenite ratios of 2.5 or 5 were required to inhibit selenite catalysis of glutathione oxidation.

The enzymic reduction of selenite to elemental selenium by yeast extracts was studied by Nickerson and Falcone (1963). The active components of their system included glutathione, TPN^+, glucose 6-phosphate, and a quinone. Selenite reduction was inhibited 49% by 10^{-3} M arsenite and the inhibition was not reversed by glutathione. Evidence was presented

that selenite was bound to a vicinal dithiol during the reduction, and the role of glutathione was believed to be the maintenance of protein dithiols in reduced form. This system did not catalyze the formation of volatile selenium (Falcone and Nickerson, 1963). The requirement for a quinone in selenite reduction may be limited to cases where elemental selenium is the product, although further purification may still reveal a role for quinones in the six-electron reduction of selenite to selenide. The purified sulfite reductase of *Escherichia coli* also reduces selenite by means of TPNH, but the apparent Michaelis constant for selenite is 0.09 M, compared to 8×10^{-6} M for sulfite (Kemp *et al.*, 1963). Rosenfeld and Beath (1948) have demonstrated the reduction of selenium in bovine tissues.

Studies on the biosynthesis of organoselenium compounds may have some bearing on the nutritional role of selenium. It is known that vitamin E and antioxidants are closely interrelated with selenium function, and it has been suggested that the primary role of selenium is also that of an antioxidant protecting sensitive cellular structures from oxidative destruction. In view of the sensitivity of many organoselenium compounds to oxidation, it should be kept in mind that selenium may have a more specific biological role as an organoselenium compound and that this compound may be protected from oxidative destruction by vitamin E or other antioxidants.

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