The Dependence on Vitamin E and Selenium of Drug Demethylation in Rat Liver Microsomal Fractions

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1. The effects of vitamin E deficiency, and of vitamin E and selenium deficiency, on rat liver microsomal aminopyrine demethylase activity were investigated. It was found that, over a wide range of substrate concentrations, the enzyme activity in preparations from deficient animals was significantly lower than that in controls. 2. Addition of antioxidants *in vitro*, either to the homogenization or to the assay media, was without significant effect on the depressed enzyme activity. Castration and alteration in dietary protein concentration were also without effect. The rate of oxidation of NADPH was, however, lower in preparations from deficient animals. 3. Lineweaver–Burk plots of the reciprocal of enzyme activity and substrate concentration showed a higher K_m value in preparations from vitamin E-deficient animals, irrespective of whether selenium was present; the V_{max} was unaffected. These parameters were unchanged when antioxidants were added *in vitro*. Induction with phenobarbitone and 3-methylcholanthrene showed large changes in K_m value which, for preparations from vitamin E-deficient animals, was higher than that for corresponding controls. 4. Examination of the synergism between NADH and NADPH as donors of reducing equivalents for aminopyrine demethylation showed that vitamin E and selenium were only minimally involved in the phenomenon. However, both the initial rate and the extent of demethylation were significantly lower in vitamin E- and selenium-deficient preparations and both nutrients were required for the restoration of full activity. 5. The significance of these results is discussed in the light of our working hypothesis.

Caygill *et al.* (1973) found that the incorporation of selenium (as ^{75}Se) into liver microsomal fractions and its conversion into selenide ($^{75}\text{Se}^{2-}$) was greater in normal rats when phenobarbitone was also administered. In vitamin E-deficient rats, no effect of phenobarbitone on the ^{75}Se incorporation and its conversion into $^{75}\text{Se}^{2-}$ was observed. Other work (Caygill & Diplock, 1973) indicated the presence of oxidant-labile non-haem iron in rat liver microsomal fractions prepared in solutions containing antioxidants, and that the presence of this non-haem iron depended on the rats being given both vitamin E and selenium in the diet. These and other observations have been incorporated into a working hypothesis (Diplock & Lucy, 1973), which proposes that a primary function of vitamin E *in vivo* may be to inhibit the oxidation of mitochondrial and microsomal selenide-containing non-haem iron proteins and that the nutritional inter-relationships between vitamin E and dietary polyunsaturated fatty acids may be due to the formation of specific complexes within normal membranes between the phytol side chain of tocopherol and some of the molecules of arachidonic acid present naturally in the membrane phospholipids.

Carpenter (1967, 1968, 1972) has shown that drug demethylation is impaired in liver microsomal fractions of rats deprived of vitamin E. The study described in the present paper was undertaken to examine whether the defective drug metabolism might be due to changes in the nature of the liver microsomal system caused by a deficiency of a selenium-containing non-haem iron protein that might function, in the location ascribed to the unknown electron carrier 'X' (Estabrook *et al.*, 1971), in microsomal electron transport.

Experimental and Results

Methods

Diets. The vitamin E-deficient and -supplemented diets and the vitamin E- and selenium-deficient or -supplemented diets were described by Caygill *et al.* (1973).

Animals. Caesarian-derived male Wistar rats (40–50 g) were obtained from a commercial breeder and treated as shown in the individual experiments.

Preparation of microsomal fractions. The method of Caygill *et al.* (1973) was used. After determination of protein in the microsomal suspension by the method of Lowry *et al.* (1951), the suspension was diluted with 0.25M-sucrose to give a final protein concentration of 2mg/ml and used within 2h of preparation, during which time it was stored at 4°C.

Aminopyrine demethylase assay. This was by modification of the method of Gilbert & Golberg (1965) and the formaldehyde produced was measured by the method of Nash (1953). The composition of the assay medium (final volume 4ml) was (mM): $MgCl_2$ (2.5); semicarbazide (0.76); Tris-HCl buffer, pH 7.4 (85.0); $NADP^+$ (0.33); isocitric acid (1.93); isocitrate dehydrogenase (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 0.5 unit; 1 ml of microsomal fraction (2mg of protein/ml) and dimethylaminopyrine (K and K Laboratories, Plainview, Hollywood, Calif., U.S.A.) at the concentrations shown in the individual experiments were also added. The following technique was found to give reproducible results and minimize random variables. The $MgCl_2$, semicarbazide, Tris buffer and substrate were mixed in 50ml flasks and stored at 4°C until required. The $NADP^+$, isocitric acid and isocitrate dehydrogenase were mixed separately immediately before incubation and added to the flasks, which were then preincubated at 37°C for 5 min. The appropriate microsomal fraction was warmed separately at 37°C for 30s and added to the flask to start the reaction. The reaction was stopped at the time indicated in each experiment by the addition of 2 ml of 1.0M-NaOH and the flasks were placed on ice immediately after the contents were mixed. When cold, the proteins were precipitated by the addition of 20% (w/v) $ZnSO_4$ solution. After centrifugation at 2500 rev./min for 5 min, formaldehyde was measured in the supernatant by the method of Nash (1953). The colour produced was measured at 412 nm in 2 cm-light-path cells.

NADH- and NADPH-cytochrome c reductase assay. Microsomal fractions were prepared by the method of Caygill *et al.* (1973) and enzyme activities measured by the method of Ernster *et al.* (1962).

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

Effect of vitamin E and selenium deficiency on liver microsomal aminopyrine demethylase activity

Preliminary experiments. Substrate concentrations in the range 0.5–1.0 μ mol of aminopyrine/incubation mixture were used to show that the rate of demethylation was lower in microsomal preparations from vitamin E-deficient or vitamin E- and selenium-deficient rats.

Optimal substrate concentration. Microsomal fractions were prepared from livers of pairs of rats given

Fig. 1. Aminopyrine demethylase in liver microsomal fraction of vitamin E-deficient and vitamin E-supplemented rats

Rats were given the vitamin E-deficient diet, or that diet supplemented with 100 mg of DL- α -tocopherol acetate/kg, for 5 months, and liver microsomal fraction was prepared as described in the text. Formaldehyde production was measured, at the initial aminopyrine concentrations indicated, during a 5 min incubation period. Each point on the Figure is the mean value $\pm$ s.d. of observations made on three pairs of livers. $\circ$, Vitamin E-deficient rats; $\bullet$, vitamin E-supplemented rats.

either the vitamin E-deficient or -supplemented diets for 5 months. Formaldehyde production was measured for each, with the following final concentrations of aminopyrine; 22, 44, 130, 260, 520 nmol/ml, and an incubation time of 5 min. The results are given in Fig. 1(a); each point represents the mean value $\pm$ s.d. of three determinations, each made with microsomal fraction from a pair of rats. It is clear that the extent of demethylation by the microsomal fraction from vitamin E-deficient rats is substantially lower than that for vitamin E-supplemented rats. In order to find the saturating substrate concentration, a

Fig. 2. Time-course of aminopyrine demethylase activity in liver microsomal fraction of vitamin E-deficient and vitamin E-supplemented rats

Experimental conditions were similar to those used in Fig. 1. Aminopyrine demethylase activity was measured with an initial substrate concentration of $25 \mu\text{mol/ml}$ and formaldehyde was determined in samples 5, 10 and 15 min after the reaction was started. ○, Vitamin E-deficient rats; □, vitamin E-deficient rats; α -tocopherol ($100 \mu\text{g/ml}$) or ethoxyquin ($43 \mu\text{g/ml}$) added to incubation medium; △, vitamin E-deficient rats; α -tocopherol ($100 \mu\text{g/ml}$) added to sucrose solutions used for isolation of microsomal fractions; ●, vitamin E-supplemented rats. Each point represents the mean of duplicate observations made on three pairs of livers. (The s.d. values were omitted for the sake of clarity.)

further experiment was done, with the same number of rats and the same incubation time, with the following final substrate concentrations; 5, 10, 15, 20, 25 and $30 \mu\text{mol/ml}$. The results (Fig. 1b) confirm those in Fig. 1(a) and show that saturation of the enzyme system is reached in microsomal fractions from both vitamin E-deficient and -supplemented rat livers at $25 \mu\text{mol/ml}$.

Effect of antioxidants on aminopyrine demethylation. In view of the fact that α -tocopherol is a powerful antioxidant capable of preventing peroxidation *in vitro* of membrane polyunsaturated phospholipids, the possibility existed that the lowered demethylation in vitamin E-deficient rat liver microsomal fraction might be due to peroxidation *in vitro*. Microsomal fractions were therefore prepared from portions of pairs of livers derived from 5-month-old vitamin E-deficient and vitamin E-supplemented rats. In addition, DL- α -tocopherol ($100 \mu\text{g/ml}$) or ethoxyquin (6-ethoxy-1,2-dihydro-2,2,3-trimethylquinoline; $43 \mu\text{g/ml}$) were suspended by sonication (Diplock

et al., 1971) in the 0.25 M -sucrose used to homogenize other portions of the livers of the vitamin E-deficient rats. Aminopyrine demethylase activity was studied by using a final substrate concentration of $25 \mu\text{mol/ml}$, and samples were taken 5, 10 and 15 min after the reaction was started and formaldehyde was determined. Other samples of microsomal fraction from vitamin E-deficient rat livers were studied in incubation media to which $100 \mu\text{g}$ of DL- α -tocopherol/ml or $43 \mu\text{g}$ of ethoxyquin/ml had been added. The results are given in Fig. 2. In a separate experiment the effect of adding ethoxyquin at the homogenization or assay stages was investigated by using microsomal fraction derived from liver of normal rats. The antioxidant was without effect on the aminopyrine demethylase activity of the microsomal preparation when it was added to the homogenization medium. When it was added to the assay a small inhibitory effect was observed but this was not of the same magnitude as that observed in liver microsomal fraction of vitamin E-deficient rats. In microsomal fractions from vitamin E-supplemented rats the production of formaldehyde was approximately linear between 5 and 15 min; in the vitamin E-deficient microsomal fraction, the extent of the demethylation was significantly different ($P < 0.01$) from the supplemented control at the 5 min point, and no further formaldehyde was produced up to 15 min. Addition of ethoxyquin to the homogenization or assay medium or of α -tocopherol to the assay medium used for the vitamin E-deficient microsomal fraction was without effect. Addition of α -tocopherol to the homogenization medium used for the vitamin E-deficient liver caused a small increase in the extent of formaldehyde production which was significantly different from the result for the microsomal fraction from vitamin E-deficient rats after 10 min ($P < 0.01$) and 15 min ($P < 0.001$).

Effect of castration. The criterion of vitamin E-deficiency that we have used in our rats has been the appearance of testicular degeneration. Orrenius *et al.* (1969) reported a lowered activity of microsomal demethylase activity in castrated rats. Vitamin E-supplemented rats were therefore castrated and allowed to recover for 6 weeks. They were then killed and liver microsomal fractions prepared in triplicate, and the aminopyrine demethylase activity, by the use of a final concentration of $25 \mu\text{mol/ml}$ and a 5 min incubation period, was compared with the microsomal demethylase activity of similar vitamin E-supplemented and -deficient rats. The activity of the microsomal fraction from the castrated rats was indistinguishable from the vitamin E-supplemented rats, both of which showed the usual difference from the vitamin E-deficient rats.

Dietary protein. Maclean & Maclean (1966) showed that liver microsomal demethylase activity

was lowered in protein-deficient rats. In view of the low protein content of our diets (8.3% of casein) an experiment was done in which the dietary protein content was raised to 13%. No difference was noted that could be attributed to the change in dietary protein content.

Functional integrity of electron transfer. It has been shown (Caygill *et al.*, 1973) that the amount of liver microsomal cytochrome *P*-450 and the full extent of the reduction by NADPH were unaltered in vitamin E deficiency. The possibility could not be excluded, however, that the rate of reduction of cytochrome *P*-450 was impaired in microsomal fractions from vitamin E-deficient animals. The activities of the NADPH- and NADH-cytochrome *c* reductase of the liver microsomal fraction were examined in triplicate pairs of 5-month-old vitamin E-deficient and -supplemented rats. No differences between the dietary groups were noted. The activity of NADH-cytochrome *c* reductase was between 0.55 ± 0.05 and 0.60 ± 0.04 unit and the activity of NADPH-cytochrome *c* reductase was between 0.052 ± 0.005 and 0.053 ± 0.007 unit. (One unit of enzyme activity is the amount of enzyme that effects the reduction of $1 \mu\text{mol}$ of cytochrome *c* in 1 min.)

The rate of oxidation of NADPH during aminopyrine demethylation was next measured. The aminopyrine demethylase assay mixture was warmed to 37°C and placed in 3 cm cuvettes in the Unicam SP.800 spectrophotometer with water at 37°C passing through the cuvette holder. Microsomal fraction, from either vitamin E-deficient or -supplemented rats, was then added to both cuvettes and pre-warmed aminopyrine solution, to give a final concentration of $25 \mu\text{mol/ml}$, was added to one cuvette only. E_{366} was measured over a 20 min period, and the decrease in absorption was taken as a measure of the oxidation of NADPH. Several determinations were made by using vitamin E-deficient and -supplemented rat liver microsomal fraction. The results of a typical experiment are given in Fig. 3, which shows a slower rate of oxidation of NADPH in the liver microsomal fraction from the vitamin E-deficient rats.

Kinetics of aminopyrine demethylation in vitamin E deficiency

Although aminopyrine demethylase is a heterogeneous multicomponent system, it is possible to obtain apparent reaction kinetics by using reversed reciprocal plots of substrate concentration and product (formaldehyde) formed. This was done by using microsomal fraction derived from rats given diets deficient in, or supplemented with, vitamin E and selenium. Twelve weanling male rats were given the vitamin E-supplemented diet for 4 months, and

Fig. 3. Rates of NADPH oxidation during aminopyrine demethylation by liver microsomal fraction of vitamin E-deficient and -supplemented rats

Experimental conditions were similar to those used in Fig. 1. The aminopyrine demethylase assay system was pre-warmed at 37°C in two jacketed 3 cm-light-path cuvettes in the spectrophotometer and microsomal fraction was added to each. Aminopyrine solution ($25 \mu\text{mol/ml}$) was added to one cuvette and the absorbance difference between the two cuvettes was measured at 366 nm during a 20 min period. Several determinations were made by using liver microsomal fraction from vitamin E-deficient (○) and -supplemented (●) rats, and the result shown is representative of these results.

60 similar rats were given the vitamin E-deficient diet for the same period of time. Twelve other rats were given the supplemented diet for 1 week and then the *Torula* yeast diet supplemented with vitamin E and selenium for a further 18 days; 12 similar rats were given the vitamin E-deficient diet for 1 week and the vitamin E- and selenium-deficient diet (*Torula* yeast) for a further 18 days. Microsomal fraction was prepared from the livers of all the rats. The livers of 12 of the vitamin E-deficient rats were homogenized in pairs in 0.25 M-sucrose solution that contained $100 \mu\text{g}$ of DL- α -tocopherol/ml suspended by sonication, and a similar amount of α -tocopherol was added to all the solutions used subsequently in the preparation of these microsomal fractions. The livers of 12 other vitamin E-deficient rats were treated similarly in media that contained $43 \mu\text{g}$ of ethoxyquin/

Table 1. *Demethylation of aminopyrine by liver microsomal fractions from normal, vitamin E-deficient and vitamin E- and selenium-deficient rats*

Aminopyrine demethylase activity was studied by the method of Gilbert & Goldberg (1965) in liver microsomal fractions obtained by the method of Caygill *et al.* (1973) from rats given appropriate diets as indicated. Final substrate concentrations of aminopyrine were 360 nmol/ml–20 μ mol/ml. The apparent K_m and V_{max} values of the system were obtained from reversed reciprocal plots of the values obtained and of the substrate concentrations. Each point represents the mean of six observations on microsomal fractions from a pooled sample of the liver from a pair of rats. Values given in the Table are means $\pm$ S.D.

	$10^4 \times K_m$ (M)	V_{max} (μ mol of formaldehyde/h per g of liver)
Vitamin E-supplemented	2.56 $\pm$ 0.03	4.9 $\pm$ 0.6
Vitamin E-deficient	8.00 $\pm$ 0.16	4.4 $\pm$ 0.7
Vitamin E-deficient, but α -tocopherol (100 μ g/ml) added at homogenization of liver	8.32 $\pm$ 0.21	4.5 $\pm$ 0.3
Vitamin E-deficient, but ethoxyquin (43 μ g/ml) added at homogenization of liver	8.17 $\pm$ 0.19	4.6 $\pm$ 0.1
Vitamin E-deficient but α -tocopherol (100 μ g/ml) added to incubation	8.08 $\pm$ 0.07	4.4 $\pm$ 0.6
Vitamin E-deficient but ethoxyquin (43 μ g/ml) added to incubation	8.11 $\pm$ 0.10	4.2 $\pm$ 0.3
Vitamin E- and selenium-supplemented	2.49 $\pm$ 0.05	4.7 $\pm$ 0.4
Vitamin E- and selenium-deficient	8.72 $\pm$ 0.13	4.7 $\pm$ 0.1

ml, i.e. an amount equimolar with 100 μ g of α -tocopherol/ml. Aminopyrine demethylase activity was measured during a 5 min period in all the microsomal fractions and a range of values of final substrate concentration from 360 nmol/ml to 20 μ mol/ml was chosen; the assay media contained 100 μ g of α -tocopherol/ml or 43 μ g of ethoxyquin/ml as appropriate. In addition one further portion of microsomal fraction, prepared without added antioxidants from vitamin E-deficient rats, was studied in assay media to which 100 μ g of DL- α -tocopherol/ml had been added, and a similar portion was studied in assay media containing 43 μ g of ethoxyquin/ml. The 5 min incubation period was chosen because it was found (Fig. 2) that there was only a very small difference in the formaldehyde production, at this time, by microsomal fraction from normal and vitamin E-deficient rats. Since only a few experiments could be done on any one day, the times of placing the rats on the respective diets were staggered, so that when they were killed they had all been receiving the diets for the appropriate time. Plots were made of the reciprocal of the substrate concentration against the reciprocal of the formaldehyde formed and apparent K_m and V_{max} values obtained. The results (Table 1) show that the apparent V_{max} value was unaffected by any of the treatments. However, the apparent K_m of the enzyme system was significantly higher in liver microsomal fraction from vitamin E-deficient rats than in corresponding adequately fed rats, and further depletion of selenium did not affect the 'deficient' value. Addition of the antioxidants either at the homogenization stage or to the assay medium was without effect on the apparent K_m of the liver microsomal fraction of vitamin E-deficient rats.

Effect of inducers of microsomal demethylases

Because we have shown (Caygill *et al.*, 1973) that phenobarbitone greatly increased the uptake of ^{75}Se and its conversion into the $^{75}\text{Se}^{2-}$ in liver microsomal fractions of vitamin E-supplemented rats, it was decided to study the effect of inducers on the vitamin E-induced alteration of aminopyrine demethylase activity. The demethylation of aminopyrine probably proceeds in two stages; the drug first binds to microsomal fraction to give a Type I binding spectrum (Remmer *et al.*, 1966; Imai & Sato, 1966) and formaldehyde is produced. The monomethylaminopyrine produced then undergoes a further demethylation reaction which produced formaldehyde and 4-aminoantipyrine. Pederson & Aust (1970) showed that reversed reciprocal plots of liver microsomal aminopyrine demethylase activity were non-linear at high substrate concentration. However, they found that pre-treatment of rats with phenobarbitone not only stimulated the demethylase activity but also produced a linear reciprocal plot with an apparent K_m of 7×10^{-4} M. Pre-treatment with 3-methylcholanthrene caused no stimulation but increased the apparent K_m by an order of magnitude. Pederson & Aust (1970) concluded that their results suggested the existence of multiple drug-metabolizing sites in liver microsomal fractions. In view of the similarity to 3-methylcholanthrene treatment of the increase in apparent K_m that we had observed in microsomal fractions from vitamin E-deficient rats, an experiment was undertaken in which vitamin E-deficient and -supplemented rats were pre-treated either with phenobarbitone or with 3-methylcholanthrene before death. The aminopyrine demethylase activity of liver

Fig. 4. Effect of phenobarbitone on kinetics of aminopyrine demethylation in liver microsomal fraction of vitamin E-supplemented and -deficient rats

Eight weanling male rats were given the vitamin E-supplemented diet for 4 months, and eight similar rats were given the vitamin E-deficient diet. Four rats in each group were given phenobarbitone solution (1 mg/ml) to drink *ad lib.* for 5 days before they were killed, and the remaining rats received water. Rats were killed on the sixth day and the aminopyrine demethylase activity in microsomal fraction from pieces of livers was studied by using the same substrate concentrations and assay conditions as those shown in Fig. 2. Each point represents the mean $\pm$ S.D. of eight observations. (a) $\blacktriangledown$, Vitamin E-deficient rats (K_m , $2.13 \times 10^{-3} \pm 0.29 \times 10^{-3}$ M; V_{max} , 9.82 ± 2.02); $\bullet$, vitamin E-deficient rats given phenobarbitone (K_m , $5.51 \times 10^{-4} \pm 0.56 \times 10^{-4}$ M; V_{max} , 36.66 ± 3.84). (b) $\triangle$, vitamin E-supplemented rats (K_m , $7.97 \times 10^{-4} \pm 2.05 \times 10^{-4}$ M; V_{max} , 11.52 ± 3.52); $\circ$, vitamin E-supplemented rats given phenobarbitone (K_m , $3.87 \times 10^{-4} \pm 0.009 \times 10^{-4}$ M; V_{max} , 34.99 ± 3.33).

microsomal fractions derived from them was then studied.

Sixteen weanling male rats were given the vitamin E-supplemented diet for 4 months, and 16 similar rats were given the vitamin E-deficient diet. Half the rats in each group were given phenobarbitone solution (1 mg/ml in water) to drink *ad lib.* for the 5 consecutive days before they were killed, and the remaining rats received water. Rats were killed on the sixth day and the aminopyrine demethylase activity in microsomal

Fig. 5. Effect of 3-methylcholanthrene on kinetics of aminopyrine demethylation in liver microsomal fraction of vitamin E-supplemented and -deficient rats

Experimental details are similar to those given in Fig. 4. In place of phenobarbitone, four rats in each dietary group were given 10 mg of 3-methylcholanthrene/kg per day in 0.25 ml of olive oil by intraperitoneal injection on each of 3 consecutive days before they were killed. Each point represents the mean $\pm$ S.D. of eight observations. (a) $\blacktriangledown$, Vitamin E-deficient rats (K_m , $1.97 \times 10^{-3} \pm 0.45 \times 10^{-3}$ M; V_{max} , 9.21 ± 1.65); $\bullet$, vitamin E-deficient rats given 3-methylcholanthrene (K_m , $7.61 \times 10^{-4} \pm 0.77 \times 10^{-4}$ M; V_{max} , 13.65 ± 0.45). (b) $\triangle$, Vitamin E-supplemented rats (K_m , $7.21 \times 10^{-4} \pm 0.45 \times 10^{-4}$ M; V_{max} , 12.68 ± 2.52); $\circ$, vitamin E-supplemented rats given 3-methylcholanthrene (K_m , $5.27 \times 10^{-4} \pm 0.61 \times 10^{-4}$ M; V_{max} , 15.02 ± 2.15).

fraction prepared from pairs of livers was studied by using the same substrate concentrations and assay conditions as those used in the preceding experiment. The results are given in Fig. 4. The experiment was then repeated with 3-methylcholanthrene as the inducing agent. All rats were given water to drink, and, on the 3 consecutive days before they were killed, half the rats received 10 mg of 3-methylcholanthrene/kg per day, which was dissolved in 0.25 ml of olive oil and administered by intraperitoneal injection. The results of the aminopyrine demethylase measurements are given in Fig. 5. In confirmation of the previous experiment, the apparent V_{max} was unaffected by the

deficiency and the apparent K_m was altered substantially. Treatment with phenobarbitone caused a large increase in the apparent V_{max} , and vitamin E had no effect on this change; 3-methylcholanthrene had no effect on the apparent V_{max} . The apparent K_m values were significantly lower after treatment with both inducing agents; however, the value obtained from the vitamin E-deficient rats was in both cases significantly higher ($P < 0.001$) than that of the corresponding vitamin E-supplemented controls.

Synergistic effects of NADH and NADPH in drug metabolism

Cohen & Estabrook (1971*a,b*) and Hildebrandt & Estabrook (1971) have demonstrated a complex interaction between the NADH-dependent and NADPH-dependent electron-transfer pathways in liver microsomal fraction during drug demethylation. In view of the possibility that the hypothetical selenide non-haem iron-containing protein 'X' may function as an accessory electron carrier in this system, we studied the synergistic effects of NADH and NADPH as donors of reducing equivalents in liver microsomal demethylation in rats deprived of vitamin E, selenium, or both vitamin E and selenium, and in normal rats. Six weanling male rats were fed on the vitamin E-supplemented diet for 5 months, and six similar rats were given the vitamin E-deficient diet for the same period. Thirty-two similar weanling male rats were given the vitamin E-deficient diet for 1 week and then divided into four groups. Rats in group (a) were given the *Torula* yeast (vitamin E- and selenium-deficient) diet for 15 days; rats in group (b) were given the *Torula* yeast diet supplemented with DL- α -tocopherol acetate (100mg/kg) and selenium (0.1 p.p.m. as sodium selenite) for 15 days; rats in group (c) were given the diet supplemented with DL- α -tocopherol acetate alone for 15 days; and rats in group (d) were given the diet supplemented with selenium only for the same period. The time of commencement of the dietary treatments was staggered to enable the experiments to be done at the right time-intervals. Aminopyrine demethylase activity was measured by the technique described above in microsomal fractions derived from pairs of livers of rats given each dietary treatment; the final substrate concentration was 8mM-aminopyrine, and the NADPH-generating system was replaced by either (final concns.) 0.111 mM-NADH, 0.090 mM-NADPH, or a mixture of 0.111 mM-NADH+0.090 mM-NADPH (see Cohen & Estabrook, 1971*a*). The results are given in Figs. 6 and 7. Two parameters were selected from the results for statistical evaluation; (a) the 'initial rate' of demethylation, being the amount of formaldehyde formed in the first minute, and (b) the 'extent' of demethylation, being the amount of

Fig. 6. Synergistic effects of NADPH and NADH in aminopyrine demethylation in liver microsomal fraction in vitamin E-supplemented and -deficient rats

Six weanling male rats were fed on the vitamin E-supplemented diet, and six similar rats were fed on the vitamin E-deficient diet, for 5 months. Aminopyrine demethylase activity was measured as previously shown; the initial substrate concentration was 8mM-aminopyrine and the NADPH-generating system was replaced by either 0.111mM-NADH, 0.090mM-NADPH or a mixture of these in the molar amounts indicated. Each point is the mean value $\pm$ S.D. of two observations made on liver microsomal fraction derived from each of three pairs of rats. (a) vitamin E-deficient rats; (b) vitamin E-supplemented rats.

formaldehyde formed after 15 min. The results of the statistical analysis are shown in Table 2. In addition, the extent of the synergism of NADH and NADPH was calculated as a percentage of the arithmetic sum of the results for the individual reduced nicotinamide nucleotides, and these results are given in Table 3.

Both the initial rate and the extent of demethylation were significantly lower (Table 2) in microsomal fraction derived from vitamin E-deficient or vitamin E- and selenium-deficient rats, when compared with the corresponding controls. Supplementation of the

Fig. 7. Synergistic effects of NADPH and NADH in aminopyrine demethylation in liver microsomal fraction of vitamin E- and selenium-deficient and -supplemented rats

Thirty-two similar weanling male rats were given the vitamin E-deficient diet for 1 week and then divided into four groups. Rats in group (a) were given the vitamin E- and selenium- deficient diet for 15 days; rats in group (b) were given the diet supplemented with DL- α -tocopherol acetate (100 mg/kg) and selenium (0.1 p.p.m.); rats in group (c) were given the diet supplemented with DL- α -tocopherol acetate (100 mg/kg); and rats in group (d) were given the diet supplemented with selenium (0.1 p.p.m.). The experimental procedure was the same as that shown in Fig. 6. Each point is the mean value $\pm$ S.D. of six observations made on liver microsomal fraction derived from each of four pairs of rats.

doubly deficient diet with vitamin E or selenium alone showed no improvement in the initial rate of demethylation; the extent of demethylation was somewhat increased by vitamin E alone, but not by selenium. Further, both the initial rate and the extent of demethylation were significantly lower when either

vitamin E or selenium were given alone than when both nutrients were supplied together, indicating a clear functional interaction between the two nutrients. The synergistic additive effect of NADH and NADPH was seen in all cases (Table 3) irrespective of dietary treatment, and the magnitude of this synergism was

Table 2. *Statistical significance of differences in initial rates and extent of aminopyrine demethylation*

The values were those used to plot the curves shown in Figs. 6 and 7. Not significant indicates $P > 0.05$

Dietary groups compared	Initial rate of demethylation		Extent of demethylation after 15 min	
	NADPH	NADPH+NADH	NADPH	NADPH+NADH
Vitamin E-deficient versus vitamin E-supplemented	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Vitamin E- and Se-deficient versus vitamin E- and Se-supplemented	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Vitamin E- and Se-deficient versus vitamin E-supplemented	Not significant	Not significant	$0.05 > P > 0.02$	$0.05 > P > 0.02$
Vitamin E- and Se-deficient versus Se-supplemented	Not significant	Not significant	Not significant	Not significant
Vitamin E and Se-supplemented versus vitamin E-supplemented	$0.01 > P > 0.001$	$0.01 > P > 0.001$	$0.02 > P > 0.01$	$P < 0.001$
Vitamin E and Se-supplemented versus Se-supplemented	$0.01 > P > 0.001$	$P < 0.001$	$0.01 > P > 0.001$	$P < 0.001$

Table 3. *Extent of synergism of NADH+NADPH in aminopyrine demethylation*

The values were those used to plot the curves shown in Figs. 6 and 7. Value marked * is significantly different from those marked †; $P < 0.01$.

Dietary Group	Extent of demethylation (μmol of formaldehyde/15 min)			Synergism as a percentage of the arithmetic sum of the separate activities
	With NADH	With NADPH	With NADH+NADPH (synergism)	
Vitamin E-deficient	0.011	0.094	0.174	166 ± 4
Vitamin E-supplemented	0.010	0.129	0.239	172 ± 2
(a) Vitamin E- and Se-deficient	0.013	0.061	0.110	$147 \pm 3†$
(b) Vitamin E- and Se-deficient+Vitamin E and Se	0.015	0.117	0.214	$162 \pm 6*$
(c) Vitamin E- and Se-deficient+Vitamin E	0.017	0.102	0.172	$144 \pm 5†$
(d) Vitamin E- and Se-deficient+Se	0.017	0.094	0.160	$144 \pm 2†$

unaffected by vitamin E deficiency. When the magnitude of the synergism was calculated for the liver microsomal fraction of vitamin E- and selenium-deficient rats it was found to be significantly lower (Table 3) than for the corresponding control values, and dietary supplementation with vitamin E or selenium individually had no effect.

Effect of cyanide. Levander *et al.* (1973a,b) showed that mitochondrial swelling *in vitro* induced by the addition of GSH and selenite was inhibited by 1 mM-cyanide. This phenomenon is related (Levander *et al.*, 1973b) to the ability of selenite to catalyse the transfer of electrons from GSH to cytochrome *c*, and may be due to the involvement of a reactive intermediate GSSe^- in the reduction, the activity of which is quenched by cyanide. This is analogous to the situation that we consider may be involved in microsomal electron transport, where a selenide-containing protein is thought to act as a donor of electrons to cytochrome P-450. Oxidative desaturation of fatty acyl-CoA by rat liver microsomal

fraction requires cytochrome *b₅* and NADH; NADPH can also function in this system as a donor of reducing equivalents, and the system is highly sensitive to cyanide. We therefore studied the effect of cyanide on the aminopyrine demethylase system in microsomal fraction of livers derived from four pairs of vitamin E-supplemented and four pairs of vitamin E-deficient rats. The experimental procedure was similar to that used in the preceding experiment, except that parallel incubations of the microsomal fractions were carried out in which cyanide (final concentration 1 mM) was added. It was found that cyanide had an inhibitory effect on aminopyrine demethylation with either NADPH or NADPH+NADH as donors of reducing equivalents. The extent of the inhibition was, however, not affected by the dietary treatments.

Discussion

The results reported here establish the existence of impaired microsomal demethylation in the livers of

rats given vitamin E-deficient diets. The original observations of this phenomenon were reported by Carpenter (1967, 1968), and these were put in question subsequently by Quaife & Friedman (1970), who were unable to demonstrate lowered codeine *O*-demethylase activity in vitamin E-deficient rat liver. However, Carpenter (1972) showed that, since codeine may be demethylated by both *O*- and *N*-demethylations and that *N*-demethylation predominates in male rat liver, it was probable that only *N*-demethylation is sensitive to the absence of α -tocopherol. We therefore chose to study in depth the *N*-demethylation of aminopyrine, which can only undergo two successive *N*-demethylations, although we have also studied the *N*-demethylation of codeine and confirmed Carpenter's (1972) observations (Diplock, 1974).

The results of our preliminary experiments confirmed the fact that the *N*-demethylase activity of liver microsomal fraction derived from vitamin E-deficient rats was substantially lower than that of vitamin E-supplemented controls over a wide range of substrate concentrations. Investigation of the possibility that the phenomenon was caused merely by peroxidation *in vitro* of the unsaturated moieties of microsomal membrane phospholipids revealed that the addition of α -tocopherol or ethoxyquin to the microsomal fraction, either during their preparation or during the assay, was without any significant effect. When the apparent kinetics of the system were measured, it was found that the apparent $V_{\max}$ was unchanged by vitamin E deficiency, or by vitamin E and selenium deficiency. However, there was a sharp increase in the apparent K_m of the system in vitamin E deficiency, and a similar value was found in rats deprived of both vitamin E and selenium. Here, also, the addition of antioxidants *in vitro* did not alter the high K_m value of the microsomal demethylase from deficient rats. It would seem legitimate to conclude therefore that non-enzymic lipid peroxidation can be dismissed as a possible cause of the phenomenon.

The possibility that an enzymic peroxidative mechanism may be involved cannot so readily be excluded. O'Brien has shown (Hrycay & O'Brien, 1971*a,b*) that the microsomal system can have peroxidase activity which is accompanied by a decrease in the associated drug demethylase activity. If lipid peroxide were formed *in vivo* in the absence of vitamin E by the enzymic mechanism proposed by McCay *et al.* (1971, 1972) then the presence of hydroperoxide in association with the microsomal membrane might be expected to give rise to a decrease in the demethylase activity of the microsomal fraction in our experiments.

The results of the experiments with phenobarbitone (Fig. 4) and 3-methylcholanthrene (Fig. 5) were surprising in that although the change in the apparent kinetics was obtained with phenobarbitone, we did not find the increase in apparent K_m in microsomal

fraction from rats given 3-methylcholanthrene that would have confirmed the results of Pederson & Aust (1970). On the contrary, although the apparent $V_{\max}$ was substantially unchanged by 3-methylcholanthrene treatment, the apparent K_m showed a decrease. Thus these experiments failed to throw any further light on the increased apparent K_m of liver microsomal aminopyrine demethylase activity. However, the significant observation to be made from these results (Figs. 4 and 5) is that, in all circumstances where the rats were pre-treated with either phenobarbitone or 3-methylcholanthrene or not pre-treated at all, there was a sharp difference in the K_m value between the vitamin E-deficient animals and their controls. If the phenomenon were caused by lipid peroxidation *in vivo*, either enzymic or non-enzymic, then it would be expected that there would be a gradual progressive of lipid peroxidation in membrane unsaturated phospholipids. If this were the case then a range of values for the apparent K_m of the system would be expected as more membrane-associated enzyme became subjected to attack by damaging free-radical species. A more probable explanation of our results is that the change in apparent K_m in vitamin E deficiency is caused by an alteration in the nature of one of the components of the system being investigated. This criterion would be expected to be satisfied by the substitution of sulphur for selenium in a non-haem iron protein in vitamin E deficiency, such as we have suggested (Caygill & Diplock, 1973), and which might lead to a change in the electron-transfer capabilities of the protein, which would be manifested as an impairment of the efficiency of drug demethylation.

Cohen & Estabrook (1971*a*) showed that the addition of NADH to a microsomal system catalysing the NADPH-dependent demethylation of aminopyrine resulted, not in an additive effect, but in a synergism where the resultant increase in demethylation was substantially greater than the sum of the demethylation resulting from the addition of either reduced nicotinamide nucleotide individually. Hildebrandt & Estabrook (1971) advanced a scheme to explain their earlier observation which fulfils two requirements: (1) the participation of cytochrome P-450 as a one-electron transfer haemoprotein in a two-electron process, and (2) a role for NADH and cytochrome b_5 in the associated oxidation-reduction process. In view of the close involvement of the unidentified component 'X', which we have suggested may be a selenide- and non-haem iron-containing protein (Caygill *et al.*, 1973), in the scheme of Hildebrandt & Estabrook (1971), we investigated the synergistic effect of NADH and NADPH, as donors or reducing equivalents for microsomal aminopyrine demethylation, in livers of vitamin E-deficient and vitamin E- and selenium-deficient rats, as well as in rats given vitamin E or selenium only.

The results of these experiments (Figs. 6 and 7; Tables 2 and 3) showed that removal of vitamin E alone from the diet did not affect the synergism. Removal of vitamin E and selenium together resulted in a small but statistically significant lowering in the extent of the synergism which was not reversed by the addition to the diet of either nutrient singly. It is difficult to assess whether this change is meaningful in physiological terms. It must be borne in mind that the vitamin E- and selenium-deficient rats used in this study were in the pre-necrotic phase of the liver disease defined by Schwarz (1962), and that shortly afterwards they would have died of the disease had they not been used in the experiments. (It should, however, be stressed that no pathological degeneration can be demonstrated in rats similar to those used for these experiments, by either light or electron microscopy.) Thus the small impairment in the NADH+NADPH synergism that we observed may be an early manifestation of a condition that shortly afterwards may lead to severe pathological changes.

A more striking observation to be made from these experiments on the synergism is that, when both vitamin E and selenium were withdrawn, the initial rate and extent of aminopyrine demethylation was depressed to an extremely low value. Further, vitamin E or selenium singly did not restore the full activity, which was only seen when both nutrients were available together. This observation demonstrates again the close co-operativity that exists between vitamin E and selenium in liver microsomal metabolism.

The results discussed here are generally consistent with our working hypothesis (Diplock & Lucy, 1973), which contains a suggested mechanism for the changes *in vivo* that lead to the phenomena that we have studied *in vitro*. The possibility that oxidative destruction of membrane-associated phospholipids *in vivo* occurs spontaneously or by an enzymic mechanism (McCay *et al.*, 1971) cannot be excluded as a possible cause. If α -tocopherol functions in the manner that we have suggested (Diplock & Lucy, 1973) to form a physicochemical interaction between its side chain and arachidonyl moieties of membrane phospholipids, then oxidative destruction of α -tocopherol (as suggested by McCay *et al.*, 1971) might not necessarily occur, since the antioxidant (hydroxyl) function of tocopherol need not be involved. The failure of Green *et al.* (1967) to demonstrate increased destruction of small amounts of ^{14}C -labelled α -tocopherol in vitamin E-deficient rats given large amounts of dietary polyunsaturated fatty acids might thus be explained. Further, the possibility of accelerated breakdown of membrane-associated phospholipids by endogenous phospholipases must be considered; the expected alteration in the membrane architecture in vitamin E deficiency would be

expected to lead to an increased accessibility of the substrate, polyunsaturated fatty acyl residues of membrane phospholipids, to the enzyme.

The probable consequence of peroxidation or of increased phospholipase-induced membrane turnover would be that non-haem iron proteins, located in more polar regions of membranes, might themselves become exposed to attack by molecular O_2 . The O_2^- radicals so generated (cf. Orme-Johnson & Beinert, 1969) might then accelerate the turnover of selenide, containing non-haem iron proteins in particular, because of their greatly increased susceptibility to oxidation, compared with sulphur-containing proteins. The substitution in these circumstances of sulphide for selenide that we have postulated (Caygill & Diplock, 1973), based on our observation of a more stable form of non-haem iron in liver microsomal fraction of vitamin E-deficient rats, might be expected to have the consequence of a sharp change in the kinetics of reactions associated with the electron-transfer chains that contain the non-haem iron protein.

Vitamin E is thus seen as having three possible closely related roles: (i) by acting in the manner we have described (Diplock & Lucy, 1973) to provide a more compact membrane architecture so that attack by O_2 or phospholipases on membrane-associated polyunsaturated fatty acyl residues of phospholipids is prevented; (ii) by functioning in a limited manner only as a scavenger for any lipid peroxide radicals that are formed; and (iii) to act as a scavenger for oxygen radicals in polar regions of the membrane among sensitive proteins that have an electron-transfer function.

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