The Effect of Vitamin E on the Oxidation State of Selenium in Rat Liver

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1. ^{75}Se as $\text{Na}_2^{75}\text{SeO}_3$ was administered orally to rats under different nutritional conditions. 2. The selenium found in the liver subcellular organelle fractions was present in at least three oxidation states: acid-volatile selenium, assumed to be selenide, zinc-hydrochloric acid-reducible selenium, assumed to be selenite, and higher oxidation states of selenium and organic derivatives, called selenate for convenience. 3. The proportion of the total selenium present as selenide is susceptible to oxidation *in vitro*, which can be prevented by the addition of antioxidants *in vitro*. 4. The proportion of selenide is also directly related to the vitamin E status of the rats, and treatment of vitamin E-deficient rats with vitamin E results in an increase in the proportion of selenide. 5. Freezing the liver *in situ* before preparation of the organelle fractions did not alter the susceptibility of the selenide proportion to dietary vitamin E, indicating that the observed effects occur *in vivo* and not as a result of oxidation *post mortem*. 6. Intravenous administration of $\text{Na}_2^{75}\text{SeO}_3$ to rats whose alimentary tract was partially sterilized by neomycin treatment, gave a similar result to that in paragraph 4, indicating that the reduction of selenite to selenide probably occurs *in vivo*, and that intestinal micro-organisms are not responsible. 7. Treatment of vitamin E-deficient rats with silver produced a fall in the total ^{75}Se content of the liver, an effect only partially reversed by vitamin E administration. The proportion of the total selenium present as selenide was also lowered by the treatments with silver, and vitamin E significantly reversed this trend in most cases. 8. These results are consistent with the hypothesis that the active form of Se may be selenide and that the selenide may form part of the active centre of an uncharacterized class of catalytically active non-haem-iron proteins that are protected from oxidation *in vivo* by vitamin E.

In an extensive examination of the antioxidant hypothesis for the mode of action of vitamin E and selenium, Green, Diplock, Bunyan, McHale & Muthy (1967) and Diplock, Bunyan, McHale & Green (1967a) were unable to find any evidence that the absence of vitamin E and selenium from rat and chick tissues was accompanied by the proliferation of lipid peroxidation that would be expected if the vitamin were acting as a lipid antioxidant *in vivo* (cf. Tappel, 1962). After a series of experiments, in which a number of classical vitamin E deficiency states was examined, these authors (Green *et al.* 1967; Diplock *et al.* 1967a) concluded that the antioxidant hypothesis could no longer be regarded as viable (see Green & Bunyan, 1969).

Ideally any hypothesis about the mode of action of vitamin E should explain its relationships with

sulphur-containing amino acids, with selenium and with polyunsaturated fatty acids (the interaction of vitamin E with vitamin A being regarded as a special case of its relationship to unsaturated lipids), as well as the fact that certain actions of vitamin E can be mimicked by synthetic antioxidants. We have formulated a new hypothesis for the mode of action of vitamin E that is considered to satisfy these criteria (Diplock, Baum & Lucy, 1968). According to this hypothesis, α -tocopherol may function as a membrane-bound redox substance. The redox function of the molecule, being localized in the hydrophilic chromanol ring structure, would be expected to be associated with polar rather than non-polar residues of membrane proteins; in a region of membrane having a bilayer structure, the redox function would presumably be capable of acting at or near the membrane surface. We suggest that the redox function is directed toward oxidation-sensitive active centres of membrane-associated proteins that contain either sulphur or selenium or

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both. Likely candidates for catalytically active compounds containing such potentially sensitive active sites are the non-haem-iron proteins. Proteins that contain sulphide as part of their iron-bearing active centre are well known and have been documented thoroughly. We have suggested that, in addition to protecting the sulphide in such proteins, the redox function of α -tocopherol may also protect a further, and as yet undocumented, class of non-haem-iron proteins that contain selenide at their active centre. It is known that sulphide can be replaced *in vitro* by selenide in at least one non-haem-iron protein (Tsibris, Namtredt & Gunsalus, 1968), and we understand (W. Orme-Johnson, personal communication) that the selenide in such modified proteins is exceptionally susceptible to autoxidation.

The present paper describes experiments designed to determine whether subcellular organelles from rat liver contain selenium in the selenide form, whether such selenide is susceptible to autoxidation *in vitro*, and whether the proportion of selenide to other oxidation states of the element is affected by the presence of vitamin E in the animal's diet.

EXPERIMENTAL

Diets. The vitamin E-deficient diet used was formulated to contain the minimal amount of selenium necessary to prevent dietary liver necrosis. The composition of the diet was: casein (low vitamin content, Genatosan Ltd., Loughborough, Leics., U.K.), 8.30%; lard, 10.00%; glucose, 75.97%; salt mixture, 5.33%; vitamin mixture, 0.40%. The salt mixture supplied, in g/kg: NaH_2PO_4 , 26; CaCO_3 , 18.2; KCl , 3.5; Na_2CO_3 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4; ferric citrate, 0.15; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06; KI , 0.003; NaF , 0.00025; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.002; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, 0.0007; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02. The vitamin mixture supplied, in mg/kg: thiamin, 9; riboflavin, 19; nicotinic acid, 90; pyridoxine, 9; calcium pantothenate, 90; *p*-aminobenzoic acid, 90; choline dihydrogen tartrate, 900; menaphthone sodium bisulphite, 0.28. Vitamins A and D were added as a stabilized powder to give 10.8 and 1.6 i.u./g of diet respectively.

The *Torula* yeast diet had the following composition: *Torula* yeast (Lake States Yeast and Chemical Division of St Regis Paper Co., Rhinelander, Wis., U.S.A.), 30.00%; sucrose, 48.4%; glucose, 18.0%; salt mixture, 3.2%; vitamin mixture, 0.4%. The salt mixture supplied, in g/kg: CaCO_3 , 17.5; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 6.5; KCl , 3.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.0; FeCl_3 , 0.15; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06; KI , 0.0003; NaF , 0.00025; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.002; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, 0.0007; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02. The vitamin mixture was the same as that used for the vitamin E-deficient diet.

Animals, feeding regimens and dosage. Caesarian-derived weanling male Wistar rats (40–50 g) were purchased from a commercial breeder and fed on the vitamin E-deficient diet for periods ranging from 2 to 4 months, as indicated

in the individual experiments. In Expts. 2 and 3, where extensive pre-depletion of selenium was desirable, the *Torula* yeast diet was supplied for the 15 days immediately preceding and during the administration of the radio-active selenium. The ^{75}Se was purchased as $\text{Na}_2^{75}\text{SeO}_3$ (from The Radiochemical Centre, Amersham, Bucks., U.K.) and administered orally in water. α -Tocopherol was dissolved in methyl oleate and administered orally with a ball-ended dosing needle passed into the oesophagus; the daily dose in all the experiments was 10 mg of α -tocopherol in 0.25 ml of oil. In Expt. 4, rats treated with silver received where indicated a daily oral dose of 1.93 mg of silver acetate in 1.0 ml of water, and were given a 0.15% (w/v) solution of silver acetate to drink.

Cell fractionation. The method of Hogeboom (1955) was used. Where appropriate, the 0.25M- and 0.34M-sucrose solutions employed contained 5 mM-mercaptoethanol and 100 μg of DL- α -tocopherol/ml, the latter being emulsified in a small portion of the solution and subsequently diluted with the remainder to give the appropriate concentration; mercaptoethanol was stored under N_2 . The particulate fractions were resuspended in 0.25M-sucrose either with or without antioxidants, as appropriate, for radioisotope counting.

Radioisotope counting. The ^{75}Se radioactivity was counted in 3 ml portions of the supernatant, or of resuspended particulate fractions, in a 2 in Ekco well-crystal γ -isotope counter at 78% efficiency. After the total ^{75}Se radioactivity had been counted, the fractions were acidified with 1 ml of HCl , N_2 was passed for 10 min, and the samples were re-counted; the difference between the first and second counts was attributed to acid-labile volatile selenide. Zn dust was then added to each fraction and N_2 was passed again for 10 min before re-counting; the difference between the second and third counts was attributed to selenite, and the remaining non-volatile selenium was for convenience called selenate. In separate experiments, it was shown that the passage of N_2 without acidification did not drive off any selenium; further, the dilution due to the addition of the acid did not of itself affect the radioisotope count. The addition of mercaptoethanol and/or α -tocopherol was without effect on the acid-volatility of selenium. Se may be trapped in AgNO_3 solution (A. T. Diplock & C. Thomas, unpublished work).

Nature of volatile selenium. Attempts were made to trap the volatile ^{75}Se radioactivity in lead acetate solution, but these were unsuccessful. Recent experiments, in collaboration with Mr C. Thomas, have shown that the selenium is very sensitive to oxidation and that, unless precautions are taken rigorously to exclude oxygen, it is not possible to trap the volatile material since it is deposited on the walls of the gas-train employed. A gas-train was designed in which the surface to which the volatile material was exposed was kept to a minimum. In a model experiment with H_2 gas to displace the material liberated from a sample of $\text{Na}_2^{75}\text{SeO}_3$, by the addition of Zn dust and HCl , it was found that at least 80% of the volatile ^{75}Se formed could be trapped in AgNO_3 solution. The remaining 15–20% of ^{75}Se was probably elemental selenium deposited on the walls of the gas-train. AgNO_3 was chosen, since the Ag^+ ion has an exceptionally high affinity for selenide; the material trapped in the silver nitrate solution contained all the radioactivity lost from the sample of $\text{Na}_2^{75}\text{SeO}_3$ and it was not further volatile

on acidification. This result is considered to show that the volatile H_2^{75}Se is trapped as $\text{Ag}_2^{75}\text{Se}$. The metallic selenides are very like the sulphides (Sidgwick, 1950b) and it is known that very sparingly soluble sulphides, e.g. CuS , PbS and Ag_2S (Sidgwick, 1950a), are not dissolved by strong acids (Partington, 1951).

When a similar technique was applied to liver fractions containing the volatile selenium, the results were variable; about 50% of the volatile ^{75}Se was usually trapped in the AgNO_3 solution, and was not further volatile on acidification. It is thought that the remainder of the ^{75}Se , not trapped in the AgNO_3 solution, may have been lost by deposition of elemental selenium on the walls of the gas-train. Experiments are in progress to attempt to decrease still further the path-length of the apparatus, since, in these experiments with tissue fractions, the amounts of volatile ^{75}Se are so small. At present we cannot exclude the possibility that the untrapped volatile material may represent alkyl selenides of low molecular weight which are released from the proteins by lowering the pH. However, more than 50% of the volatile ^{75}Se would appear to be H_2^{75}Se derived from tissue selenide.

Statistical analysis. The statistical significance of the results was evaluated by using the Student's *t* test.

Experiment 1. Twelve rats were fed on the vitamin E-deficient diet for 2 months and dosed with ^{75}Se on each of the terminal 7 days before being killed; the total amount of ^{75}Se administered was $217\mu\text{Ci}$ (equivalent to $361\mu\text{g}$ of Se). The rats were killed in three lots of four and the livers cooled at once in ice-cold 0.25M-sucrose containing mercaptoethanol and α -tocopherol. Each lot of four livers was chopped and the resultant mince divided into two parts. One part was homogenized and fractionated in sucrose solutions containing the antioxidants, the other part being treated in sucrose to which no antioxidants had been added. The cell debris, mitochondrial and microsomal fractions were resuspended and portions of these and the supernatant fractions subjected to the counting procedures. The results of this experiment are given below, and in Tables 1 and 2.

Experiment 2. Rats (24) were given the vitamin E-deficient diet for 2 months and the *Torula* yeast diet for a further 2 weeks to deplete their tissue selenium concentration further. They were then divided into two groups of 12 rats and the *Torula* yeast diet was continued for a further 3 days. On each of these 3 days, rats in one group received an oral dose of α -tocopherol and the rats in the other group an equivalent amount of methyl oleate. ^{75}Se was also administered at the same time, the total amount given being $68\mu\text{Ci}$ (equivalent to $36\mu\text{g}$ of Se) and the rats were killed on day 4 in four lots of three rats per group. The livers were treated in the same way as in Experiment 1, fractionated either in the presence or absence of antioxidants and the fractions counted for radioactivity. After radioactivity counting, fractions from similar treatment groups were pooled and dialysed against 20 vol. of 0.25M-sucrose, in all cases containing the antioxidants. After dialysis, samples of the dialysed material were subjected to the radioactivity-counting procedure for assessment of retention of the acid volatility. The results of this experiment are given below, and in Tables 3 and 4.

Experiment 3. Rats (48) were given the vitamin E-deficient diet for 3 months. They were then given the *Torula* yeast diet for a further 2 weeks and divided into

two groups of 24 rats. In one group, all the rats were given three consecutive daily oral doses of α -tocopherol and the rats in the other group given an equivalent amount of methyl oleate, the *Torula* yeast diet being continued. ^{75}Se was also administered at the same time as the oils; 12 rats in each group were given the usual oral doses of $\text{Na}_2^{75}\text{SeO}_3$, a total of $61\mu\text{Ci}$ (equivalent to $33\mu\text{g}$ of Se) being received by each rat. The remaining 12 rats in each group received a single intravenous injection of an equivalent amount of ^{75}Se in 0.9% NaCl on the second of the 3 days; the injections were made into the external jugular vein, a small incision being made under ether anaesthesia and closed subsequently with a suture. All these rats had received, on each of the 2 days before having the intravenous injections, an oral dose of 10 mg of neomycin sulphate in 0.5 ml of water, and their drinking water was replaced with a solution of neomycin sulphate (1 mg/ml of water). No infection was noted in the 36 h remaining before the rats so treated were killed in four lots of three rats and their livers fractionated in media containing antioxidants and the fractions counted for radioactivity as usual. At the same time, the rats that had received oral doses of ^{75}Se were anaesthetized with ether in lots of three rats and their livers sampled with tongs cooled to -70°C with solid CO_2 . A piece of frozen liver was weighed from each liver in each lot and the three pieces were dropped into a volume of 0.25M-sucrose containing antioxidants to give a 10% mixture. As soon as the liver had thawed adequately, it was homogenized and fractionated in the usual way. The fractions from both parts of this experiment were then subjected to the usual radioactivity-counting procedures. The results of this experiment are given below, and in Table 5.

Experiment 4. Rats (52) were given the vitamin E-deficient diet for 5 months. At 15 days before the rats were to be killed for fractionation of their livers, they were divided into four groups and the diet was continued until they were killed. Group A (three lots of three rats) received six consecutive daily doses of ^{75}Se ($30.8\mu\text{Ci}$; $8.1\mu\text{g}$ of Se) on days 9–14 and 0.1 ml of methyl oleate on days 1–14. Group B (three lots of three rats) received the same ^{75}Se dosage as group A rats, and α -tocopherol on days 1–14. Group C (three lots of three rats with eight additional rats to test the toxicity of silver) received the same ^{75}Se dosage as group A, and silver (both orally and in the drinking water) on days 11–14; those rats being examined for toxicity received the silver dosage each day after day 15 until all the rats in this group had died. Group D (three lots of three rats, with eight additional rats to test the toxicity of silver) received the same treatments as the rats in group C, with the addition of dosage with α -tocopherol on days 1–14. All the rats, except those in the toxicity test, were killed on day 15 and their livers fractionated, in lots of three, in the presence of the antioxidants. The fractions and samples of the homogenates were subjected to the radioactivity-counting procedures, and the results are given below and are tabulated in Tables 6 and 7.

In addition, all fractions were dialysed individually against 20 vol. of 0.25M-sucrose containing antioxidants, and the contents of the dialysis bag were examined for total selenium and acid-volatile material remaining. In the toxicity study, all the rats in groups C died between day 18 and day 26, and post-mortem examination showed

extensive liver necrosis. No rats in group D had died by day 26, and at post mortem the livers appeared normal, although they were not examined histologically.

RESULTS AND DISCUSSION

Experiment 1. This was a pilot experiment designed to test whether there was selenide present in rat liver cell fractions and whether the content of selenide was dependent on the presence of antioxidants in the isolating medium. The distribution of ^{75}Se among the rat liver subcellular fractions was not affected by the addition of antioxidants to the isolating medium (Table 1). However, when the oxidation states of the ^{75}Se were examined (Table 2), it was found that, in the mitochondrial and supernatant fractions, the proportion of selenium present as selenide was significantly increased when anti-

oxidants were present in the sucrose solutions used for isolation of the fractions. In the mitochondrial fraction there was a significant increase in the proportion of selenite, whereas in the supernatant fractions there was a decrease, when antioxidants were added. The microsomal fraction showed a similar trend to the mitochondrial fraction, but the differences observed were not statistically significant. The result of this experiment shows that the selenium present in rat liver is present in at least three forms, and that the extent of reduction of this selenium, i.e. the quantity of selenide, observed depends on whether or not it is protected by antioxidants during isolation of the subcellular fractions.

Experiment 2. This experiment was designed to investigate the effect of dietary vitamin E on the proportion of selenide in rat liver cell fractions, and to confirm the susceptibility of the selenide to the presence of antioxidants *in vitro* observed in Expt. 1. Neither dietary supplementation with vitamin E nor the addition *in vitro* of antioxidants affected the recovery or intracellular distribution of total ^{75}Se (Table 3). In the mitochondrial fraction (Table 4), the addition of antioxidants *in vitro* significantly increased the proportion of selenide found, and the administration of α -tocopherol to the rats significantly increased even further the proportion of selenium present as selenide. The increases in selenide appear to have been at the expense of both selenite and selenate. Similar results were obtained for the microsomal fractions (Table 4); both antioxidants *in vitro* and α -tocopherol *in vivo* increased the proportions of selenide found and here these increases were accompanied by a significant fall in the proportions of selenite. In the supernatant fraction (Table 4) the addition of antioxidants *in vitro* caused a significant increase in the proportion of selenide found, but the administration *in vivo* of α -tocopherol was without effect on the proportion of selenide observed.

When the mitochondrial, microsomal and supernatant fractions isolated in antioxidant-containing medium from the vitamin E-deficient and supplemented rat liver were subjected to dialysis, 97%

Table 1. *Experiment 1. Distribution of ^{75}Se among rat liver cell fractions*

Twelve 2-month-old vitamin E-deficient rats were given a total of $217\mu\text{Ci}$ of ^{75}Se ($361\mu\text{g}$ of Se) on 7 consecutive days and killed on the 8th day in three lots of four rats. Livers were homogenized and fractionated in sucrose solutions, with or without antioxidants (mercaptoethanol, 5 mM; D- α -tocopherol, $100\mu\text{g}/\text{ml}$, here and in all other tables) as indicated. The particulate fractions were resuspended in 0.25 M-sucrose solution containing antioxidants, and portions of these and of the soluble fractions subjected to the radioactivity-counting procedures described in the text.

	Liver homogenized with antioxidants	Liver homogenized without antioxidants
Mean total liver ^{75}Se ($10^{-4} \times \text{d.p.s./liver}$)	28.4	28.6
Mean % of total liver ^{75}Se in:		
Mitochondrial fraction	7.2 ± 0.7	8.0 ± 1.2
Microsomal fraction	18.7 ± 2.3	21.0 ± 3.6
Supernatant fraction	41.9 ± 4.1	41.0 ± 1.7
Debris fraction	32.1 ± 8.9	30.0 ± 7.6

Table 2. *Experiment 1. Apparent oxidation state of selenium in rat liver cell fractions*

Experimental details are given in Table 1. Results are expressed as % of the total ^{75}Se in the fraction (mean $\pm$ S.D.). Numbers in parentheses are numbers of observations. Values marked * differ significantly from those marked † ($P > 0.001$) for directly comparable values read horizontally. Values marked ‡ differ significantly from those marked § ($P > 0.01$) for directly comparable values read horizontally. Values marked || differ significantly from those marked ¶ ($P > 0.05$) for directly comparable values read horizontally.

	Liver homogenized with antioxidants			Liver homogenized without antioxidants		
	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate
Mitochondrial fraction (3)	$15.5 \pm 1.3^*$	$33.1 \pm 7.2^\ddagger$	$51.3 \pm 6.2 $	$3.1 \pm 1.6^\ddagger$	$19.2 \pm 6.2§$	$77.6 \pm 5.1¶$
Microsomal fraction (3)	14.8 ± 1.5	33.2 ± 8.9	52.0 ± 7.5	11.5 ± 3.9	21.7 ± 3.2	66.7 ± 7.1
Supernatant fraction (3)	$31.6 \pm 4.3^\ddagger$	$9.1 \pm 2.0^\ddagger$	59.3 ± 2.2	$14.5 \pm 2.0§$	$28.8 \pm 4.4§$	56.6 ± 5.7

Table 3. *Experiment 2. Distribution of ^{75}Se among rat liver cell fractions*

A group (24) of 2-month-old vitamin E-deficient rats were given the *Torula* yeast diet for 2 weeks and then were given a total of 68 μCi of ^{75}Se (36 μg of Se) on 3 consecutive days, during which half the rats received a daily oral dose of α -tocopherol and the remaining half a dose of methyl oleate. All rats were killed on the 4th day in lots of three rats, and the livers homogenized and fractionated in sucrose solutions with or without antioxidants as indicated. The particulate fractions were resuspended in 0.25M-sucrose solution containing antioxidants, and samples of these and of the soluble fractions were subjected to the radioactivity-counting procedures described in the text.

	Vitamin E-deficient rats		Vitamin E-supplemented rats	
	Liver homogenized with antioxidants	Liver homogenized without antioxidants	Liver homogenized with antioxidants	Liver homogenized without antioxidants
Mean total liver ^{75}Se ($10^{-4} \times \text{d.p.s./liver}$)	13.5 $\pm$ 1.2	14.4 $\pm$ 0.2	13.3 $\pm$ 1.9	12.2 $\pm$ 2.2
Mean % of total liver ^{75}Se in:				
Mitochondrial fraction	23.3 $\pm$ 3.6	26.5 $\pm$ 2.8	28.7 $\pm$ 3.4	27.2 $\pm$ 2.1
Microsomal fraction	14.4 $\pm$ 0.7	13.2 $\pm$ 0.3	15.5 $\pm$ 1.0	13.1 $\pm$ 0.8
Supernatant fraction	23.7 $\pm$ 1.1	22.4 $\pm$ 0.9	23.3 $\pm$ 1.9	25.3 $\pm$ 1.5
Debris fraction	38.6 $\pm$ 4.2	37.9 $\pm$ 5.3	32.5 $\pm$ 3.7	34.4 $\pm$ 6.0

of the ^{75}Se in the supernatant fractions passed through the dialysis membrane. In the mitochondrial fractions, 20–25% of the ^{75}Se passed through the dialysis membrane and this was largely selenite. Similarly, in the microsomal fractions, 10% of ^{75}Se , largely selenite, passed through the membrane.

The results of this experiment demonstrate four main features: first, that selenium has been shown to be present in rat liver as selenide, selenite and a higher oxidation state (or another form not volatile in the presence of acid and zinc dust); secondly, a greater proportion of the ^{75}Se is present as selenide when the animal receives vitamin E; thirdly, the selenide is very susceptible to oxidation *in vitro*; and fourthly, although the mitochondrial and microsomal selenide appears to be largely protein-bound, that in the supernatant fraction is not. In other experiments it was found that either mercaptoethanol or α -tocopherol used separately was not as effective in protecting the selenide *in vitro* as the mixture of the two antioxidants.

Experiment 3. Two questions arose from the results of Expt. 2. First, it was conceivable that the observed smaller proportion of selenide in the liver organelles of rats that did not receive vitamin E might be caused by autooxidation occurring immediately *post mortem*, i.e. before the addition *in vitro* of antioxidants. In this experiment, an attempt was made to avoid oxidation *post mortem* by freezing the liver *in situ* in the living animal. Table 5 shows that when the livers were sampled by freezing *in situ* in the living animal, homogenates from vitamin E-supplemented animals had a proportion of selenide significantly greater than in the homogenates from vitamin E-deficient animals. The homogenate was examined in this experiment, since it was realized

that subfractionation of liver that had been frozen to -70°C would be unsatisfactory. However, an attempt was made to subfractionate these livers, and the results are given in Table 5. In the mitochondrial fraction, a very large scatter was found in the proportion of selenide, presumably caused by lysis of the mitochondria on freezing. In the microsomal and supernatant fractions, there was a larger proportion of selenide found in the livers of the vitamin E-supplemented rats compared with the vitamin-deficient animals, a difference that appears to be largely mirrored by a corresponding decrease in the proportion of selenite. These results are considered to show that, when precautions against autooxidation of selenide *post mortem* are taken, the proportion of selenide found in liver organelles from vitamin E-supplemented rats remains significantly greater than that found in corresponding organelles from vitamin E-deficient animals. It seems improbable that any significant oxidation *in vitro* of selenide could have occurred while the liver remained frozen; the liver was thawed in sucrose solution containing the antioxidants, and homogenized as soon as this became practicable. It remains possible that, during the period of thawing, before the selenide was in contact with the antioxidants, some autooxidation of the selenide may have occurred in those tissues that contained little endogenous vitamin E, but this seems unlikely.

Table 5 also gives the results of the second part of this experiment. Investigations on sulphur metabolism, which may be similar to the metabolism of selenium, indicate that reduction of sulphite to sulphide does not occur in animal tissues, and indeed reduced sulphur is generally acquired in the form of the thiol groups of amino acids, and their subsequent metabolic fate is oxidative. It is

Table 4. *Experiment 2. Apparent oxidation state of selenium in rat liver cell fractions*

Experimental details are given in Table 3. Results are expressed as % of the total ^{75}Se in the fraction (mean $\pm$ s.d.). Numbers in parentheses are numbers of observations. Values marked * differ significantly from those marked † ($P > 0.001$) for directly comparable values read horizontally. Values marked ‡ differ significantly from those marked § ($P > 0.01$) for directly comparable values read horizontally. Values marked ¶ differ significantly from those marked ¶ ($P > 0.05$) for directly comparable values read horizontally.

	Liver homogenized with antioxidants						Liver homogenized without antioxidants					
	Vitamin E-deficient rats			Vitamin E-supplemented rats			Vitamin E-deficient rats			Vitamin E-supplemented rats		
	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate
Mitochondrial fraction (4)	25.5 $\pm$ 1.6*	25.2 $\pm$ 1.5	49.3 $\pm$ 2.9	36.1 $\pm$ 5.1†	20.0 $\pm$ 13.7	48.9 $\pm$ 9.5	10.7 $\pm$ 1.9§	25.2 $\pm$ 4.9	64.1 $\pm$ 6.5	12.9 $\pm$ 1.1§	34.6 $\pm$ 6.7	52.5 $\pm$ 5.6
Microsomal fraction (4)	30.2 $\pm$ 1.7*	16.1 $\pm$ 2.3	53.7 $\pm$ 3.9	43.1 $\pm$ 1.7†	3.6 $\pm$ 2.1	53.2 $\pm$ 1.4	16.8 $\pm$ 5.5¶	21.5 $\pm$ 2.2	61.7 $\pm$ 3.3	22.5 $\pm$ 5.8¶	17.3 $\pm$ 3.9	60.6 $\pm$ 15.3
Supernatant fraction (4)	14.1 $\pm$ 1.5†	17.4 $\pm$ 3.5	68.5 $\pm$ 4.5	18.3 $\pm$ 2.4*	11.9 $\pm$ 2.9	70.3 $\pm$ 5.2	8.1 $\pm$ 0.5§	10.3 $\pm$ 0.9	81.7 $\pm$ 0.7	6.1 $\pm$ 0.5†	7.6 $\pm$ 0.9	86.3 $\pm$ 0.7

Table 5. *Experiment 3. Apparent oxidation state of selenium in rat liver cell fractions*

A group (48) of 3-month-old vitamin E-deficient rats were given the *Torula* yeast diet for 2 weeks. Half of these rats were treated with neomycin sulphate and used for the study of the intravenous incorporation of ^{75}Se into liver subcellular fractions, and the remaining rats were used to study the effect of freezing the liver *in situ* to -70°C on the oxidation states of orally administered ^{75}Se in the subcellular organelles; full details are given in the text. All livers were homogenized with antioxidants in the isolating medium. Results are expressed as a percentage of the total ^{75}Se in the fraction (mean $\pm$ s.d.). The numbers in parentheses are numbers of observations. Values marked * differ significantly from those marked † ($P > 0.001$) for directly comparable values read horizontally. Values marked ‡ differ significantly from those marked § ($P > 0.01$) for directly comparable values read horizontally. Values marked ¶ differ significantly from those marked ¶ ($P > 0.05$) for directly comparable values read horizontally.

	^{75}Se given orally						^{75}Se given intravenously					
	Vitamin E-deficient rats			Vitamin E-supplemented rats			Vitamin E-deficient rats			Vitamin E-supplemented rats		
	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate
Homogenate (4)	6.0 $\pm$ 1.1†	21.0 $\pm$ 4.1	72.8 $\pm$ 4.5	13.0 $\pm$ 2.4§	22.8 $\pm$ 0.9	64.2 $\pm$ 2.8¶	14.9 $\pm$ 0.1*	33.7 $\pm$ 2.1*	51.4 $\pm$ 2.1	28.7 $\pm$ 0.8†	22.1 $\pm$ 0.9†	49.0 $\pm$ 3.5
Mitochondrial fraction (4)	27.2 $\pm$ 6.2	12.5 $\pm$ 5.3	60.3 $\pm$ 11.7	13.4 $\pm$ 8.0¶	14.3 $\pm$ 8.1	72.7 $\pm$ 8.8	17.4 $\pm$ 0.9*	30.9 $\pm$ 5.0†	51.8 $\pm$ 5.8	46.2 $\pm$ 3.7†	6.2 $\pm$ 2.0§	46.5 $\pm$ 0.7
Microsomal fraction (4)	21.7 $\pm$ 5.7†	18.3 $\pm$ 3.9†	59.9 $\pm$ 7.5	43.6 $\pm$ 3.4§	5.5 $\pm$ 0.2§	50.9 $\pm$ 2.3	6.2 $\pm$ 0.4†	10.5 $\pm$ 0.5	88.2 $\pm$ 9.2	21.3 $\pm$ 2.8§	5.9 $\pm$ 4.1	72.1 $\pm$ 7.4
Supernatant fraction (4)	12.2 $\pm$ 3.9	9.4 $\pm$ 3.0	78.1 $\pm$ 1.3	19.1 $\pm$ 0.9¶	4.2 $\pm$ 2.0	78.6 $\pm$ 1.6						

Table 6. *Experiment 4. Distribution of ^{75}Se among rat liver cell fractions*

A number (36) of vitamin E-deficient rats were divided into four groups, containing three lots of three rats, 15 days before they were killed. Group A received a total of $30.8\mu\text{Ci}$ of ^{75}Se ($8.1\mu\text{g}$ of Se) on days 9–14 and 0.1 ml of methyl oleate on days 1–14. Group B received the same ^{75}Se dosage as group A rats, and $\text{D-}\alpha$ -tocopherol on days 1–14. Rats in groups C and D received the same dosage as those in groups A and B respectively, and in addition they were given silver (both orally and in their drinking water) on days 11–14. All rats were killed on day 15, in three lots of three rats/group, and their livers were homogenized in the presence of antioxidants. The particulate fractions were resuspended in 0.25 M-sucrose solution containing antioxidants and portions of these and of the soluble fractions subjected to the radioactivity-counting procedures described in the text. A further 16 rats were also used, eight additional rats in groups C and eight in group D, to determine silver toxicity (see the text). Values marked * differ significantly from those marked † ($P > 0.001$) for directly comparable values read horizontally. Values marked ‡ differ significantly from those marked § ($P > 0.01$) for directly comparable values read horizontally.

	Vitamin E-deficient rats		Vitamin E-supplemented rats	
	Untreated (group A)	Silver-treated (group C)	Untreated (group B)	Silver-treated (group D)
Mean total liver (homogenate) ^{75}Se ($10^{-3} \times \text{d.p.s./liver}$)	$23.4 \pm 5.3^*$	$5.4 \pm 0.7^{\dagger\dagger}$	$21.4 \pm 1.8^*$	$11.0 \pm 0.1^{\dagger}\S$
Mean % of total liver ^{75}Se in:				
Mitochondrial fraction	$10 \pm 2^*$	$15 \pm 6^{\dagger}$	$8 \pm 2^*$	$19 \pm 1^{\dagger}$
Microsomal fraction	9 ± 2	12 ± 3	10 ± 1	7 ± 2
Supernatant fraction	$53 \pm 2^*$	$29 \pm 10^{\dagger}$	$49 \pm 2^*$	$16 \pm 2^{\dagger}$
Debris fraction	28 ± 3	44 ± 6	33 ± 2	58 ± 6

necessary to suppose, therefore, that a specific, unique mechanism exists in animal tissues for the reduction of selenite, administered orally, to the selenide that we have detected in liver subcellular fractions. The possibility that intestinal micro-organisms might be responsible for the reduction of selenite to selenide, which was then absorbed, could not be overlooked, and the second part of Expt. 3 was designed to test this possibility. When the selenite was administered intravenously and the intestinal aerobic micro-organisms were largely eliminated by treatment with neomycin (Horth *et al.* 1966), selenide was found in the liver subcellular organelles. In general, the results (Table 5) reproduce substantially those obtained in Expt. 2; both the proportions of selenide and the magnitude of the difference between organelles derived from vitamin E-deficient or vitamin E-supplemented animals are very similar to those found in the earlier experiment. If one assumes that the small population of anaerobic micro-organisms remaining in the alimentary tract after neomycin treatment (Horth *et al.* 1966) were not responsible for the reduction of the selenite, then it can be concluded that an endogenous mechanism is responsible for the reduction to selenide.

Experiment 4. The toxicity of silver to vitamin E-deficient rats was first described by Shaver & Mason (1951) and studied in more detail by Diplock, Green, Bunyan, McHale & Muthy (1967b). The latter authors described a massive necrosis of the liver after administration of silver acetate to

vitamin E-deficient rats, and suggested that the disease was, superficially at least, similar to dietary liver necrosis induced in rats by the combined deficiency of vitamin E and selenium. In a study of the histopathology and electron microscopy of the silver-induced necrosis, Grasso *et al.* (1969) found that the condition was indistinguishable from dietary liver necrosis. They suggested that the administration of silver to vitamin E-deficient rats complexed selenium and prevented it from entering its active site, with the result that liver necrosis was produced. It was therefore necessary to determine whether the proportion of the total cell selenium present as selenide was affected by the administration of silver, and whether dietary vitamin E had any effect on this. In addition, this experiment was used to extend our observations as to whether the three oxidation states of selenium that we have delineated passed through a dialysis membrane.

It is possible with all the results of this experiment to make four comparisons (Table 6): comparison of group A with group B gives the effect of dietary vitamin E; comparison of group C with group D gives the effect of dietary vitamin E when silver was administered concomitantly; comparison of group A with group C and group B with group D gives the effect of silver administration in vitamin E-deficient or vitamin E-supplemented rats respectively.

The results for the distribution of ^{75}Se among the liver fractions are given below and in Table 6.

Considering first the homogenate, administration of silver substantially lowered the total amount of ^{75}Se in the liver, whereas the vitamin E treatment significantly reversed this decrease, although the amount of ^{75}Se found in the vitamin E-treated livers was not as great as that in the livers of the vitamin E-treated rats that received no silver. In the mitochondrial fractions there was little difference in the amount of ^{75}Se found when vitamin E was administered; significantly more ^{75}Se was found in this fraction when silver was given and vitamin E did not affect this increase. Grasso *et al.* (1969) found a proliferation of lysosomes in the livers of vitamin E-deficient rats treated with silver, and, since the mitochondrial fraction in our experiments probably contains lysosomes and autophagic vacuoles, it is probable that the silver-induced increase in ^{75}Se was caused by accumulation of Ag_2Se in these contaminating bodies. In the livers of the vitamin E-deficient rats, the microsomal fractions appeared to contain more ^{75}Se when silver was given, and supplementation with vitamin E reversed this, although none of these effects was statistically significant. In the supernatant fraction a highly significant lowering of the ^{75}Se content was observed when silver was given, and vitamin E did not affect this. The sum of the ^{75}Se recovered from the fractions was 83.94% of the ^{75}Se in the original homogenates. These results are interpreted as being indicative of a specific role for selenide in the mitochondrial and microsomal fractions: as selenide has been rendered biologically unavailable by the silver treatment, a greater proportion of the total available selenium seems to have become incorporated into these organelles at the expense of the soluble fraction.

The apparent oxidation state of the Se and the dialysis results are given in Table 7; no dialysis results were obtained for the homogenates. The results are complex, and some of the small changes observed, although they may have statistical significance, may not be important in biological terms. In the homogenate, the proportion of selenide was significantly increased when the rats were supplemented with vitamin E; a lower proportion of selenide was observed in the silver-treated liver homogenates than in the untreated ones, and vitamin E supplementation caused a significant increase in the proportion of selenide in these rats. Similar general trends were found in the mitochondrial, microsomal and supernatant fractions; in the microsomal fraction, silver treatment did not affect the proportion of selenide when the rats were also given vitamin E, whereas in the absence of vitamin E, silver treatment caused a small fall in the proportion of selenide.

The dialysis results (Table 7) confirm the observations in Expt. 2. Most of the selenide in the mito-

Table 7. *Experiment 4. Apparent oxidation state of selenium in rat liver cell fractions*

Experimental details are given in Table 6. Results are expressed as % of the total ^{75}Se in the fraction (mean $\pm$ S.D.) or as a percentage of the Se lost on dialysis (mean $\pm$ S.D.). Numbers in parentheses are numbers of observations. Values marked * differ significantly from those marked † ($P > 0.001$) for directly comparable values read horizontally. Values marked ‡ differ significantly from those marked § ($P > 0.01$) for directly comparable values read horizontally.

	Vitamin E-deficient rats						Vitamin E-supplemented rats					
	Untreated (group A)			Silver-treated (group C)			Untreated (group B)			Silver-treated (group D)		
	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate	Selenide	Selenite	Selenate
Homogenate (3)												
% of total	13.9 $\pm$ 0.5*	17.1 $\pm$ 2.3	69.0 $\pm$ 2.7	9.4 $\pm$ 1.2†	21.6 $\pm$ 3.6	69.1 $\pm$ 4.7	23.9 $\pm$ 7.1†	26.4 $\pm$ 4.0	49.6 $\pm$ 10.0	17.7 $\pm$ 3.7§	20.7 $\pm$ 3.7	61.6 $\pm$ 4.8
Mitochondrial fraction (3)												
% of total	8.2 $\pm$ 1.2*†	13.5 $\pm$ 3.0†	78.4 $\pm$ 3.9	4.7 $\pm$ 1.1*§	23.8 $\pm$ 3.9§	71.5 $\pm$ 3.4	20.9 $\pm$ 0.9†	12.2 $\pm$ 3.7	66.9 $\pm$ 4.6†	11.2 $\pm$ 1.8†§	26.2 $\pm$ 2.5	62.6 $\pm$ 4.2
% lost on dialysis	7 $\pm$ 3	77 $\pm$ 18	59 $\pm$ 7	0	62 $\pm$ 16	58 $\pm$ 5	0	31 $\pm$ 10	42 $\pm$ 10	12 $\pm$ 7	83 $\pm$ 4	48 $\pm$ 19
Microsomal fraction (3)												
% of total	11.6 $\pm$ 1.7†	30.4 $\pm$ 4.9	58.0 $\pm$ 6.2	9.6 $\pm$ 2.3*	28.9 $\pm$ 5.2	64.9 $\pm$ 5.2	23.3 $\pm$ 3.7§	25.9 $\pm$ 4.6	50.9 $\pm$ 5.0	26.3 $\pm$ 1.6†	26.7 $\pm$ 7.5	47.2 $\pm$ 7.2
% lost on dialysis	9 $\pm$ 1	76 $\pm$ 4	76 $\pm$ 0	3 $\pm$ 1	64 $\pm$ 7	44 $\pm$ 1	15 $\pm$ 2	78 $\pm$ 6	58 $\pm$ 3	10 $\pm$ 1	63 $\pm$ 10	33 $\pm$ 6
Supernatant fraction (3)												
% of total	5.4 $\pm$ 2.0†	25.7 $\pm$ 3.3	68.9 $\pm$ 4.8	1.0 $\pm$ 0.4†	28.5 $\pm$ 8.1	70.5 $\pm$ 8.1	23.0 $\pm$ 5.0§	23.4 $\pm$ 4.7	56.9 $\pm$ 8.5	13.8 $\pm$ 3.4§	28.7 $\pm$ 3.7	67.6 $\pm$ 13.2
% lost on dialysis	30 $\pm$ 1	80 $\pm$ 1	55 $\pm$ 5	97 $\pm$ 3	98 $\pm$ 4	47 $\pm$ 12	79 $\pm$ 1	81 $\pm$ 2	62 $\pm$ 10	100 $\pm$ 0	81 $\pm$ 1	70 $\pm$ 3

chondrial and microsomal fractions did not pass through the dialysis membrane, irrespective of the dietary treatment of the rats from which the fractions were derived. This is interpreted as indicating that the selenide in these fractions is protein bound, in contrast with the selenite and selenate, which appeared generally more freely diffusible. The selenide in the supernatant fraction almost all passed through the dialysis membrane in the fractions from the rats in groups B, C and D. The lower proportion of diffusible selenide in group A is difficult to explain. In the experiment to test the toxicity of silver, seven of the eight rats used had died by day 20 and examination *post mortem* showed frank necrosis both in the seven that died and in the survivor. None of the rats given silver and vitamin E had died by day 20.

Three main generalizations may be made from the results of Expt. 4. First, the administration of silver to rats, irrespective of their vitamin E status, results in a lowering of the uptake or retention of selenium by the liver. Secondly, the proportion of the total selenium present as selenide is lowered by silver treatment, and this tendency is generally reversed by the administration of vitamin E. Thirdly, the greater part of the mitochondrial and microsomal selenide appeared to be protein-bound. These results support the suggestion of Grasso *et al.* (1969) that silver competes with the active centres of non-haem-iron proteins for the available selenide by forming a complex with it. We suggest that Ag_2Se may be formed and we have found (A. T. Diplock and C. Thomas, unpublished work) that Ag_2Se does not yield a volatile selenium derivative on acidification. The lower total amount of ^{75}Se found may be due to the formation of an insoluble

silver salt of selenium in the small intestine, and other experiments, not reported here, have indicated a greater faecal excretion of ^{75}Se in silver-treated rats.

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