The Nature of the Acid-Volatile Selenium in the Liver of the Male Rat

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1. The properties of rat liver acid-volatile selenium have been compared with those of H_2Se and $(\text{CH}_3)_2\text{Se}$. 2. In model experiments oxidation-sensitive H_2^{75}Se was trapped quantitatively under anaerobic conditions in 0.1 M- AgNO_3 , and $(\text{CH}_3)_2^{75}\text{Se}$ was trapped quantitatively in 8 M- HNO_3 . The acid-labile selenium of a liver homogenate, and of a microsomal fraction, was found to behave quite unlike $(\text{CH}_3)_2^{75}\text{Se}$ and in a manner indistinguishable from H_2^{75}Se . 3. It was concluded that the acid-volatile material is certainly not $(\text{CH}_3)_2\text{Se}$ and that it is probably H_2Se . 4. The significance of these findings is discussed in relation to current knowledge about the metabolism and detoxication of selenium, and a scheme is proposed which incorporates this knowledge with recent observations on the interactions between trace amounts of selenium and tocopherol, and the production of acute selenium deficiency by Ag^+ in vitamin E-deficient rats.

Diplock *et al.* (1971) showed that, when rats were given small amounts of $\text{Na}_2^{75}\text{SeO}_3$, a substantial proportion of the ^{75}Se present in the liver subcellular organelles was an acid-volatile material tentatively identified as selenide. It was further shown that the proportion of the selenium that was acid-volatile was smaller in vitamin E-deficient rats than in normal rats, and that the acid-volatile selenium was very susceptible to oxidation *in vitro*. Caygill *et al.* (1971) presented evidence suggesting that the acid-volatile selenium was particularly associated, in adequately fed rats, with the mitochondria and the smooth-surfaced endoplasmic reticulum and that this association was lost when vitamin E was removed from the diet of the animals; the acid-volatile selenium was also largely protein-bound. These results have been taken to indicate that a major function of vitamin E *in vivo* is to prevent the oxidation of the acid-volatile selenium, which is thought to represent a form of the element that is biologically active in trace amounts. It has been further proposed that the acid-volatile selenium may form a part of the active centre of certain non-haem iron-containing proteins which are protected from oxidation *in vivo* by vitamin E, and a general theory for the mode of action of vitamin E and selenium has been advanced that incorporates these proposals (Diplock & Lucy, 1973).

Some evidence was presented by Diplock *et al.* (1971) that the acid-volatile selenium was selenide (Se^{2-}). However, these results were preliminary only, and the present paper gives the results of further experiments that have been carried out to determine the nature of the acid-volatile selenium. It was of

particular importance to determine whether the selenium that was volatilized on acidification was H_2Se or an alkyl selenide, since Ganther (1966) has shown that, in mouse liver homogenates, toxic amounts of $\text{Na}_2^{75}\text{SeO}_3$ are converted into $(\text{CH}_3)_2^{75}\text{Se}$ which, if it were also present in rat liver, might be released on acidification in our experiments.

Experimental

Animals, diets and dosage

Caesarian-derived weanling male Wistar rats (40–50 g) were purchased from a commercial breeder and maintained on the Oxoid 41B pellet diet for 4 months in wire-floored cages. The rats were then killed and homogenates of their livers prepared as described below for the model experiments on $(\text{CH}_3)_2\text{Se}$. Other rats, to be used for the identification of the acid-volatile selenium, were given three consecutive daily doses of $\text{Na}_2^{75}\text{SeO}_3$ in 0.1 ml of 0.9% (w/v) NaCl ($50 \mu\text{Ci} \equiv 9 \mu\text{g}$ of Se per dose), killed on the fourth day, and their livers homogenized as described.

Radioisotopes and radioisotope counting

The specific radioactivity of the $\text{Na}_2^{75}\text{SeO}_3$ (The Radiochemical Centre, Amersham, Bucks., U.K.) used in all the experiments described was $5.56 \mu\text{Ci}/\mu\text{g}$ of Se. ^{75}Se radioactivity was counted in the Wallac GTL-300 automatic γ -isotope counter, with 5.08 cm (2 in) crystal, at a counting efficiency of 78%. Each measurement was made in duplicate or triplicate and the values given are means of these observations.

Model experiments with H_2Se

Preliminary experiments, development of apparatus. Reactions (1) and (2) were investigated:

A great deal of time was spent in investigating appropriate conditions and a suitable apparatus in which H_2Se produced by treating Na_2SeO_3 with Zn dust and conc. HCl (reaction 1) could be trapped quantitatively in 0.1M- $AgNO_3$ solution (reaction 2). The difficulty appeared to be the ease with which H_2Se is oxidized to elemental Se. Glass apparatus was found to be unsatisfactory because: (i) it is difficult to work, and the fine capillaries needed for these experiments were very fragile; (ii) it has a surface on which elemental Se is readily deposited from H_2Se , a fact that could be demonstrated by counting the radioactivity in shattered pieces of the apparatus; (iii) once contaminated with elemental Se the apparatus proved impossible to clean.

Nylon and polypropylene were found to be unsuitable because selenium was also readily deposited on their surface. The apparatus shown in Fig. 1 was used in all the experiments. The polystyrene vials (Luckham Ltd., Labro Works, Burgess Hill, Sussex, U.K.) (internal diam. 12.7mm and length 89.7mm) were fitted with a polythene stopper. The tube for the carrier gas was a disposable glass Pasteur pipette, and a no. 20 disposable hypodermic needle passed through the stopper for the addition of acid and was equipped with a fine polythene capillary reaching to the bottom of the vial. The polythene connecting tubing (2.18mm internal diam., 3.25mm external diam., 170mm long) was obtained from Portex Ltd., Hythe, Kent, U.K. The size of the vials, and the length of the connecting tube given, were found by experiment to be optimum and critical. The advantages of this apparatus were that it was entirely disposable and only used once, and the vials could be placed directly in the γ -isotope counter.

The rate at which the acid was added to the mixture of Zn dust and $Na_2^{75}SeO_3$, and the bubbling rate of the carrier gas used, were also found to be important. When the amount of acid-volatile selenium in tissue fractions was measured (Diplock *et al.*, 1971), it was found necessary to add the acid rapidly to achieve consistent results; this was thought to be due to the fact that, unless the volatile material was released rapidly, there was a greater chance of its oxidation. Similarly, with the model experiments, it was essential to add the acid as rapidly as possible, otherwise the amount of the $Na_2^{75}Se$ formed that was converted into elemental ^{75}Se and left in the reaction vial could be very high. Once formed, the $H_2^{75}Se$ is so susceptible to oxidation that it was found necessary to use a rapid

Fig. 1. Apparatus for identification of acid-volatile selenium

Full details are given in the Experimental section.

flow of carrier gas to purge it from the reaction vial; once purged, however, it was also essential to avoid so fast a flow rate that the $H_2^{75}Se$ was not trapped in the second vial. A flow rate of 200ml/min gave satisfactory results and was used in all subsequent experiments; the flow rate was determined by placing the outlet tube from the trapping vial in a beaker of water, and inverting a burette filled with water over the outlet tube so that the bubbles of gas were trapped in the burette.

The standard conditions for Expts. 1-3, described in the next section, were as follows. A quantity of $Na_2^{75}SeO_3$ in 2 ml of water was placed in the reaction vial and the ^{75}Se radioactivity determined by triplicate counting. About 350mg of Zn dust was then added together with four drops of octanol and the vial connected as shown in Fig. 1. The gas delivery tube was connected to the appropriate gas supply and a

1 ml tuberculin hypodermic syringe containing 1 ml of conc. HCl was connected to the needle. Then 3 ml of the appropriate trapping agent was placed in the trapping vial and carrier gas passed for at least 10 min to purge the system of O₂. The acid was then added by depressing the plunger of the syringe as rapidly as possible, and passage of carrier gas continued for a further 10 min. The end of the connecting tube was washed with 1 ml of water and the ⁷⁵Se radioactivity was determined by triplicate counts in the trapping vial, and also the residual ⁷⁵Se radioactivity was measured in the reaction vial. It was found possible to do three experiments simultaneously, by using a manifold to distribute the carrier gas. The octanol was added to prevent frothing; four drops of octanol were also added to the tissue fractions described in our previous publications (Diplock *et al.*, 1971; Caygill *et al.*, 1971), but mention of this fact was inadvertently omitted from the description of the experiments.

Investigation of carrier gases and different trapping agents for H₂Se. Expt. 1. The suitability as carrier gas of H₂ and O₂-free N₂ was investigated in a number of experiments and the result of a typical experiment is shown in Table 1.

Expt. 2. It was thought possible that the small loss

of ⁷⁵Se radioactivity from the AgNO₃ trapping vial in Expt. 1 might be due to contamination of the terminal end of the connecting tube with ⁷⁵Se. This was shown to be the case in several experiments, the results of one of which are shown in Table 2.

Expt. 3. In this experiment the usefulness of several different agents for trapping H₂Se was examined. AgNO₃ solution had already been shown to be an excellent trapping agent and this was compared with 8 M-HNO₃, shown by Ganther (1966) to be an excellent agent for trapping (CH₃)₂Se, and with chloroform, since this had been suggested to us as a good trapping agent for (CH₃)₂Se (T. Sadeh, personal communication); the 'solubility' of H₂Se in water was also investigated, although the results given probably partly represent oxidation of H₂Se to elemental Se in the water rather than true solubility of H₂Se. The results of this experiment are given in Table 3.

Model experiments with (CH₃)₂Se

In these experiments, the system described by Ganther (1966) for studying the biosynthesis of (CH₃)₂Se *in vitro* in a cell-free system derived from

Table 1. *Expt. 1. Model experiments with H₂⁷⁵Se*

A portion (0.027 mg) of Se (as Na₂⁷⁵SeO₃) was placed in the reaction vial (see the text and Fig. 1) and diluted to 2 ml with water. Zn dust (350 mg) and four drops of octanol were added, carrier gas was passed for 10 min, and 1 ml of conc. HCl added rapidly. Passage of gas was continued for 10 min and volatile products were collected in the trapping vial in 3 ml of 0.1 M-AgNO₃ and the ⁷⁵Se radioactivity was counted in the trapping vial and in the reaction vial. Numbers of observations are given in parentheses and values given are mean values ± s.d.

	Carrier gas	
	H ₂	N ₂
Initial ⁷⁵ Se radioactivity (c.p.s.)	43 ± 3 (3)	43 ± 4 (3)
Loss of ⁷⁵ Se radioactivity on Zn+HCl treatment (%)	89.3 ± 2.7	88.5 ± 1.3
Recovery of ⁷⁵ Se radioactivity in AgNO ₃ (% of ⁷⁵ Se lost from reaction vial)	40.2 ± 3.8	89.5 ± 0.8

Table 2. *Expt. 2. Model experiments with H₂⁷⁵Se*

A portion (0.27 ng) of Se (as Na₂⁷⁵SeO₃) was placed in the reaction vial and the experiment was carried out as for Expt. 1., with N₂ as carrier gas. The terminal 4 cm of connecting tube (see Fig. 1) was cut into 0.5 cm lengths and the ⁷⁵Se radioactivity was also determined in the pieces. Numbers of observations are given in parentheses and values given are mean values ± s.d.

Initial ⁷⁵ Se radioactivity (c.p.s.)	437 ± 22 (5)
Loss of ⁷⁵ Se radioactivity on Zn+HCl treatment (%)	91.2 ± 3.1
Recovery of ⁷⁵ Se radioactivity in AgNO ₃ (% of ⁷⁵ Se lost from reaction vial)	88.9 ± 3.8
Recovery of ⁷⁵ Se radioactivity in terminal 4 cm of connecting tube (% of ⁷⁵ Se lost from reaction vial)	6.7 ± 0.3
Total recovery of ⁷⁵ Se radioactivity (% of ⁷⁵ Se lost from reaction vial)	95.7 ± 1.5

Table 3. Expt. 3. Model experiments with $H_2^{75}Se$

A portion (0.098 ng) of Se (as $Na_2^{75}SeO_3$) was placed in the reaction vial and the experiment was carried out as for Expt. 1, by using N_2 as carrier gas and the various trapping agents shown. In the fifth part of the experiment a second trapping vial, containing 3 ml of 0.1 M- $AgNO_3$ solution, was connected in series after the first, containing 3 ml of 8 M- HNO_3 . Numbers of observations are given in parentheses and values given are mean values $\pm$ S.D.

	Trapping agents				
	0.1 M- $AgNO_3$	8 M- HNO_3	Water	Chloroform	8 M- HNO_3 then 0.1 M- $AgNO_3$
Initial ^{75}Se radioactivity (c.p.s.)	158 $\pm$ 2 (3)	158 $\pm$ 4 (3)	161 $\pm$ 4 (3)	157 $\pm$ 6 (3)	—
Loss of ^{75}Se radioactivity on Zn + HCl treatment (%)	83.7 $\pm$ 2.2	91.2 $\pm$ 5.6	87.0 $\pm$ 1.0	85.3 $\pm$ 2.0	—
Recovery of ^{75}Se radioactivity in trapping agent shown (% of ^{75}Se lost from reaction vial)	87.7 $\pm$ 5.6	21.7 $\pm$ 1.8	13.5 $\pm$ 2.1	38.5 $\pm$ 6.0	147 $\pm$ 4 (3) 84.0 $\pm$ 2.7
					18.3 $\pm$ 3.2 95.6 $\pm$ 5.2*

* Shown as % of volatile ^{75}Se remaining after passage through 8 M- HNO_3 then through 0.1 M- $AgNO_3$.

mouse liver was used, with some modifications, for rat liver.

Preparation of extracts and reaction mixture. Adequately fed rats (4 months old) were killed and their livers removed and placed at once in ice-cold 0.25 M-sucrose–0.1 mM-EDTA. Homogenates were prepared in the same medium (1 g of liver + 9 ml of medium) in the homogenizer described previously (Caygill *et al.*, 1971) and centrifuged at 9000g for 10 min; the supernatant fraction from this centrifugation was used for the experiments.

Incubations were carried out in the apparatus shown in Fig. 1; the carrier-gas tube was cut off 0.5 cm above the level of the fluid in the reaction vial, since it was found impracticable to pass N_2 through the incubation mixture containing the tissue fraction owing to foaming, and it was not possible to add octanol because it might inactivate the enzymes present. The reaction vial was placed in a shaking water bath (60–80 excursions/min) at 37°C and the trapping vial was placed at room temperature above the water surface.

The reaction mixture contained, in μ mol: sodium phosphate buffer, pH 6.25, 100; GSH, 60; $MgCl_2$, 40; ATP, 12; S-adenosyl-L-methionine, 2; CoA, 0.6; EDTA, 3; $NADP^+$, 0.4; glucose 6-phosphate, 6; 6 units of glucose 6-phosphate dehydrogenase [Boehringer Corp. (London) Ltd.; London W.5, U.K.]. This mixture was prepared freshly before each experiment and gassed for 15 min with O_2 -free N_2 before being placed in the reaction vial together with 0.4 ml of liver supernatant (equivalent to 40 mg of liver) previously gassed with N_2 for 15 min to give a final volume of 3.0 ml. The system was gassed with N_2 for 10 min and 0.15 μ mol of $Na_2^{75}SeO_3$ in 0.1 ml of sodium phosphate buffer, pH 6.25, was passed through the hypodermic needle used in the previous experiments for the addition of acid. The collection of the volatile products in various trapping agents is described in the individual experiments. The ^{75}Se radioactivity was measured in the $Na_2^{75}SeO_3$ added to the reaction vial, in the residue in the reaction vial after the experiments and in the various trapping agents employed. The $Na_2^{75}SeO_3$ was diluted with Na_2SeO_3 to give the appropriate specific radioactivity for these experiments.

Expt. 4. The four trapping agents utilized in the experiments with H_2Se were investigated here. Collection of the volatile selenium was allowed to proceed for 15 min, then 0.5 ml of 5 M- $NaOH$ was added through the hypodermic needle to stop the reaction, and gas passed for a further 5 min. Each trapping agent was studied twice in quadruplicate and the results are given in Table 4.

Expt. 5. The previous experiment was unsatisfactory in that there was a large variation in the amount of ^{75}Se volatilized under apparently identical conditions. A further experiment was therefore done

Table 4. Expt. 4. Model experiments with (CH₃)₂Se

Na₂⁷⁵SeO₃ (0.15 μmol) was added to the reaction vial containing the mixture described in the text. Collection of volatile ⁷⁵Se was for 15min, with N₂ as carrier gas, in the trapping agents indicated. After addition of 0.5 ml of 5M-NaOH, the collection was continued for 5 min. Numbers of observations are given in parentheses and values given are mean values ± s.d.

	Trapping agents			
	8M-HNO ₃	0.1M-AgNO ₃	Water	Chloroform
10 ⁻⁴ × ⁷⁵ Se radioactivity added (c.p.s.)	4.14 ± 0.05 (8)	4.16 ± 0.09 (8)	4.15 ± 0.09 (8)	4.16 ± 0.09 (8)
10 ⁻⁴ × ⁷⁵ Se radioactivity volatilized (c.p.s.)	1.51 ± 0.27	0.82 ± 0.19	0.86 ± 0.01	1.04 ± 0.09
10 ⁻⁴ × ⁷⁵ Se radioactivity recovered (c.p.s.)	1.31 ± 0.11	0.18 ± 0.05	0.17 ± 0.02	0.34 ± 0.06
⁷⁵ Se radioactivity recovered (% of ⁷⁵ Se radioactivity volatilized)	83.6 ± 11.7	22.4 ± 4.2	20.7 ± 2.0	33.2 ± 7.3

Fig. 2. Expt. 5. Model experiments with (CH₃)₂Se

Na₂⁷⁵SeO₃ (0.15 μmol) was added to the reaction vial containing the mixture described in the text. Collection of volatile ⁷⁵Se with N₂ as carrier gas was for 5min periods as follows:

Time-interval (min)	0-5	5-10	10-15	15-20	20-25	25-30	30-35	35-40	40-45
Trapping agent	HNO ₃	AgNO ₃	Water	HNO ₃	AgNO ₃	Water	HNO ₃	AgNO ₃	Water

The experiment was done in quadruplicate, and each point represents the mean of four observations and the bar the s.d. of the mean of the observations. ●, 8M-HNO₃; ▲, 0.1M-AgNO₃; ■, water.

in which the collection period was shortened to 5 min, and the three trapping agents HNO_3 , AgNO_3 solution and water were studied sequentially by using the same incubation mixture. Thus HNO_3 was the trapping agent employed in the first 5 min period, AgNO_3 solution in the second and water in the third; the cycle was then repeated twice for a total of nine 5 min collection periods. The results of this experiment are presented in Fig. 2. The recovery of the total ^{75}Se radioactivity volatilized was estimated by drawing a line between the three points obtained with 8M- HNO_3 and taking the sum of the ^{75}Se radioactivity for the nine time-intervals. By using this technique, the recovery of the ^{75}Se volatilized (expressed as a percentage of the ^{75}Se lost from the reaction vial) was $92.8 \pm 3.3\%$ for the four observations made.

Experiments with the acid-volatile selenium of rat liver

Expt. 6. Three pairs of 4-month-old adequately fed rats were given three consecutive daily intraperitoneal doses of $\text{Na}_2^{75}\text{SeO}_3$ ($50 \mu\text{Ci} \equiv 9 \mu\text{g}$ of Se) in 0.1 ml of 0.9% (w/v) NaCl solution and killed on the 4th day. The livers were removed and placed at once in ice-cold 0.25M-sucrose solution containing $100 \mu\text{g}$ of α -tocopherol/ml (Roche Products Ltd., London W.1, U.K.) and 5mm-mercaptoethanol as described previously (Diplock *et al.*, 1971). Homogenates (10%, w/v) were prepared from each pair of livers in the same medium and used without preliminary centrifugation in the experiments. Portions (0.5–1.0 ml) of the homogenates were diluted to 3 ml with the 0.25M-sucrose- α -tocopherol-mercaptoethanol solution, placed in the reaction vial (Fig. 1) and ^{75}Se radioactivity was measured. Oxygen-free N_2 was passed for 10 min, with the delivery tube passing to the bottom of the vial so that the gas bubbled through the suspension; foaming was prevented by the addition of four drops of octanol. Then 1 ml of conc. HCl was added rapidly through the hypodermic needle with a syringe and gas was passed for a further 10 min. The volatile selenium produced from each homogenate was passed through 3 ml of 0.1M- AgNO_3 solution, and the ^{75}Se radioactivity remaining in the reaction vial, and trapped in the second vial, was measured. The procedure was then repeated twice, first with 8M- HNO_3 as trapping agent, and then with water. The entire sequence of experiments was then repeated so that the values given in Table 5 (Trial 1) are means of duplicate observations on each of the three homogenates.

In two subsequent trials, triplicate and quadruplicate pairs of rats were given the same ^{75}Se dosage as in Trial 1, and the trapping of the acid-volatile selenium was investigated, by using 0.1M- AgNO_3 solution and 8M- HNO_3 only as trapping agents. The results of Trials 2 and 3 are also given in Table 5.

Table 5. Expt. 6. Identification of acid-volatile protein-bound selenium from rat liver homogenates

Trapping agents	Trial 1			Trial 2			Trial 3		
	0.1M- AgNO_3	8M- HNO_3	Water	0.1M- AgNO_3	8M- HNO_3		0.1M- AgNO_3	8M- HNO_3	
Initial ^{75}Se radioactivity in homogenate (c.p.s.)	627 $\pm$ 13 (6)	663 $\pm$ 7 (6)	650 $\pm$ 4 (6)	1017 $\pm$ 13 (6)	1077 $\pm$ 21 (6)		1529 $\pm$ 15 (8)	1401 $\pm$ 47 (8)	
^{75}Se radioactivity volatilized on acid treatment (% of initial ^{75}Se radioactivity)	16 $\pm$ 0	17 $\pm$ 1	18 $\pm$ 0	19 $\pm$ 2	20 $\pm$ 2		14 $\pm$ 0	13 $\pm$ 1	
^{75}Se radioactivity trapped in agent specified on acid treatment (% of ^{75}Se radioactivity volatilized)	91 $\pm$ 6	8 $\pm$ 2	9 $\pm$ 3	91 $\pm$ 2	11 $\pm$ 3		91 $\pm$ 2	3 $\pm$ 0	

In Trials 1 and 2 triplicate pairs, and in Trial 3 quadruplicate pairs, of adequately fed rats were given three consecutive daily intraperitoneal doses of $\text{Na}_2^{75}\text{SeO}_3$ ($50 \mu\text{Ci} \equiv 9 \mu\text{g}$ of Se) in 0.1 ml of 0.9% (w/v) NaCl and killed on the fourth day. Then 10% (w/v) homogenates were prepared in 0.25M-sucrose solution containing $100 \mu\text{g}$ of α -tocopherol/ml and 5mm-mercaptoethanol, as described in the text. Samples (0.5 ml) of the homogenates were placed in the reaction vial (Fig. 1). N_2 was passed for 10 min and 1 ml of HCl added. Carrier gas was passed for a further 10 min and trapping of the acid-volatile products was investigated in the agents shown. Values given are mean values $\pm$ S.D., with numbers of observations given in parentheses.

Expt. 7. This experiment was similar to Expt. 6, except that liver microsomal fractions were studied. Two identical trials were done, by using quadruplicate pairs of adequately-fed rats, and dosage and preparation of homogenates was similar to that employed in Expt. 6. A microsomal fraction was then prepared by the method of Hogeboom (1955). Samples (3 ml) of the resuspended microsomal fraction were placed in the reaction vial and the trapping of the acid-volatile selenium was examined as in Expt. 6. In addition, in Trial 1, a further experiment was done in which the microsomal suspension was acidified with 1 ml of 50% (v/v) H_2SO_4 , and the volatile selenium trapped in 0.1 M- AgNO_3 solution. The results of these experiments are given in Table 6.

Susceptibility of acid-volatile selenium to oxidation in vitro

Expt. 8. It has been shown (Diplock *et al.*, 1971) that α -tocopherol is capable of protecting acid-volatile selenium from oxidation when it is added to the diet of rats, and that α -tocopherol and mercaptoethanol together are able to protect it *in vitro*. It was, however, also decided to determine whether α -tocopherol alone effects a similar protection *in vitro*, and the present experiment was therefore done to study this possibility. A group (12) of weanling male Wistar rats were given the vitamin E-deficient diet for 3 months and the *Torula* yeast diet for a further 2 weeks (Diplock *et al.*, 1971). They were then continued on the *Torula* yeast diet for a further 3 days, on each of which they received an oral dose of 0.25 ml of olive oil containing 10 mg of α -tocopherol, and, at the same time, 0.1 ml of a solution of $\text{Na}_2^{75}\text{SeO}_3$ ($50 \mu\text{Ci} \equiv 9 \mu\text{g}$) in 0.9% (w/v) NaCl intraperitoneally. On the fourth day the rats were killed in four lots of three rats and the livers placed at once in ice-cold 0.25 M-sucrose solution containing $100 \mu\text{g}$ of α -tocopherol/ml and 5 mM-mercaptoethanol (Diplock *et al.*, 1971). The livers in each lot were then removed from the sucrose solution, blotted, minced in a beaker with scissors and three homogenates made from each lot; one homogenate was made in 0.25 M-sucrose containing no antioxidants, a second was made in a similar medium containing $100 \mu\text{g}$ of α -tocopherol/ml which had been dispersed by sonication, and a third in the medium with both α -tocopherol and mercaptoethanol. A mitochondrial and a microsomal fraction was prepared from each, and the quantity of acid-volatile selenium measured by the methods previously described (Diplock *et al.*, 1971); the results are given in Table 7.

Results and Discussion

A major route of selenium detoxication is the formation of the volatile methylated derivative

Table 6. Expt. 7. Identification of acid-volatile protein-bound selenium from rat liver microsomal fractions

Trapping agents	Trial 1			Trial 2		
	0.1 M- AgNO_3	8 M- HNO_3	0.1 M- AgNO_3 *	0.1 M- AgNO_3	8 M- HNO_3	0.1 M- AgNO_3
Initial ^{75}Se radioactivity in microsomal fraction (c.p.s.)	789 $\pm$ 13 (8)	797 $\pm$ 12 (8)	776 $\pm$ 15 (8)	219 $\pm$ 7 (8)	201 $\pm$ 0 (8)	201 $\pm$ 0 (8)
^{75}Se radioactivity volatilized on acid treatment (% of initial ^{75}Se radioactivity)	14 $\pm$ 3	14 $\pm$ 0	9 $\pm$ 2	14 $\pm$ 1	13 $\pm$ 1	13 $\pm$ 1
^{75}Se radioactivity trapped in agent specified on acid treatment (% of ^{75}Se radioactivity volatilized)	87 $\pm$ 6	<1	90 $\pm$ 6	84 $\pm$ 5	<1	<1

* In this experiment the microsomal fraction was acidified with 50% (v/v) H_2SO_4 .

Table 7. Expt. 8. *Susceptibility of acid-volatile selenium to oxidation in vitro*

Vitamin E-deficient rats (12; 3 months old) were given the *Torula* yeast diet for 2 weeks and then were given three consecutive daily doses of 10mg of α -tocopherol orally, and 50 μ Ci (9 μ g) of ^{75}Se as $\text{Na}_2^{75}\text{SeO}_3$ intraperitoneally. The rats were killed on the fourth day, and the livers fractionated in lots of three rats in 0.25M-sucrose solutions with or without antioxidants as indicated. The fractions were subjected to the counting procedures described previously (Diplock *et al.*, 1971) for the measurement of acid-volatile selenium. Values given are mean values $\pm$ s.d. By Student's *t* test, the values for acid-volatile selenium in the presence of either antioxidant were greater ($P > 0.01$) than the corresponding value when no antioxidant was added. The c.p.s. were converted into d.p.s. by using the known counting efficiency (78%) as indicated in the Experimental section.

	Liver homogenized without antioxidants	Liver homogenized with 100 μ g of α -tocopherol/ml	Liver homogenized with 100 μ g of α - tocopherol/ml and 5mm-mercaptoethanol
$10^{-4} \times \text{Total } ^{75}\text{Se}$ in the mito- chondrial fraction (d.p.s./liver)	33.1 $\pm$ 4.4	32.7 $\pm$ 4.2	33.1 $\pm$ 4.4
Acid-volatile ^{75}Se (% of total ^{75}Se)	13.6 $\pm$ 1.1	37.1 $\pm$ 3.4	37.8 $\pm$ 5.7
$10^{-4} \times \text{Total } ^{75}\text{Se}$ in the microsomal fraction (d.p.s./liver)	15.9 $\pm$ 0.9	16.2 $\pm$ 0.8	17.3 $\pm$ 2.2
Acid-volatile ^{75}Se (% of total ^{75}Se)	13.8 $\pm$ 2.9	31.6 $\pm$ 3.3	34.3 $\pm$ 7.1

$(\text{CH}_3)_2\text{Se}$ (McConnell & Portman, 1952; Ganther, 1966) or the urinary derivative $(\text{CH}_3)_3\text{Se}^+$ (Byard, 1969). The present experiments sought to determine whether the acid-volatile selenium in rat liver sub-cellular fractions (Diplock *et al.*, 1971) is H_2Se derived from tissue selenide, or whether it is $(\text{CH}_3)_2\text{Se}$ which is released from the tissue on acidification. Conditions were therefore established in which H_2Se and $(\text{CH}_3)_2\text{Se}$, generated in a controlled manner, could be trapped quantitatively by passage through, respectively, 0.1M- AgNO_3 and 8M- HNO_3 , and the behaviour of the acid-volatile selenide originating from rat liver was then studied under similar conditions.

It was found that H_2Se , in the trace amounts utilized, was exceptionally susceptible to oxidation and that in similar experiments using larger amounts, a red deposit, presumed to be elemental Se, was formed. Important factors in preventing oxidation of the H_2S were the material of which the apparatus was constructed, the rate at which the acid was added to the mixture of $\text{Na}_2^{75}\text{SeO}_3$ and Zn dust, the rate at which the carrier gas (O_2 -free N_2) was passed, and the path-length through which the H_2Se passed before it was trapped in the AgNO_3 solution. The apparatus shown in Fig. 1 was ultimately designed in which quantitative recovery of H_2S in 0.1M- HgNO_3 could be achieved.

In Expt. 1 (Table 1) it was found that, whereas recovery of the H_2Se as Ag_2Se was almost quantitative when N_2 was used, H_2 was not suitable for this purpose and that when H_2 was used, there was extensive deposition of ^{75}Se radioactivity on the walls of the apparatus. O_2 -free N_2 was therefore used in all

subsequent experiments. The results of Expt. 2 (Table 2) establish the results of Expt. 1 and show further that some of the loss experienced when N_2 was employed is attributable to the deposition of ^{75}Se radioactivity on the wall of the terminal length of the connecting tube.

$(\text{CH}_3)_2\text{Se}$ can be trapped quantitatively by passage through 8M- HNO_3 (Challenger, 1935; Ganther & Baumann, 1962; Ganther, 1966). The results of Expt. 3 (Table 3) show that, in contrast to 0.1M- AgNO_3 , 8M- HNO_3 is a poor trapping agent for H_2Se , only slightly better than water; chloroform was found to trap only about one-third of the H_2Se . In view of the fact that 8M- HNO_3 is a strong oxidizing agent, it is surprising that the H_2Se was not oxidized to Se during its passage through the acid; perhaps the oxidation of H_2Se to Se requires O_2 , the presence of which was carefully excluded in these experiments. When the gas emerging from the 8M- HNO_3 vial was further passed through a vial containing 0.1M- AgNO_3 (Table 3), the remaining H_2Se was trapped quantitatively.

Ganther (1966) studied the production of $(\text{CH}_3)_2\text{Se}$ by cell-free systems derived from mouse liver. A similar system was employed with rat liver in the present experiments (Expt. 4) and it was found (Table 4) that a volatile ^{75}Se -containing product, presumed to be $(\text{CH}_3)_2^{75}\text{Se}$, was produced, which was trapped quantitatively in 8M- HNO_3 . When 0.1M- AgNO_3 , water and chloroform were studied, it was found (Table 4) that the $(\text{CH}_3)_2^{75}\text{Se}$ was only poorly trapped in all three agents.

The results of Expt. 4 (Table 4) appeared to indicate that, as the liver cell extract aged, its ability

to convert Na_2SeO_3 into $(\text{CH}_3)_2\text{Se}$ was diminished. In Expt. 5 $(\text{CH}_3)_2^{75}\text{Se}$ was trapped sequentially for 5 min periods only, with the same sample of liver cell extract, in the three trapping agents 8M- HNO_3 , 0.1M- AgNO_3 and water. The results (Fig. 2) confirm those of the previous experiment.

The nature of the acid-volatile selenium of rat liver was next examined. When a homogenate of the liver of rats given $\text{Na}_2^{75}\text{SeO}_3$ was studied (Expt. 6), it was found (Table 5) that the volatile ^{75}Se produced on acidification could be trapped quantitatively in 0.1M- AgNO_3 and was scarcely trapped at all in 8M- HNO_3 . The proportion of the ^{75}Se that was acid volatile (13–20%) was of the order that we usually find in rats whose selenium and vitamin E reserves have not been depleted before their being re-fed with α -tocopherol and $\text{Na}_2^{75}\text{SeO}_3$ (Diplock *et al.*, 1971).

A microsomal fraction, prepared from the livers of rats treated similarly to those used in Expt. 6, was also examined (Expt. 7). The acid-volatile ^{75}Se was also trapped nearly quantitatively in 0.1M- AgNO_3 and not at all in 8M- HNO_3 (Table 6). An additional experiment with this microsomal preparation (Trial 1, Table 6) shows that 50% (v/v) H_2SO_4 is almost as effective as conc. HCl in causing the volatilization of ^{75}Se .

The experiments reported here establish that the

acid-volatile protein-bound selenium of rat liver is not $(\text{CH}_3)_2\text{Se}$. It is not entirely certain, however, that the acid-volatile selenium is Se^{2-} that gives rise to H_2Se on acidification, since the volatile selenide has not been characterized. A major difficulty in undertaking this characterization would be the exceptional lability to oxidation of this acid-volatile compound, and the small amount (less than 1 ng-atom of Se) that is produced. However, the behaviour of the acid-volatile selenium is so similar to that of H_2Se that, for the purposes of the discussion that follows, it will be assumed that it is H_2Se .

The proposal of Hofmeister (1894) that toxic amounts of selenium are converted into $(\text{CH}_3)_2\text{Se}$, which is exhaled, has been confirmed by McConnell & Portman (1952) and Ganther (1966). McConnell (1952) has also further demonstrated that some protection against selenium toxicity is afforded by methyl donors. Hopkins *et al.* (1966) have shown that at sub-acute or physiological doses of administered selenium, the major excretory product is a metabolite in urine identified by Byard (1969) as trimethyl selenide, $(\text{CH}_3)_3\text{Se}^+$; a further metabolite in urine, designated X_2 by Byard & Baumann (1967), has not been characterized. $(\text{CH}_3)_2\text{Se}$ appears to be formed only at, or near to, toxic amounts of selenium intake (Ganther *et al.*, 1966), when the urinary route of elimination is

apparently unable to discharge the larger amounts of selenium.

The proposal of Painter (1941) that there could be a direct reaction between selenious acid and thiols (reaction 3):

has been confirmed by Ganther (1968). It is possible that a part of the toxicity of selenium in rats is due to the formation of the RSSeSR species, called by Ganther (1968) a selenotrisulphide. The occurrence of non-specific reactions of this kind among enzyme proteins might form cross-linked products devoid of enzyme activity. The reaction has been shown to occur with pancreatic ribonuclease (Ganther & Corcoran, 1969) and the product was without enzyme activity.

The mechanism of the formation of methylated derivatives proposed by Ganther (1970, 1971) involves (i) a non-enzymic reaction between GSH and selenite in a similar manner to reaction (3); (ii) conversion of the resultant selenotrisulphide by an NADPH-linked reaction involving glutathione reductase into a labile reduced intermediate selenol which (iii) is methylated by *S*-adenosylmethionine (AdoMet) to give $(\text{CH}_3)_2\text{Se}$ (reaction 4):

Alternatively, Ganther (1971) suggests that the selenol is first converted into H_2Se which is then methylated. It is further suggested here that the normal biosynthesis of selenide-containing proteins may require the functioning of the first two of the three detoxication reactions proposed by Ganther (1970) for the biosynthesis of $(\text{CH}_3)_2\text{Se}$ and that the labile intermediate selenol GSSeH (or H_2Se itself) is the precursor of selenide in such non-haem iron-containing proteins.

The role of α -tocopherol with regard to selenium remains a matter for speculation. The toxicity of Ag^+ to vitamin E-deficient rats (Shaver & Mason, 1951; Diplock *et al.*, 1967; Grasso *et al.*, 1969), was explained (Diplock *et al.*, 1971) by the exceptional affinity of Ag^+ for Se^{2-} . Apparently, in addition to preventing the oxidation of the protein-bound selenide, α -tocopherol prevents the interference by Ag^+ in the metabolism of selenium. Either α -tocopherol prevents the reaction of Ag^+ with selenide *in situ* in the protein, or Ag^+ interferes with the production of the precursor selenol, GSSeH . In the latter case, α -tocopherol must protect the selenol from Ag^+ , and also possibly from oxidation; indeed the protective effect of α -tocopherol may be extended to the entire NADPH-glutathione reductase system. The points made in this discussion are summarized in Scheme 1.

In Expt. 8 we investigated the suggestion of Ganther (1970, in his discussion), that the protein-bound selenide of rat liver subcellular organelles might be formed *in vitro*, as a decomposition product of selenotrisulphide formed by reaction between selenite and the mercaptoethanol added to the 0.25 M-sucrose solution used for homogenization. The results (Table 7) show that when only α -tocopherol was present as an antioxidant in the homogenization medium a similar proportion of the selenium was found to be present in the selenide form as when α -tocopherol and mercaptoethanol were both present, and in both cases this was significantly greater than in the organelles prepared without the addition *in vitro* of antioxidants. The possibility of interaction between selenite and mercaptoethanol was thus excluded in this experiment, and it is unlikely that the protein-bound selenide was formed *in vitro*.

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