Studies on Selenium Incorporation into, and Electron-Transfer Function of, Liver Microsomal Fractions from Normal and Vitamin E-Deficient Rats given Phenobarbitone

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1. The incorporation of ^{75}Se from $\text{Na}_2^{75}\text{SeO}_3$ into the liver endoplasmic reticulum of rats given phenobarbitone was investigated by using a zonal centrifuge technique. 2. It was found that, in rats deprived of vitamin E, or of vitamin E and selenium, phenobarbitone was without effect on the incorporation of ^{75}Se or on its conversion to $^{75}\text{Se}^{2-}$. When vitamin E was given at the same time as the phenobarbitone and ^{75}Se , there was a large increase in the amount of ^{75}Se and $^{75}\text{Se}^{2-}$ found in the smooth reticulum. It is concluded that there may be a specific vitamin E-dependent role for selenium and selenide in the smooth endoplasmic reticulum, and it is suggested, in the light of these and other observations, that the selenide may form a part of the active centre of a non-haem iron-containing protein 'X', that may function in microsomal electron transport. 3. Measurements of the contents of cytochromes *P*-450 and *b*₅ in liver microsomal fractions of rats given vitamin E-deficient, and vitamin E- and selenium-deficient diets, showed that haemoprotein biosynthesis is unimpaired in these rats and phenobarbitone treatment resulted in the expected increase in the haemoproteins. 4. When the reduction of cytochrome *P*-450 by NADH and NADPH was measured, no difference was found between normal and deficient animals. 5. These results are discussed in relation to current knowledge of microsomal electron transfer.

When rats were given a small amount (about 20–40 μg) of $\text{Na}_2^{75}\text{SeO}_3$, a significant proportion of the ^{75}Se isolated from liver organelles was converted into an acid-labile form (Diplock *et al.* 1968, 1971*a*) which has been identified as selenide ($^{75}\text{Se}^{2-}$) (Diplock *et al.*, 1973). The selenide was protein-bound and particularly associated with the mitochondria and smooth endoplasmic reticulum, and its presence in significant amounts in these organelles was dependent on the presence of vitamin E in the diet of the rats (Caygill *et al.*, 1971). These and other observations have been incorporated in a hypothesis (Diplock & Lucy, 1973) for the mode of action of vitamin E and selenium. This proposes that a primary function of vitamin E *in vivo* may be to inhibit the oxidation of selenide-containing proteins, and that the nutritional inter-relationships between vitamin E and dietary polyunsaturated fatty acids may be due to the occurrence of specific complexes within normal intracellular membranes between the vitamins and some of the molecules of polyunsaturated phospholipids. It is further proposed that the selenide may form a part of the active centre of an uncharacterized class of non-haem iron proteins that may have functional activity in mitochondria and in the smooth endoplasmic reticulum. The existence of a non-haem iron protein in the smooth endoplasmic reticulum

is subject to doubt (Miyake *et al.*, 1967), but recent work (Caygill & Diplock, 1973) indicates the presence of oxidant-labile non-haem iron in rat liver microsomal fractions prepared in solutions containing antioxidants, and that this non-haem iron is dependent on the presence of selenium and vitamin E in the diet given to the animals.

In the present work the incorporation of ^{75}Se into the liver smooth endoplasmic reticulum and its conversion into selenide were investigated in vitamin E-deficient and -supplemented rats given phenobarbitone. In addition, the electron-transfer function of the microsomal fractions was examined. Preliminary publication of these results has already been made (Diplock *et al.*, 1971*b*; Jeffery & Diplock, 1972).

Experimental and Results

Methods

Diets. The vitamin E-deficient diet (Diplock *et al.*, 1971*a*), which contains a minimal amount of selenium required to prevent liver necrosis, was used. The vitamin E-supplemented diet was made by adding 100 mg of DL- α -tocopheryl acetate/kg to the deficient diet. The *Torula* yeast diet contained negligible amounts of vitamin E and selenium; where indicated,

it was supplemented with 100mg of DL- α -tocopheryl acetate/kg and 0.1p.p.m. of selenium as sodium selenite.

Animals. Caesarian-derived male Wistar rats (40–50g) were obtained from a commercial breeder. Their subsequent treatment is described in the individual experiments.

Zonal centrifugation. Homogenization of samples from pairs of rat livers was as described previously (Caygill *et al.*, 1971). The homogenate was centrifuged for 10min at 2000g and the supernatant fraction applied to the MSE BXIV titanium rotor on the MSE Superspeed 50 centrifuge as described before (Caygill *et al.*, 1971). The mitochondrial and lysosomal fractions (run 1) were discarded, and, after the usual 'washing' procedure (run 2), the 16–18h centrifugation (run 3) was carried out at 30000rev./min in a further continuous sucrose gradient (20–60%, w/w), to separate the microsomal fractions. At the end of the centrifugation 60×10ml fractions were displaced from the rotor by using 600ml of 60% (w/w) sucrose. Tubes 1–19, which contained the soluble proteins, were discarded. The smooth endoplasmic reticulum (peak tubes 34–36) was separated from the rough endoplasmic reticulum (peak tubes 54–56) by this procedure [see Caygill *et al.* (1971) for a discussion of the validity of the separation].

Preparation of microsomal fractions. A 25% (w/v) liver homogenate was made in 0.25M-sucrose by using four passes of the nylon-glass homogenizer described previously (Caygill *et al.*, 1971). The homogenate was centrifuged in an 8×50ml angle rotor at 10000g for 10min to remove mitochondria, nuclei, debris etc. The supernatant fraction was decanted, and 10ml portions were layered on a discontinuous gradient consisting of an upper layer of 10ml of 0.3M-sucrose containing 0.23M-NaCl to remove haemoglobin, and a lower layer of 4ml of 0.4M-sucrose in a 50ml centrifuge tube (Peterman & Pavlovic, 1963). The tubes were centrifuged in an 8×50ml angle rotor at 130000g for 60min and the pellets were resuspended in 0.25M-sucrose. The suspensions were combined and their protein contents measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard. The microsomal suspensions were then diluted with 0.25M-sucrose to give a final protein concentration of 2mg/ml, and used within 6h of preparation, during which time they were stored at 4°C.

Radioactivity counting (Expts. 1 and 2). This was done by using the Wallac GTL-300 γ -isotope counter on all the fractions 20–60 obtained from the zonal centrifugation. The selenide was then measured as before (Caygill *et al.*, 1971; Diplock *et al.*, 1973).

Biochemical measurements. Cytochrome P-450 and cytochrome b_5 were measured by the methods of

Omura & Sato (1964a,b). Succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase was measured as described previously (Caygill *et al.*, 1971). The reduction of cytochrome P-450 by NADH and NADPH was studied as follows. The reduced nucleotides were dissolved immediately before use in 0.5M-Tris-HCl buffer, pH 7.4, to give a concentration of 10mg/ml. Microsomal suspension (2.5ml) was placed in each of two matched cuvettes and 0.5mg of NADH added to each. The experimental cuvette was gassed with CO for 30s and a difference spectrum from 400 to 500nm plotted in a Unicam SP800 spectrophotometer. NADPH (0.3mg) was then added to both cuvettes, the gassing of the experimental cuvette with CO repeated, and the spectrum plotted again. A few crystals of sodium dithionite were then added to both cuvettes, the experimental cuvette was gassed with CO, and the spectrum plotted again. The spectrum obtained with sodium dithionite was used to determine the total cytochrome P-450 (Omura & Sato, 1964b) and the percentage of this total amount reducible by NADH and NADPH calculated. In preliminary trial experiments it was found that 0.5mg of NADH and 0.3mg of NADPH were slightly in excess of the amounts necessary to effect the reduction of the cytochrome P-450. In some experiments, as a precaution, a small additional amount of NADH and NADPH was added and no further reduction was observed.

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

Presentation of results. A similar technique was adopted to that described previously (Caygill *et al.*, 1971) and the results obtained for the two or three experiments done with each dietary treatment were very similar. The results shown are plotted from the means of the observations obtained in the similar experiments. In addition, the upper trace in Figs. 1 and 2 (----) shows the $^{75}\text{Se}^{2-}$ in each tube as a percentage of the total ^{75}Se in that tube.

Effect of vitamin E deficiency and phenobarbitone on ^{75}Se incorporation

Weanling rats were given the vitamin E-deficient diet for 3 months.

Treatment (a). Pairs of rats were each given five consecutive daily oral doses of 3mg of DL- α -tocopherol in 0.1ml of olive oil, and at the same time they also received an intraperitoneal injection of approx. 50 μCi of $\text{Na}_2^{75}\text{SeO}_3$ (about 8 μg of Se) in 0.1ml of 0.9% NaCl; the precise amount of the ^{75}Se varied with the specific radioactivity of individual batches of $\text{Na}_2^{75}\text{SeO}_3$, purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Immediately after the first injections a solution (1mg/ml) of phenobarbitone was given to the rats in place of their usual drinking

water, and this treatment was continued until the rats were killed on the fourth day. The mean daily water intake of the rats was 10ml.

Treatment (b). Pairs of rats were treated in the same manner as those given treatment (a), except that the α -tocopherol dosage was omitted. Each pair of rats was killed on the sixth day after commencement of the ^{75}Se dosage and portions of the livers were subjected to fractionation in the zonal centrifuge. Pairs of rats given each dietary treatment were studied on two or three separate occasions.

Phenobarbitone given to vitamin E-deficient rats resulted (Fig. 1b) in a tendency to increased selenium incorporation in the smooth endoplasmic reticulum, but the amount of selenide was unaffected. When, however, vitamin E was re-fed at the same time as the phenobarbitone was given (Fig. 1a), there was a very large increase ($P > 0.01$) in the amount of selenium found in the smooth endoplasmic reticulum, with 14% of the total amount in tubes 20–60 found in the peak tube (tube 35). The amount of selenide was also greatly increased ($P > 0.001$) and it is noteworthy that the proportion of the total selenium present as selenide (28%) (upper trace, Fig. 1a), was twice the proportion found when no vitamin E was given (upper trace, Fig. 1b).

Effect of vitamin E and selenium deficiency, and phenobarbitone on ^{75}Se incorporation

Weanling rats were given the vitamin E-deficient diet for 7 days, and the selenium- and vitamin

E-deficient (*Torula* yeast) diet for a further 14–16 days. Treatments (a) and (b) were identical with the corresponding treatments in the previous experiment and pairs of rats were used for each experiment.

Treatment (c). The selenium- and vitamin E-deficient diet was supplemented with vitamin E and selenium, and the rats were thereafter given phenobarbitone in their drinking water (1mg/ml) for the 5 days before death.

Treatment (d). This was similar to treatment (c) except that the phenobarbitone was not given. The pairs of rats were killed on the sixth day after commencement of the ^{75}Se dosage, and portions of their livers were subjected to fractionation as before; the procedure was repeated two or three times for each dietary treatment.

In a previous publication (Caygill *et al.*, 1971) reference was made to the friable nature of livers of rats given the doubly deficient diet. Further preliminary investigation in the present series of experiments revealed that the membrane instability was largely confined to the mitochondrial fractions, and that, provided that the 'washing' procedure (run 2) was included, reproducible results could be obtained with the endoplasmic reticulum (run 3). To determine whether the microsomal fractions were contaminated with mitochondrial fragments, preliminary experiments were also done to investigate the presence of a mitochondrial marker enzyme, succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-tetrazolium reductase, in the microsomal fractions; contamination was only rarely encountered and even when present it only accounted in total for 5% of the

Fig. 1. Distribution of selenium (^{75}Se ; $\cdots$), selenide ($^{75}\text{Se}^{2-}$; —) and selenide expressed as a percentage of the total selenium (---) 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. Rats given both diets were also given phenobarbitone. Details of the diets, dosage with vitamin E, ^{75}Se and phenobarbitone, and the methods of fractionation and measurement of ^{75}Se are given in the Experimental and Results section.

activity of the enzyme in a mitochondrial fraction derived from the same livers.

When phenobarbitone was given to vitamin E- and selenium-deficient rats, little effect was observed (Fig. 2*b*). Re-feeding with vitamin E, however, showed (Fig. 2*a*) a very large rise ($P > 0.01$) in the amount of selenium in the smooth endoplasmic reticulum, and in the proportion ($P > 0.01$) of the selenium that was present as selenide [26% in the peak tube (tube 36)]. A similar result was obtained when phenobarbitone was given to rats given the diet supplemented with selenium and vitamin E (Fig. 2*c*), although here the percentage of selenide in the peak tube (tube 34) was even higher (31%).

Cytochrome contents, and reduction of cytochrome P-450 by NADH and NADPH, in liver microsomal fractions of rats given phenobarbitone

(a) *Normal and vitamin E-deficient rats.* A group (36) of weanling rats was given the vitamin E-deficient diet for 3 months, and another group (36) were given the vitamin E-supplemented diet for 3 months. During the 5 days before their death, half the rats given each diet were given phenobarbitone solution (1mg/ml) to drink. Microsomal fractions were prepared from pairs of rats given each of the four treatments, and the cytochrome P-450 and b_5 contents, and the extent of reduction of cytochrome P-450 by NADH and NADPH, were measured.

Fig. 2. Distribution of selenium (^{75}Se ; $\cdots$), selenide ($^{75}\text{Se}^{2-}$; —), and selenide expressed as a percentage of the total selenium (---) in the microsomal fractions of rat liver

Rats were given the following dietary treatments: (a) vitamin E- and selenium-deficient diet, re-fed with vitamin E; (b) vitamin E- and selenium-deficient diet; (c) and (d), vitamin E- and selenium-deficient diet with added vitamin E. Rats given treatments (a), (b) and (c) were also given phenobarbitone. Details of the diets, dosage with vitamin E, ^{75}Se and phenobarbitone, and the methods of fractionation and measurement of ^{75}Se are given in the Experimental and Results section.

The results of these experiments (Table 1) show that there was no effect of vitamin E deficiency on the contents of cytochromes *P*-450 and *b*₅ in liver microsomal fractions, whether the animals were treated with phenobarbitone or not. The extent of reduction of cytochrome *P*-450 by NADPH was similar in rats given both diets, and was unaffected by phenobarbitone. The extent of reduction of cytochrome *P*-450 by NADH was, however, significantly smaller in rats given the deficient diet, and significantly larger in the same rats given phenobarbitone.

(b) *Normal and vitamin E- and selenium-deficient rats.* Weanling rats were given the vitamin E-deficient diet for 7 days and then divided into six groups and treated as follows. Treatment (a): six rats were given the selenium- and vitamin E-deficient (*Torula* yeast) diet supplemented with selenium and vitamin E, for a further 14–16 days. Treatment (b): ten rats were given the selenium- and vitamin E-deficient diet for a further 14–16 days. Treatment (c): 18 rats were given the selenium- and vitamin E-deficient diet supplemented with vitamin E only for a further 14–16 days. Treatment (d): 12 rats were given treat-

ment (b) and phenobarbitone (1 mg/ml) in their drinking water for the 5 days before death. Treatment (e): 18 rats were given treatment (c) and phenobarbitone in their drinking water for the 5 days before death.

The rats were killed in pairs and microsomal fractions prepared from their livers; the contents of cytochromes *P*-450 and *b*₅, and the extent of the reduction of cytochrome *P*-450 by NADH, followed by NADPH, were measured in the microsomal fractions.

Depletion of rats of selenium as well as vitamin E gave similar results (Table 2) to those obtained with the vitamin E-deficient rats. Here, however, treatment with phenobarbitone resulted in no increase in the proportion of the cytochrome *P*-450 reducible by NADH in the deficient rats.

(c) *Time-course of phenobarbitone induction in normal and vitamin E-deficient rats.* A group (64) of weanling rats was given the vitamin E-supplemented diet for 3 months, and another group (96) was given the vitamin E-deficient diet for the same period. All rats were then given phenobarbitone (1 mg/ml) in their drinking water, and after 1 day

Table 1. *Contents of cytochromes P-450 and b₅, and extent of reduction of cytochrome P-450 by NADH and NADPH, in liver microsomal fractions from normal and vitamin E-deficient rats*

For details see under 'Biochemical measurements'. Each dietary treatment was for nine pairs of rats and values are means $\pm$ s.d. *, Significantly different from †, $P > 0.01$.

Dietary treatment	Cytochrome <i>P</i> -450 (nmol/mg of microsomal protein)	Cytochrome <i>b</i> ₅ (nmol/mg of microsomal protein)	Reduction by NADH (%)	Further reduction by NADPH (%)
Normal	0.75 $\pm$ 0.04	0.43 $\pm$ 0.03	9.0 $\pm$ 0.2*	43.0 $\pm$ 2.0
Normal given phenobarbitone	1.36 $\pm$ 0.01	—	10.5 $\pm$ 4.1*	57.7 $\pm$ 3.2
Vitamin E-deficient	0.71 $\pm$ 0.05	0.41 $\pm$ 0.04	6.3 $\pm$ 0.1†	44.3 $\pm$ 3.5
Vitamin E-deficient given phenobarbitone	1.56 $\pm$ 0.30	—	16.3 $\pm$ 3.9†	62.6 $\pm$ 6.4

Table 2. *Contents of cytochromes P-450 and b₅, and extent of reduction of cytochrome P-450 by NADH and NADPH, in liver microsomal fractions from normal and vitamin E- and selenium-deficient rats*

For details see under 'Biochemical measurements'. Values given are means ($\pm$ s.d.) and the number of determinations made is given in parentheses. *, Significantly different from †, $P > 0.01$.

Dietary treatment	Cytochrome <i>P</i> -450 (nmol/mg of microsomal protein)	Cytochrome <i>b</i> ₅ (nmol/mg of microsomal protein)	Reduction by NADH (%)	Further reduction by NADPH (%)
(a) Normal (3)	0.74 $\pm$ 0.03	0.57 $\pm$ 0.06	10 $\pm$ 0*	39 $\pm$ 1
(b) Vitamin E- and selenium-deficient (5)	0.75 $\pm$ 0.04	0.55 $\pm$ 0.07	17 $\pm$ 3†	38 $\pm$ 3
(c) Selenium-deficient (9)	0.70 $\pm$ 0.04	0.59 $\pm$ 0.09	18 $\pm$ 3†	40 $\pm$ 4
(d) Vitamin E- and selenium-deficient given phenobarbitone (6)	1.88 $\pm$ 0.11	0.69 $\pm$ 0.08	10 $\pm$ 2	42 $\pm$ 6
(e) Selenium-deficient given pheno- barbitone (9)	1.69 $\pm$ 0.09	0.71 $\pm$ 0.06	10 $\pm$ 2	48 $\pm$ 4

Table 3. Contents of cytochromes *P*-450 and *b*₅, and extent of reduction of cytochrome *P*-450 by NADH and NADPH, in liver microsomal fractions from normal and vitamin E-deficient rats during 6 days' treatment with phenobarbitone

Experimental details are given in the text. Values are means $\pm$ S.D.

No. of days of treatment with phenobarbitone (no. of observations in parentheses)	Cytochrome <i>b</i> ₅ (nmol/mg of microsomal protein)	Cytochrome <i>P</i> -450			
		Content (nmol/mg of microsomal protein)	Reduction by dithionite (%)	Reduction by NADH (%)	Further reduction by NADPH (%)
Vitamin E-supplemented rats					
1 (8)	0.57 ± 0.26	0.61 ± 0.07	100	8.7 ± 2.1	34.2 ± 5.5
2	—	—	—	—	—
3 (7)	0.88 ± 0.21	1.52 ± 0.27	100	8.7 ± 3.1	41.3 ± 5.7
4 (8)	0.86 ± 0.13	1.58 ± 0.40	100	9.1 ± 2.2	43.8 ± 9.7
5	—	—	—	—	—
6 (7)	0.72 ± 0.02	1.55 ± 0.32	100	7.6 ± 1.4	40.3 ± 4.3
Vitamin E-deficient rats					
1 (8)	0.45 ± 0.04	0.73 ± 0.04	100	5.8 ± 1.3	39.7 ± 8.6
2 (8)	0.43 ± 0.05	1.09 ± 0.08	100	5.9 ± 2.4	49.0 ± 9.5
3 (7)	0.70 ± 0.02	1.54 ± 0.23	100	7.4 ± 3.4	41.5 ± 7.9
4 (8)	0.71 ± 0.13	1.63 ± 0.39	100	5.2 ± 0.3	43.8 ± 6.6
5 (8)	0.85 ± 0.03	1.50 ± 0.06	100	7.1 ± 1.7	48.3 ± 5.0
6 (7)	0.63 ± 0.03	1.89 ± 0.10	100	5.2 ± 2.8	38.8 ± 10.0

eight pairs of rats given each diet were killed and the contents of cytochromes *P*-450 and *b*₅, and the extent of reduction of the cytochrome *P*-450 by NADH, followed by NADPH, were measured in liver microsomal fractions. Thereafter rats were killed at daily intervals as shown in Table 3, each observation shown being made on a pair of rats.

The results of this experiment (Table 3) show no differences between the vitamin E-deficient and vitamin E-supplemented rats.

Discussion

In a previous study of the incorporation of ⁷⁵Se from doses of Na₂⁷⁵SeO₃ into rat liver subcellular fractions (Caygill *et al.*, 1971) it was found that adequately fed rats showed a specific concentration of selenium and of selenide in the smooth-endoplasmic-reticulum fractions. This was not observed in vitamin E-deficient rats and the normal distribution pattern was restored by re-feeding the vitamin E-deficient rats for a few days with vitamin E. In view of the hypothesis that has been advanced that the selenide may be located in the active centre of a non-haem iron-containing protein that may function in the NADPH-dependent electron-transfer chain of liver smooth endoplasmic reticulum (Caygill *et al.*, 1971; Diplock & Lucy, 1973), it was decided to study the effect of phenobarbitone, which has an inductive effect on the

haemoproteins of the system (Estabrook & Cohen, 1969), on the incorporation of selenium into the smooth endoplasmic reticulum. The results (Figs. 1 and 2) of the two experiments reported here, in which the effect of phenobarbitone on ⁷⁵Se incorporation was studied, show that when vitamin E was present in the diet there was a large amount of ⁷⁵Se and ⁷⁵Se²⁻ incorporated into the smooth endoplasmic reticulum. This observation may be interpreted as indicating that there is a vitamin E-dependent role for selenium in drug metabolism in the liver smooth endoplasmic reticulum and is consistent with our suggestion that selenium may have a functional role in a non-haem iron-containing protein ('X') that may function between the flavoprotein and cytochrome *P*-450 in the NADPH-dependent electron-transfer chain of rat liver microsomal fractions (Estabrook *et al.*, 1971a). In view of the probable close association between such an hypothetical non-haem iron protein and cytochrome *P*-450, it was thought possible that the amount of the cytochrome, as measured by the CO-binding spectrum of the dithionite-reduced haemoprotein, might be altered in vitamin E deficiency, and that the expected rise in the content of the cytochrome on phenobarbitone administration might not occur. Measurements of the content of cytochrome *P*-450 in liver microsomal fractions of vitamin E-deficient and -supplemented rats (Table 1) and in vitamin E-

and selenium-deficient rats (Table 2) showed that the vitamin was without effect; further, the rise in the content of the haemoprotein on phenobarbitone administration was also unaffected (Tables 1 and 2). No effect of vitamin E deficiency was found on the cytochrome b_5 contents (Tables 1 and 2).

At the time that these experiments were being completed, we became aware of the work of Nair and co-workers, who found large differences in the contents of cytochromes b_5 and $P-450$ in liver microsomal fractions of vitamin E-deficient and vitamin E-supplemented rats, which they attributed to a defect in haem biosynthesis (Murty *et al.*, 1970; Nair, 1972a; Caasi *et al.*, 1972; Hauswirth & Nair, 1972). Because of this conflict, a further experiment was done in which the contents of cytochromes b_5 and $P-450$ were measured in vitamin E-deficient and -supplemented rats on each of the 6 consecutive days after the commencement of daily phenobarbitone administration. The results (Table 3) show that there was no difference in the content of the cytochromes caused by vitamin E deficiency. It is therefore concluded that haem biosynthesis in our rats was unimpaired, in contrast with the results obtained by Nair and co-workers, and in agreement with the work of Carpenter (1972). It has been suggested (Nair, 1972b) that the diet used by Nair and co-workers is marginally insufficient in iron, and that it is the combined effect of deficiency of vitamin E and low dietary iron that produced the effects of vitamin E deficiency on haem biosynthesis. However, the diet used by Nair and co-workers (described by Weglicki *et al.*, 1968) contains approximately the same amount of iron as that recommended by the National Academy of Sciences, National Research Council Publication no. 990, and an amount similar to that present in our vitamin E-deficient diet. Further, Becking (1972) measured contents of cytochromes $P-450$ and b_5 in the livers of iron-deficient rats, and found the values unaltered by the deficiency.

When the extent of reduction of cytochrome $P-450$ by both NADPH and NADH was studied it was found (Tables 1 and 2) that neither vitamin E deficiency nor vitamin E and selenium deficiency had any consistent effect on the reduction by NADPH or NADH. However, it must be borne in mind that the rate of reduction of cytochrome $P-450$ was not measured in the experiments reported here.

The stoichiometry of the pigments within the endoplasmic reticulum has been discussed by Estabrook *et al.* (1971b), who concluded that the molar concentrations of flavoprotein, cytochrome b_5 and cytochrome $P-450$ were of the order 1, 10 and 30 respectively. It is noteworthy in this context that the molar concentration of non-haem iron that we have reported in rat liver microsomal fractions

(Caygill & Diplock, 1973) is of the same order as that of the cytochromes b_5 or $P-450$ that we have reported for similar rats in the present paper, although the non-haem iron content was not significantly increased by phenobarbitone treatment. This observation is consistent with the suggestion (Estabrook *et al.*, 1971b) that protein 'X' may function as a mobile carrier of electrons. It should also be noted that our observations on microsomal non-haem iron (Caygill & Diplock, 1973) led us to suggest that, in vitamin E deficiency, an oxidant-labile iron-selenide protein may be replaced by a more stable iron-sulphide protein. If this is so, the iron-sulphide protein would be expected to be fully functional in electron transfer, which would thus be unimpaired in deficiency of vitamin E.

In the special context of the liver endoplasmic reticulum, vitamin E is envisaged, according to the hypothesis of Diplock & Lucy (1973), as promoting membrane stability by physical interactions between the phytyl side chain of tocopherol and polyunsaturated fatty acyl chains of membrane phospholipids. Secondly, the oxidation-reduction function of the phenolic hydroxyl groups of tocopherol is thought to be directed towards the selenide-containing membrane-bound non-haem iron-containing protein. It is possible that the incorporation of selenium into membrane-associated proteins is dependent on a certain organization of membrane phospholipids. The heat-stable factor required for liver microsomal function (Lu *et al.*, 1969) was identified as phosphatidylcholine (Strobel *et al.*, 1970; Lu & West, 1972), although the validity of this interpretation of the observation has been questioned (Cater *et al.*, 1972). The proliferation of smooth-endoplasmic-reticulum membranes with their associated selenide-containing protein consequent on phenobarbitone treatment would thus be expected to require an increased availability of phosphatidylcholine and of α -tocopherol to form the association that we have suggested (Diplock & Lucy, 1973) with polyunsaturated fatty-acyl chains of the phospholipids. In this connexion, it is noteworthy that Marshall & McLean (1971) have demonstrated an absolute requirement for dietary linoleic acid for the induction of rat liver microsomal cytochrome $P-450$, although in the present experiments the additional presence of tocopherol was not required for the induction of the microsomal haemoproteins.

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