The Effect of Vitamin E on the Intracellular Distribution of the Different Oxidation States of Selenium in Rat Liver

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1. The liver intracellular distribution of ^{75}Se , $^{75}\text{Se}^{2-}$ and $^{75}\text{SeO}_3^{2-}$ formed from orally administered $\text{Na}_2^{75}\text{SeO}_3$ was studied in rats given four different dietary treatments. 2. Subcellular fractionation was done by using sucrose density gradients in a B XIV zonal centrifuge rotor, and conditions were established so that separation of lysosomal, mitochondrial, smooth- and rough-surfaced endoplasmic reticulum, and soluble fractions was achieved. 3. Marker enzymes acid phosphatase, succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase and glucose 6-phosphatase were used, together with electron microscopy, to establish the identity of the fractions. 4. The dietary treatments investigated were: (a) vitamin E-deficient diet for 3 months, re-fed with vitamin E during the terminal 5 days; (b) vitamin E-deficient diet; (c) adequate diet; (d) vitamin E- and selenium-deficient diet, re-fed with vitamin E during the terminal 5 days. 5. In adequately fed rats, selenide was particularly associated with the mitochondrial fractions; in vitamin E-deficient rats, little selenide was found and the buoyant density of the mitochondria was increased, whereas re-feeding with vitamin E showed a restoration of the normal pattern. In vitamin E- and selenium-deficient rats, re-fed with vitamin E, there was no tendency for selenide to be localized in the mitochondria. 6. In the microsomal regions of the gradients, adequately fed rats showed a concentration of selenide, particularly in the smooth endoplasmic reticulum fractions, and to a lesser extent in the rough endoplasmic reticulum fractions. This was not observed in vitamin E-deficient rats, and the normal pattern was restored on re-feeding with vitamin E, both in rats given the vitamin E-deficient diet and the vitamin E- and selenium-deficient diet. 7. Some selenide was also found in the soluble fractions, when vitamin E was present, and a substantial proportion of this selenide was found to pass through a dialysis membrane. 8. These results are taken to support our hypothesis that the active form of selenium may be selenide located in non-haem iron-containing proteins, and that the function of vitamin E may be to protect the selenide from oxidation.

Diplock, Baum & Lucy (1971) showed that the selenium found in rat liver organelles is present in three or more oxidation states tentatively identified as selenide, selenite and higher oxidation states of selenium. The proportion of the total selenium present as selenide was found to be dependent on (a) the presence of antioxidants (α -tocopherol and mercaptoethanol) in the media used for isolating the organelles, and (b) the presence of vitamin E in the animals' diet, significantly more selenide being found in the liver organelles derived from vitamin E-supplemented rats than from vitamin E-deficient rats. It was further demonstrated that the selenide present in the mitochondrial and microsomal frac-

tions was non-diffusible, indicating that it was protein-bound, in contrast with the selenide found in the soluble fraction, which was diffusible.

The fractionation procedures used in our earlier paper (Diplock *et al.* 1971) were necessarily simple, to allow statistical evaluation of the results obtained in experiments on liver organelles from rats having differing amounts of vitamin E in their diets. An investigation with a carefully defined fractionation procedure with greater resolution has now been carried out and the results are reported here. In the present work, a technique for the total fractionation of liver cells by using the MSE B XIV titanium zonal centrifuge rotor has been

developed. This technique has been employed in a detailed examination of the intracellular distribution of the three oxidation states of selenium in the livers of rats that had received different dietary treatments.

EXPERIMENTAL

Diets. The vitamin E-deficient diet and the *Torula* yeast diet have been described before (Diplock *et al.* 1971). The former is formulated to contain the minimal amount of selenium necessary to prevent liver necrosis, whereas the latter is selenium- and vitamin E-deficient. The adequate diet was the Oxoid 41B pellet diet.

Animals, feeding regimen and dosage. Caesarian derived male Wistar rats (40–50g) were obtained from a commercial breeder. They were treated subsequently in one of four ways.

Vitamin E-deficient rats, re-fed with vitamin E [dietary treatment (a)]. Weanling rats were given the vitamin E-deficient diet for 3 months. Pairs of rats were then each given five consecutive daily oral doses of 3mg of DL- α -tocopherol in 0.1 ml of olive oil, and on days 3, 4 and 5 they also each received an intraperitoneal injection of approx. 50 μ Ci of Na₂⁷⁵SeO₃ (about 8 μ g of Se) in 0.1 ml of 0.9% NaCl; the precise amount of ⁷⁵Se varied with the specific radioactivity of individual batches of Na₂⁷⁵SeO₃, purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Each pair of rats was killed on day 6 and portions of their livers were subjected to fractionation in the zonal centrifuge.

Vitamin E-deficient rats [dietary treatment (b)]. Weanling rats were treated exactly as those receiving treatment (a), except that the five oral doses of α -tocopherol in olive oil were replaced by dosage with 0.1 ml of olive oil alone.

Adequately fed rats [dietary treatment (c)]. Weanling rats were given the Oxoid 41B pellet diet for 3 months. Pairs of rats were then each given three consecutive daily intraperitoneal injections of approx. 50 μ Ci of Na₂⁷⁵SeO₃ in 0.1 ml of 0.9% NaCl, and killed on day 4.

Vitamin E- and selenium-deficient rats re-fed with vitamin E [dietary treatment (d)]. Weanling rats were given the vitamin E-deficient diet for 7 days, and the selenium- and vitamin E-deficient (*Torula* yeast) diet for 14 days. Pairs of rats were then given five consecutive daily oral doses of 3mg of DL- α -tocopherol in 0.1 ml of olive oil, and on days 3, 4 and 5 they also received an intraperitoneal injection of approx. 50 μ Ci of Na₂⁷⁵SeO₃ (about 8 μ g of Se) in 0.1 ml of 0.9% NaCl. The rats were given the selenium- and vitamin E-deficient diet during the dosage period, and killed on day 6 after beginning dosage. In preliminary experiments it was found that, when weanling rats were introduced at 28 days of age to the selenium- and vitamin E-deficient diet, there was a high initial mortality. When the initial 7-day period of feeding with the casein diet was adopted, death due to liver necrosis occurred between days 21 and 30 after the commencement of feeding with the *Torula* yeast diet (the majority of the rats dying at day 25). It is therefore considered that the re-feeding with α -tocopherol was commenced at about the time that the 'biochemical lesion' (i.e. respiratory decline) observed by Schwarz (1962) was becoming established.

Cell fractionation. (a) Homogenization. To achieve reproducibility of the subcellular fractionation, a standardized and carefully controlled procedure was used to homogenize the tissue. A modification (Dingle & Barrett, 1969) of the homogenizer described by Aldridge, Emery & Street (1960) was employed, in which a conical nylon pestle engages in the nylon base of a glass tube. Pressure is applied mechanically by a spring-loaded motorized shaft, which can give a predetermined number of passes of the pestle through the suspension of liver to be homogenized. Experiment showed that the greatest degree of uniformity was achieved by using four passes of the pestle. A 1g sample of liver was homogenized in 25 ml of 0.25M-sucrose in 0.05M-tris chloride buffer, pH 7.4, containing 5mM-mercaptoethanol and 100 μ g of DL- α -tocopherol/ml, which was suspended by sonication. This mixture of antioxidants was always included in all solutions used in these experiments. The homogenate was centrifuged for 10 min at 2000g, and the 2000g supernatant applied to the zonal centrifuge rotor.

(b) Zonal centrifugation. The MSE BXIV titanium rotor was used on the MSE Superspeed 50 centrifuge. For run 1, the rotor was loaded with the following sucrose density gradient: 150 ml of 12% (w/w) sucrose, 400 ml of a continuous gradient from 20% (w/w) sucrose to 60% (w/w) sucrose, and 100 ml of 60% (w/w) sucrose as cushion; the loading speed was 2000 rev./min. The 2000g supernatant (20 ml) was then introduced at the centre of the rotor followed by 2 $\times$ 20 ml of 4% (w/w) sucrose as overlay. The rotor was accelerated until it reached a speed of 20000 rev./min, when it was switched off and allowed to decelerate to 2000 rev./min, where it was held for unloading. To remove selectively the lysosomal and mitochondrial regions of the gradient and leave the less-dense microsomal and supernatant regions in the rotor, a procedure was devised in which the heavier portion of the gradient was displaced in the reverse direction (i.e. from the outer edge) from that normally used for unloading. By using 450 ml of 1% (w/w) sucrose to displace the lysosomal and mitochondrial regions, 45 $\times$ 10 ml fractions were pumped out in the reverse direction and collected. By careful selection of pumping speed, turbulence was avoided and the microsomal-supernatant region remained undisturbed, its position now being near the periphery of the rotor. A new gradient, consisting of 400 ml of a continuous gradient from 20% (w/w) sucrose to 60% (w/w) sucrose, was then introduced, followed by 50 ml of 60% (w/w) sucrose, displacing the 1% (w/w) sucrose used previously to displace the mitochondrial-lysosomal regions. The microsomal-supernatant region was thus restored to its original position near the centre of the rotor, which was then accelerated (run 2) to 30000 rev./min and held at this speed for 5 min before being decelerated to the unloading speed of 2000 rev./min. The reversed unloading procedure described above was now repeated, with the 1% (w/w) sucrose; this was again displaced by a further continuous gradient from 20% (w/w) sucrose to 60% (w/w) sucrose, followed by the cushion of 60% (w/w) sucrose. For run 3 the rotor was accelerated to 30000 rev./min and held at this speed for 16–18 h (i.e. overnight). At the end of this time, 60 $\times$ 10 ml samples were displaced in the normal direction from the rotor by using 600 ml of 60% (w/w) sucrose.

Livers from rats given each of the four dietary treatments (a-d) were examined two or three times by the zonal centrifugation procedure.

Enzyme assays. Acid phosphatase and succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase were always measured in alternate fractions from run 1 and in representative fractions from runs 2 and 3. Glucose 6-phosphatase was measured in alternate microsomal and supernatant fractions (run 3), and in representative fractions from run 1 and run 2. These enzymes were selected as markers because the assays are relatively inexpensive and they can be carried out with confidence on a large number of samples. Total acid phosphatase was measured by a modification of the method of Torriani (1960) as follows. The incubation mixture was 0.5 ml of 20 mM-*p*-nitrophenyl phosphate in 0.1 M-acetate buffer, pH 4.2, 0.01 ml of 10% (w/v) Triton X-100 in 0.1 M-acetate buffer, pH 4.2, and 0.5 ml of enzyme solution. The mixture was incubated at 37°C for 10 min, the reaction stopped by the addition of 2 ml of 0.2 M-glycine-NaOH buffer, pH 10.8, containing 0.1 M-Na₃PO₄, and the yellow colour read at 400 nm.

Succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase was measured by a modification (Hinton, Burge & Hartman, 1969) of the method of Pennington (1961) as follows. The incubation mixture was 0.4 ml of 0.25% 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in 125 mM-sodium phosphate buffer, pH 7.2, 0.1 ml of 500 mM-sodium succinate or 500 mM-sodium malonate, pH 7.2, and 0.5 ml of enzyme solution. The mixture was incubated at 37°C for 30 min and the reaction stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. Then 4 ml of ethyl acetate was added and the pink colour extracted and read at 490 nm. Simultaneous observations were made on tubes containing either malonate or succinate, and the difference in colour (ΔE) between these tubes was taken as the enzyme activity.

Glucose 6-phosphatase was measured by a modification of the method of Appelmans, Wattiaux & De Duve (1955). A suspension of microsomes (0.2 ml) in 0.05 M-tris chloride buffer, pH 7.4, was incubated, either with 5 μ l of buffer or with 5 μ l of 0.1 M-citric acid, for 10 min at 37°C. To each was added 0.2 ml of 16 mM-glucose 6-phosphate in 0.1 M-cacodylate buffer, pH 6.5, and the mixture incubated for 30 min at 37°C. The reaction was stopped by the addition of 1.6 ml of 10% (w/v) trichloroacetic acid and the precipitated material removed by centrifugation. The supernatant (0.4 ml) was incubated with 0.4 ml of the phosphate reagent (Chen, Toribara & Warner, 1956) for 1.5 h and the blue colour was read at 800 nm.

Radioactivity counting. This was carried out as a routine by using the Wallac GTL-300 γ -isotope counter, in all fractions from the zonal centrifuge runs, by the method described by Diplock *et al.* (1971) and the designations of selenide and selenite are as defined in that paper.

Electron microscopy. Samples of liver from rats given either dietary treatment (a) or the vitamin E-deficient diet, supplemented with 100 mg of α -tocopherol acetate/kg for 3 months, were taken for electron microscopy. The pieces of liver were trimmed into small blocks (1 mm³) and fixed for 60 min at 4°C in 0.1 M-cacodylate-buffered osmium tetroxide fixative, and treated subsequently by

the technique of Grasso *et al.* (1969). The results of this investigation are presented in Plate 1. For electron microscopy of the liver cell fractions being studied, a pair of rats was subjected to dietary treatment (a) and their livers were fractionated by the zonal technique. The contents of tubes 26-30 (run 1), 34-40 (run 1), 34-42 (run 3), 46-50 (run 3) and 52-58 (run 3) respectively were combined and diluted to give a final concentration of approx. 0.25 M-sucrose. The mitochondrial and lysosomal fractions were centrifuged at 12000g for 20 min and the microsomal fractions were centrifuged at 100000g for 1 h. The pellets were collected and fixed immediately in 4% formalin in 0.01 M-acetate buffer, pH 7.0, for 16 h at 4°C, after which they were post-stained with osmic acid and treated as described above. The results of this investigation are shown in Plate 2. Each micrograph in Plates 1 and 2 is representative of the entire preparation as judged by exhaustive examination in the AEI-EM6B microscope of the greater part of each preparation.

Special techniques for identification of microsomes. Rats given dietary treatment (c) were given Na₂⁷⁵SeO₃ as described earlier. In addition, 30-36 h before death, they were given 100 μ Ci of sodium [6-¹⁴C]orotate and, 30 min before death, 400 μ Ci of [*Me*-³H]choline chloride; both doses were injected intraperitoneally. After homogenization and fractionation of the livers by the zonal centrifugation technique, samples of all fractions from run 3 were prepared for counting of radioactivity by the following technique.

Samples (0.1 ml) were applied to glass-fibre discs that were then washed four times with 0.2 M-HClO₄ to remove unincorporated labelled compounds. The discs were then placed in NH₃ vapour, to neutralize residual HClO₄, dried and the radioactivity was counted in a Nuclear-Chicago liquid-scintillation counter, by using 5 ml of toluene-based scintillator. The observed counts were corrected for quenching by using correction curves obtained from standard mixtures of ¹⁴C and ³H. Interference due to the ⁷⁵Se also present was eliminated by careful selection of the channel settings.

RESULTS

Presentation of results. The total amount of selenium found in the liver cell fractions varied widely from experiment to experiment, even though the dose of ⁷⁵Se had been the same in each case. It is probable that the proportion of the selenium administered intraperitoneally that is absorbed, and also the rate of excretion, is dependent on factors beyond experimental control, and some preliminary experiments indicated that starving the animals before or after dosage did not affect the observed variability. To examine and compare the results both of similar and different dietary treatments on the relative distribution of selenium, selenite and selenide it was therefore necessary to adopt the following procedure. For the lysosomal and mitochondrial regions of the gradient (Figs. 1a-1d), the amount of ⁷⁵Se found in each of tubes 20-60 (inclusive) (run 1) was summed, and the amount of ⁷⁵Se, ⁷⁵SeO₃²⁻ and ⁷⁵Se²⁻ was expressed for each

Fig. 1. Distribution of selenium (.....), selenite (---), selenide (—), acid phosphatase activity (E_{400}) (---) and succinate-2-*p*-iodophenyl-3-nitrophenyl-5-phenyltetrazolium reductase activity (ΔE_{490}) (-.-.) in the mitochondrial and lysosomal fractions of rat liver. Rats were given the following dietary treatments; (a) vitamin E-deficient diet, re-fed with vitamin E; (b) vitamin E-deficient diet; (c) adequate diet; (d) vitamin E- and selenium-deficient diet, re-fed with vitamin E. Details of the diets and the method of fractionation are given in the Experimental section.

tube as a percentage of the total and plotted as such. For the microsomal fractions (Figs. 2a-2d), a similar procedure was adopted for tubes 20-60 (run 3); for the soluble fraction (Figs. 3a-3d) the ^{75}Se in tubes 1-20 inclusive (run 3) was summed, and the amount of ^{75}Se , $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ expressed as a percentage of this value. When this procedure was adopted it was found that for any one dietary treatment there was a remarkable reproducibility between the two or three experiments carried out, both in the qualitative profiles of the various curves plotted and in the quantitative relationships between the different oxidation states of selenium. We have therefore only presented the results from one experiment for each dietary treatment, as the conclusions that we have drawn apply equally to the other experiments.

The enzyme assay values are presented directly as E for acid phosphatase and ΔE , i.e. the difference between the E value obtained by using malonate as substrate and that by using succinate as substrate, for succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase. Glucose 6-phosphatase is not shown graphically and the results obtained are discussed below.

Identity of the fractions at each region of the gradient.
The identity of the subcellular organelles or fragments may be deduced from an examination of the electron micrographs, and from the results of the marker enzyme measurements. Electron-microscopic examination of the contents of tubes 24-28 (Fig. 1a) showed a heterogeneous mixture of cell components among which the lysosomes appear (Plate 2a) to have been concentrated. However, it is also possible to distinguish pieces of rough endoplasmic reticulum and it is clear that, because of this heterogeneity, any deductions made about the selenium content of this region must be treated with caution. There is a tendency for the acid-phosphatase activity to form a peak or shoulder at around tubes 28-30, and this coincides with the region where a concentration of lysosomes was seen with the electron microscope; the tendency to form a peak is, moreover, greater in the liver from vitamin E-deficient animals re-fed with vitamin E (Fig. 1a) than in liver when this re-feeding was omitted (Fig. 1b). Acid-phosphatase activity was also usually detected in tubes 34-42 (Fig. 2) and is thought to be due to association of the enzyme with the smooth endoplasmic reticulum vesicles (see below). A large

(a)

(b)

EXPLANATION OF PLATE I

Representative electron micrographs of rat liver preparations. (a) Section from the liver of a rat given the vitamin E-deficient diet, supplemented with 100 mg of α -tocophenyl acetate/kg, for 3 months; (b) section from the liver of a rat given dietary treatment (a) (vitamin E-deficient diet for 3 months, re-fed with α -tocophenol for 5 days before death). Magnification $\times 45\,000$.

(a)

(b)

(c)

(d)

EXPLANATION OF PLATE 2

Representative electron micrographs of rat liver cell fractions from a rat given dietary treatment (a) (vitamin E-deficient for 3 months, re-fed with α -tocophenol for 5 days before death). (a) Lysosomal-debris fraction, corresponding to tubes 26–30 (Fig. 1c); (b) mitochondrial fractions, corresponding to tubes 34–40 (Fig. 1c); (c) smooth endoplasmic reticulum fractions, corresponding to tubes 34–42 (Fig. 2c); (d) rough endoplasmic reticulum, corresponding to tubes 52–58 (Fig. 2c). Magnification $\times 90\,000$.

Fig. 2. Distribution of selenium (····), selenite (---) and selenide (—) in the microsomal fractions of rat liver. Rats were given the following dietary treatments: (a) vitamin E-deficient diet, re-fed with vitamin E; (b) vitamin E-deficient diet; (c) adequate diet; (d) vitamin E- and selenium-deficient diet, re-fed with vitamin E. Details of the diets and the method of fractionation are given in the Experimental section.

amount of acid phosphatase activity was always found in the soluble fractions, presumably due to free enzyme activity released on rupture of the lysosomes during homogenization.

Electron-microscopic examination of the contents of tubes 34-40 (Fig. 1a) revealed mostly mitochondria and mitochondrial fragments (Plate 2b). When the preparation was scanned in the electron microscope, it was not possible to see any lysosomes, nor was there appreciable contamination with acid phosphatase activity when the fraction was examined. The electron-dense matrix showed evidence of damage although the inner and outer membranes and, to a large extent, the cristae appeared intact. This disturbed appearance of the

isolated mitochondria may be attributable to two causes. First, the obvious osmotic and mechanical shocks to which the tissue fragments are subjected during preparation of the mitochondria will be expected to have a detrimental effect. Secondly, there may be changes in the mitochondrial structure *in vivo* that lead to an increased fragility. Thus Djaczenko, Grabska, Urbanowicz & Pezzi (1969) have noted alterations in the appearance of the liver mitochondria of vitamin E-deficient rats, such as swelling and alterations in the arrangement of cristae, extending the observations of Sulkin & Sulkin (1962). The liver section shown in Plate 1(b) is representative of many such preparations we have examined, and a consistent feature is

swelling and loss of electron density of the mitochondrial matrix, and possibly also a decrease in the number of cristae compared with liver of vitamin E-supplemented rats (Plate 1a). Dr B. Ketterer (personal communication) has observed that similar changes in the mitochondrial structure of the livers of vitamin E-deficient rats are the first changes to occur; however, when vitamin E was re-fed, the mitochondria quickly became normal. Molenaar, Vos, Jager & Hommes (1971) have noted abnormalities in the electron density of the outer mitochondrial membranes from the intestinal brush border and the liver (I. Molenaar & J. Vos, personal communication) of vitamin E-deficient ducklings.

The succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase activities in Figs. 1(a), 1(c) and 1(d) are concentrated in tubes 28–50, and consistently show a bicuspid distribution. Reports of two populations of mitochondria, separable by differences in their buoyant density, have appeared in the literature (Pollak & Munn, 1970) and our results confirm these observations. On comparison of Fig. 1(a) with Fig. 1(b), it is striking that, in the vitamin E-deficient tissue, there is a shift in the buoyant density of the mitochondria toward the heavy end of the gradient, a feature that was observed consistently with rats given this dietary treatment. Re-feeding with vitamin E reversed this shift (Fig. 1a) to the normal buoyant density (Fig. 1c). These results appear to offer biochemical confirmation of the electron-microscopic observations of enlarged mitochondria in liver cells of vitamin E-deficient rats. It is, moreover, noteworthy that on re-feeding with vitamin E (Fig. 1a) the heavier of the two populations of mitochondria, as judged by enzyme activity, is less apparent, whereas the lighter is more apparent, in contrast with the normal situation (Fig. 1c) where the peaks are approximately equal height. It may be speculated that on re-feeding with vitamin E there is a surge of mitochondrial re-synthesis leading to an increase in the total population of the less-dense mitochondria. Succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase activity was also detected in the microsomal fractions (tubes 34–42; Fig. 2d) presumably through association of the enzyme, liberated by rupture of the mitochondria, with the smooth-surfaced vesicles, and this is considered to provide further evidence of the fragility of the liver mitochondria of vitamin E- and selenium-deficient animals.

Electron microscopy of the contents of the tubes in the microsomal region of the gradient gave a clearer result. Plate 2(c) shows a representative area of the preparation from tubes 34–42 (Fig. 2a) and appears to consist of vesicles derived from smooth endoplasmic reticulum. No rough mem-

branes could be seen when the entire preparation was scanned. This is in sharp contrast with Plate 2(d), which shows a representative area of the preparation from tubes 52–58 (Fig. 2a) and consists entirely of vesicles derived from rough endoplasmic reticulum. The region of the gradient between these two preparations was also examined (tubes 46–50; Fig. 2a) and this consisted of a mixture, in approximately equal proportions, of vesicles derived from the smooth- and rough-surfaced reticulum.

To establish more conclusively the nature of the contents of tubes 34–42 and 52–58 in the microsomal region of the gradient, a further experiment was carried out. Adequately fed rats, the selenium profiles from which are shown in Fig. 2(c), were also dosed with [^3H]choline and [^{14}C]orotate. [^3H]Choline has been shown (Hallinan, Nagley, Murty, Bennett & Grant, 1969) to be incorporated rapidly into all membranes of the endoplasmic reticulum, and [^{14}C]orotate would be expected (Lowe, Reid & Hallinan, 1970) to be incorporated rapidly into rRNA. The ^3H and ^{14}C radioactivities in all the tubes were counted, and the $^{14}\text{C}/^3\text{H}$ ratio is shown in Fig. 3; the selenium profile is also shown. As expected, the ^{14}C radioactivity is at a minimum at around tube 38 (where it was only slightly more than the background count) and rose to a maximum at tube 54. ^3H radioactivity was found throughout the gradient. The peak of ^{14}C radioactivity at tube 25 was unexpected and cannot be explained with certainty. It may represent some ribosomal breakdown products, or possibly tRNA. The results of

Fig. 3. Distribution of selenide (—) and $^{14}\text{C}/^3\text{H}$ radioactivity ratio (---) in the microsomal fractions of the liver of rats given the adequate diet [dietary treatment (c)]. Details of the diet, the method of administration of [^{14}C]orotate and [^3H]choline, and of the method of fractionation are given in the text.

this experiment confirm the electron-microscopic observation that tubes 34–42 (Fig. 2) contain vesicles derived from smooth-surfaced reticulum, whereas tubes 52–58 (Fig. 2) contain vesicles derived from rough-surfaced reticulum.

Glucose 6-phosphatase activities were measured in all the tubes shown in Figs. 2(a)–(d). The ΔE values were very low but, in general, there was an approximately even distribution of the enzyme in tubes 34–56. No glucose 6-phosphatase activity was detected in the soluble or lysosomal-mitochondrial fractions.

Figs. 4(a)–4(d) show the results obtained for the soluble protein fraction of the homogenates. No morphological studies were made, and the enzyme activities detected in this region have already been considered.

Intracellular distribution of selenium, selenite and selenide. (a) Lysosomal-mitochondrial fractions. In the lysosomal-mitochondrial region of the gradient (Figs. 1a–1d) considerable differences were noted in the distribution of selenium between the different dietary treatments. Considering first the rats given a normal diet [treatment (c); Fig. 1c], the distribution of ^{75}Se is uniform throughout the lysosomal and mitochondrial regions. The distribution of $^{75}\text{SeO}_3^{2-}$ is not specific to any particular region, in contrast with that of $^{75}\text{Se}^{2-}$, which shows an association with the lighter of the two peaks of mitochondrial enzyme activity.

Substantial amounts of $^{75}\text{Se}^{2-}$ were also found in the lysosomal region, possibly due to the presence of selenide in mitochondrial and microsomal fragments contained in autophagic vacuoles. In vitamin E-deficient rats (Fig. 1b) relatively small amounts of ^{75}Se were found associated with the mitochondria, whose increased buoyant density, relative to normal, has been discussed, and the distribution of $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ is non-specific. Substantial amounts of ^{75}Se , $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ were found in the lysosomal region and this is interpreted as indicative either of a role for selenium in lysosomes, or of an increased rate of breakdown of selenium-containing cell components in vitamin E-deficient rat liver. The second of these possibilities seems more likely when the results of the experiments on liver from vitamin E-deficient rats re-fed with vitamin E (Fig. 1a) are considered. The vitamin E treatment has resulted in the mitochondrial enzyme activities returning to normal, as already discussed, and this is accompanied by the association of substantial amounts of ^{75}Se , $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ with, in particular, the lighter of the two mitochondrial peaks. It is also noteworthy that there is here an almost complete absence of $^{75}\text{Se}^{2-}$ from the regions of peak acid-phosphatase activity. It is thought that these results indicate a specific role for selenium, and

particularly of selenide, in liver mitochondria, and that the absence of $^{75}\text{Se}^{2-}$ from the lysosomal region indicates that when vitamin E is given to rats after a period of depletion the biosynthesis of new mitochondria may be rapid, whereas the process of degradation such as was observed in the normal rat liver may be temporarily suspended.

The results shown in Fig. 1(d) represent the profiles of ^{75}Se , $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ obtained when liver from rats given dietary treatment (d) was examined. By means of parallel nutritional experiments, an attempt was made to ensure that the rats were in the pre-necrotic state, as defined by Schwarz (1962), when the biochemical lesion of respiratory decline in liver slices may be observed but the histology to light-microscope examination appears normal. Electron-microscopic examination of livers at this stage (Piccardo & Schwarz, 1958; Grasso *et al.* 1969) has revealed some abnormalities, particularly of the mitochondria. In the rats used in the present experiments, vitamin E and trace amounts of ^{75}Se were given over a period of 3 days. In contrast with the liver mitochondria of the rats given dietary treatment (a), where the same amounts of vitamin E and ^{75}Se were given, there was no tendency for the ^{75}Se , $^{75}\text{SeO}_3^{2-}$ or $^{75}\text{Se}^{2-}$ to be localized particularly in the mitochondrial region. Evidently the morphology of the mitochondria was not grossly disturbed, since the position of the peaks, and the amount, of succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase activity are normal. It is probable that because the livers of rats given dietary treatment (d) have been found previously to show discernible changes in mitochondrial morphology, it may be necessary to re-feed with vitamin E for a longer period than 3 days to restore the mitochondria more fully, in particular with regard to the incorporation of selenide into whatever site it occupies. It is, moreover, significant that the lysosomal region includes a substantial amount of $^{75}\text{Se}^{2-}$, indicating perhaps that the turnover of intracellular constituents in the livers of rats given this dietary treatment may be rapid.

No attempt was made to study selenium- and vitamin E-deficient rats not re-fed with vitamin E [i.e. a parallel treatment to (b)]. It was found in earlier fractionation studies (Diplock *et al.* 1971; A. T. Diplock, unpublished work) that the livers of such rats are so friable that fractionation is unsatisfactory and varies widely from experiment to experiment. This treatment was therefore omitted from the present work.

(b) Microsomal fractions. In the microsomal region of the gradient (Figs. 2a–2d) the rats given dietary treatment (c), the normal adequate diet, showed a peak of ^{75}Se in the smooth-surfaced endoplasmic reticulum. $^{75}\text{Se}^{2-}$ was concentrated largely

Fig. 4. Distribution of selenium (·····), selenite (---) and selenide (—) in the soluble fractions of rat liver. Rats were given the following dietary treatments; (a) vitamin E-deficient diet, re-fed with vitamin E; (b) vitamin E-deficient diet; (c) adequate diet; (d) vitamin E- and selenium-deficient diet, re-fed with vitamin E. Details of the diets and the method of fractionation are given in the Experimental section.

in the smooth-surfaced region, with a substantial additional peak in the rough vesicular region and a small peak between these two regions of major concentration of selenide. Distribution of $^{75}\text{SeO}_3^{2-}$ was similar, although the peak in the rough vesicular region was less pronounced. Deprivation of vitamin E (Fig. 2b) gave rise to a much altered distribution of ^{75}Se . In addition, the small amounts of $^{75}\text{SeO}_3^{2-}$ and $^{75}\text{Se}^{2-}$ detected were distributed in a non-specific fashion throughout the gradient, the peaks of $^{75}\text{Se}^{2-}$ radioactivity observed in the preparation derived from normal rats being completely absent. Re-feeding with vitamin E (Fig. 2a) restored substantially the normal profile: the ^{75}Se radioactivity now formed two peaks, one in the smooth-surfaced and another in the rough-surfaced vesicular regions. $^{75}\text{Se}^{2-}$ formed two substantial peaks, in the same regions as the ^{75}Se peaks, and the small peak of radioactivity, intermediate between these two, observed in the adequately fed rats did not reappear on re-feeding with vitamin E. When rats given dietary treatment (d) (vitamin E- and selenium-deficient) were re-fed with vitamin E (Fig. 2d) the ^{75}Se radioactivity appeared largely concentrated in the smooth-surfaced endoplasmic reticulum, and only a small peak was obtained in the rough vesicular region. This distribution was mirrored by the distribution of the $^{75}\text{Se}^{2-}$, and the peak tube (no. 37) in the smooth-surfaced vesicular region contained the highest proportion of ^{75}Se as $^{75}\text{Se}^{2-}$ that we have observed in these experiments (50.0% compared with 29.9% for the comparable tube from vitamin E-deficient rats re-fed with vitamin E).

These results indicate clearly a specific role for selenium, and particularly for selenide in both smooth-surfaced and rough-surfaced endoplasmic reticulum. The failure of formation of $^{75}\text{Se}^{2-}$ from the $^{75}\text{SeO}_3^{2-}$ given to vitamin E-deficient rats not re-fed with vitamin E shows a high degree of dependence upon vitamin E for the conversion that occurred when vitamin E was re-fed to such depleted animals. It was also found that the $^{75}\text{Se}^{2-}$ was bound tightly to the membranes of both of smooth and rough reticulum, and could not be removed by dialysis.

(c) Soluble fractions. The distribution of ^{75}Se among the proteins of the soluble fraction from the liver of rats given dietary treatment (c) (adequate diet) shows (Fig. 4c) a single peak of radioactivity, and this was also shown by the distribution of $^{75}\text{Se}^{2-}$. Similar results were found for dietary treatments (a) (vitamin E-deficient, rats re-fed with vitamin E) and (d) (selenium- and vitamin E-deficient, rats re-fed with vitamin E). In all three cases it was found that a variable but substantial proportion of the $^{75}\text{Se}^{2-}$ was lost on dialysis, whereas some of the $^{75}\text{Se}^{2-}$ remained bound to

the soluble proteins of the fractions. When the re-feeding with vitamin E was omitted (Fig. 2b) no peak of ^{75}Se or $^{75}\text{Se}^{2-}$ was obtained, the distribution of all three being non-specific. It is possible that the $^{75}\text{Se}^{2-}$ found in tubes 1-4 may represent soluble $^{75}\text{Se}^{2-}$ before it has become incorporated into proteins.

DISCUSSION

The results presented above confirm and amplify earlier work in this laboratory (Diplock *et al.* 1971). Selenide is clearly a major component of the total selenium of rat liver subcellular organelles. In particular, selenide is present in the mitochondria isolated from the livers of normal rats, but not in the liver mitochondria of vitamin E-deficient rats. Mitochondria obtained from depleted animals re-fed with vitamin E contain selenide. We have suggested (Diplock, Baum & Lucy, 1968) that selenide may form a part of the active centre of a class of non-haem iron-containing proteins, distinguished from those known to form a part of the mitochondrial respiratory chain (Beinert & Lee, 1961) by having their sulphide replaced by selenide. In the absence of vitamin E, the turnover of selenide-containing proteins would be expected to accelerate if, as we have postulated, the function of α -tocopherol *in vivo* is to protect the demonstrably highly labile selenide from oxidation. Where dietary selenium is adequate to satisfy the increased demand for selenide, no liver lesion develops. We suggest that liver necrosis results when selenium is withdrawn from the diet because of a metabolic failure caused by the inadequacy of a selenide-depleted mitochondrial respiratory chain. Evidence cited earlier (Schwarz, 1962) suggests that the earliest observable biochemical lesion in liver necrosis involves mitochondrial oxidative phosphorylation. Thus in the present experiments repletion of selenium- and vitamin E-depleted animals with vitamin E alone did not show (Fig. 1d) any improvement in the mitochondrial selenide formed from very small doses of $^{75}\text{SeO}_3^{2-}$, and the presence of selenide in the lysosomal fractions may possibly indicate an increased turnover of selenide. It has also been speculated (Diplock *et al.* 1971) that α -tocopherol may stabilize membranes *in vivo*; it is possible that the failure to replace mitochondrial selenide in selenium- and vitamin E-deficient animals re-fed with vitamin E is caused partly by an underlying instability of the architecture of the mitochondrial membranes and partly by the inability of α -tocopherol, in the short re-feeding period, substantially to reverse the drastic depletion of selenide from membrane-bound proteins.

The existence of an NADPH₂-dependent electron-transfer chain in rat liver microsomes is well established; this is usually regarded as being largely present in the smooth-surfaced endoplasmic reticulum, but is probably also present in the rough-surfaced reticulum. The purpose of the electron-transfer system in liver microsomes is to provide energy for certain steroid hydroxylations, and for the mixed-function oxidase system responsible for the metabolism of foreign compounds. Speculation about the possible presence in the liver microsomal electron-transfer chain of a non-haem iron-containing protein led Miyake, Mason & Landgraf (1967) to study liver microsomes from rabbits treated with phenobarbital; they were unable to detect an electron-spin resonance signal (g 1.94) comparable with that reported (Beinert & Lee, 1961) in adrenal cortical mitochondria, and concluded that the presence of a non-haem iron-containing protein in the microsomal mixed-function oxidase system was unlikely. However, this work was carried out with a crude microsomal preparation and Miyake *et al.* (1967) point out that: 'It is possible that some interaction between the iron and contiguous electron transport components of liver microsomes so broadens the signal that it cannot be detected *in situ*'. Alternatively, if one postulates that the protein involved may be an iron-selenide protein (rather than an iron-sulphide protein) then the techniques used by Miyake *et al.* (1967) for the isolation of the liver microsomes might be expected to destroy such a potentially oxidation-sensitive species. Further, it is noteworthy that Murakami & Mason (1967), in a study of 'microsomal Fe_x', state that: 'Sulphide appears to be a ligand in (cytochrome) *P*-450 and low-spin *P*-420'. This observation was based upon the behaviour of their liver microsomal preparations when *p*-chloromercuribenzenesulphonate, or other thiol-group reagents, were added. It is equally possible that these reagents form a complex, not with sulphide, but with selenide.

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