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MALONDIALDEHYDE FORMATION IN LIVER MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF VITAMIN E- AND SELENIUM-DEFICIENT RATS AS A FUNCTION OF α-TOCOPHEROL LEVEL: THE EFFECT OF TREATMENT *IN VITRO* WITH IRON

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Weanling rats were given diets with adequate vitamin E and selenium or deprived of one or the other or both, nutrients. After 28 days, liver mitochondrial and microsomal fractions were prepared and α-tocopherol (α-T) and selenium measured. α-Tocopherol fell by eight-fold in the doubly deficient rats and selenium fell three-fold. Malondialdehyde (MDA) was found to be undetectable by a sensitive HPLC method. The fractions were subjected to peroxidative stress in *in vitro* using 0.5 mM Fe^{2+}/10 mM ADP, and MDA and α-T were measured at intervals during 30 min. The results showed that in the mitochondrial fractions there was a lag time of at least 2 min before peroxidation became significant, during which time most of the α-T was consumed. In the microsomal fraction the lag phase was very short prior to the establishment of a linear rate of peroxidation, although little α-T was used up. It was concluded that the mitochondrial fraction withstood the peroxidative challenge better than the microsomal fraction even though the initial level of α-T in the microsomal fraction was about double that in the mitochondrial fraction. Selenium deficiency had no effect on the length of the lag phase of the fractions which therefore appears to be a characteristic of mitochondrial or microsomal fractions.

KEY WORDS: Rat liver fractions, vitamin E(α-tocopherol), selenium, malonaldehyde, oxidative stress.

INTRODUCTION

Increased lipid peroxidation has been demonstrated many times in liver, or liver organelles, of rats deprived of vitamin E.[1,2] Little attention, has, however been given to similar measurements in rats deprived of selenium, either alone or in combination with vitamin E deprivation. Selenium, through its role in glutathione peroxidase, has a dual role in the protection of living cells from the detrimental effects of oxygen-derived free radicals. First, by scavenging hydrogen peroxide, it controls the generation of more reactive and toxic active oxygen species by iron-driven

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catalysis. Secondly, it catalyses the reduction of lipid hydroperoxides to hydroxy acids, thus preventing the iron-catalysed formation of lipid peroxyl and alkoxyl radicals.[3] It might be expected, therefore, that liver, or liver organelles, derived from rats deprived of both vitamin E and selenium would demonstrate exceptionally high levels of lipid peroxidation. In earlier experiments[4] in our laboratory paradoxically raised levels of phospholipid polyunsaturated fatty acids were demonstrated in liver organelles of rats deprived of vitamin E and selenium, which was interpreted as showing evidence of an increased turnover of unsaturated fatty acids in the membrane phospholipids of these doubly deficient rats.

The present paper describes work designed to investigate the levels of endogenous peroxidation in livers of rats deprived of vitamin E and selenium, either separately or together. Lipid peroxidation was assessed by measurement of malonyldialdehyde (MDA), a major secondary aldehydic metabolite of lipid peroxidation, and these values were assessed in the light of the endogenous levels of α-tocopherol (α-T) and selenium. Mitochondrial and microsomal fractions were also prepared from the livers in each dietary group, and the time course of α-T decay and MDA appearance was studied following treatment of the subcellular fractions with exogenous pro-oxidant stimulus in the form of Fe^{3+}/ADP.

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MATERIALS AND METHODS

Abbreviations

α-Tocopherol: α-T; malondialdehyde: MDA; adenosine diphosphate: ADP; high performance liquid chromatography: HPLC.

Chemicals All chemicals were purchased from Sigma Chemicals (UK), except HPLC solvents which were of HPLC grade from Rathburn Chemicals, Peebleshire. Scotland.

Diets, animals and subcellular fractionation The torula yeast-based diets used were as described previously.[5] There were two basic diets, one without added vitamin E and the other with the addition of 100 mg/kg *all rac*-α-tocopheryl acetate (Roche, U.K.). Dietary modification with respect to selenium, which in the basic diet was below our limit of detection (<0.001 mg/kg), was by the addition of selenium (0.1 mg Se/litre as Na_2SeO_3) to the distilled drinking water; the rats deprived of selenium were given distilled water to drink. By this means, four dietary conditions were achieved: (A) deficient in vitamin E and selenium; (B) deficient in selenium, supplemented with 100 mg/kg *all rac*-α-tocopheryl acetate; (C) deficient in vitamin E, supplemented with 0.1 mg/kg (0.1 ppm) selenium, and (D) supplemented with 100 mg/kg *all rac*-α-tocopheryl acetate and 0.1 mg/kg (0.1 ppm) selenium. Previous experience with the doubly deficient diet (A) has shown[5,6] that all weanling rats die from centrilobular hepatic necrosis 30–36 days after the rats are transferred to the diet.

32 Male weanling Wistar rats (Tuck, Sevenoaks, Kent) were divided randomly into four groups, each of which was given one of the dietary/drinking water treatments described above. After 28 days all the rats were killed, the livers excised and placed in ice-cold 0.9% (w/v) NaCl solution. Mitochondrial and microsomal fractions were prepared as described previously[4] and the purity of the fractions

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was determined by the marker enzyme techniques described in the same paper. More than 80% of the total succinate dehyrogenase and glucose-6-phosphatase activity was found to be in the mitochondrial and microsomal fractions respectively in all the experiments.

Incubation with Fe^{2+}/ADP Microsomal and mitochondrial fractions were resuspended in 37.2 mM/Hepes buffer pH 8.0 to give a suspension of 5 mg protein/ml and subjected to oxidative stress *in vitro* in 10 ml of a stock standard solution containing 83.5 mM KCl; 5.5 mM glucose-6-phosphate (as the sodium salt); 0.25 mM $NADP^+$, 5 mM NADH; 20 i.u. glucose-6-phosphate dehydrogenase in the Hepes buffer. The NADH was added to maintain mitochondrial electron transfer activity and the NADPH-generating system was added to maintain microsomal electron transfer, a common solution being used for the mitochondrial and microsomal incubations so that they were identical with respect to their content of minor unidentified contaminants that might affect the results. Lipid peroxidation was initiated by the addition of 0.375 ml of a solution in Hepes buffer of 10 mM ADP and 0.5 mM $FeSO_4.7H_2O$ and incubations were at 25° in a shaking water bath in which the flasks were gassed continuously with 95%O_2/5%CO_2.[7] At regular intervals 1 ml samples were taken and mixed with an equal volume of acetonitrile for MDA measurement or two volumes of ethanolic pyrogallol solution for α-T measurement.[8]

Control flasks were also set up with the Fe^{2+}/ADP omitted and incubated in similar fashion. In order to remove adventitious iron, which is present in many commonly used reagents and in distilled water, all solutions used in the incubation experiments were filtered before use through a column of Sepharose B to which transferrin prepared from hens' eggs[9] had been bound by cyanogen bromide activation.[10] The peroxidation measured was found to be negligible in these control experiments.

Measurement of MDA, α-T and selenium MDA was measured by a modification of the method of Esterbauer *et al.*[11] using a Spherisorb amino phase column (250 × 4.8 mm, from Hichrom, UK) and a mobile phase consisting of equal volumes of acetonitrile and tris buffer (0.03 M, pH 7.0), at a flow rate of 1.0 /min; 2.0 μl sample was injected using a Rheodyne syringe loading injector. MDA was detected and quantitated by reference to standards at 267 nm using a Varian UV-50 variable wavelength detector. MDA stock solutions were prepared from 1,1,3,3-tetraethoxy propane[11] and checked for purity by u.v. spectroscopy at 245 nm ($\varepsilon = 13{,}700$).

α-T was measured by the HPLC method developed in our laboratory[8] following alkaline saponification and extraction into hexane. It was found to be necessary to subject the fractions to alkaline digestion in order to achieve consistently acceptable conditions for HPLC. Recovery of α-T added at the beginning of the digestion was better than 93%. Selenium was measured by acid decomposition and fluorimetry.[12,13] Protein was measured by the method of Lowry *et al.*[14] using bovine serum albumin as standard.

RESULTS

In the liver mitochondrial fraction of vitamin E-deficient rats (Diet C, Table 1) the α-T level was lowered eight-fold, and the double deficiency of vitamin E and selenium (Diet A) caused little further lowering of the α-T level. Selenium-deficient rats had significantly more α-T in their liver mitochondria (Diet B) than in the mitochondria

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TABLE 1

α-Tocopherol and selenium content of the mitochondrial fraction of the livers of rats given four dietary treatments

Diet	α-Tocopherol (μg/mg protein)	Selenium (ng/mg protein)
[A] vitamin E and selenium-deficient	$0.017 \pm 0.006^{\S}$	$0.020 \pm 0.001^{\S}$
[B] selenium-deficient	$0.236 \pm 0.047^{*}$	$0.021 \pm 0.001^{\S}$
[C] vitamin E-deficient	$0.019 \pm 0.008^{\S}$	0.075 ± 0.001
[D] adequate in vitamin E and selenium	0.161 ± 0.043	0.066 ± 0.001

Details of the composition of the diets and of the methods used are given in the text.
Values given are means ±SD of duplicate measurements on at least 6 individual livers
*Significantly different from diet [D] value, $p < 0.05$
§Significantly different from diet [D] value, $p < 0.001$

TABLE 2

α-Tocopherol and selenium content of the microsomal fraction of the livers of rats given four dietary treatments

Diet	α-Tocopherol (μg/mg protein)	Selenium (ng/mg protein)
[A] vitamin E and selenium-deficient	$0.022 \pm 0.008^{\S}$	$0.01 \pm 0.00^{\S}$
[B] selenium-deficient	$0.488 \pm 0.052^{*}$	$0.01 \pm 0.00^{\S}$
[C] vitamin E-deficient	$0.029 \pm 0.013^{\S}$	0.05 ± 0.01
[D] adequate in vitamin E and selenium	0.334 ± 0.023	0.06 ● 0.02

Details of the composition of the diets and of the methods used are given in the text.
Values given are means ±SD of duplicate measurements on at least 6 individual livers
*Significantly different from diet [D] value, $p < 0.05$
§Significantly different from diet [D] value, $p < 0.001$

of fully supplemented rats (Diet D). Depletion of selenium, either alone (Diet B) or in combination with vitamin E deprivation ([Diet A), caused a three-fold lowering in the mitochondrial selenium content. Similar results were obtained in the microsomal fraction (Table 2) although the level of α-T, expressed per mg of protein, in the fractions from adequately fed rats was about twice that in the mitochondrial fraction of the same rats. The fall in α-T level following depletion of vitamin E (Diet C) was nearly twelve fold and the selenium level in the selenium-depleted rats (Diet B) was one sixth of that in the group adequately nourished with selenium. The extent of vitamin E and selenium depletion in the microsomal fraction of doubly deficient rats (Diet A) was apparently greater than in the mitochondrial fraction of the same animals: α-T was 10.5% of diet D in mitochondria and 6.5% of diet D in microsomal fraction, and selenium was 30% of diet D in mitochondria and 16.7% of diet D in microsomal fraction.

MDA was undetectable in the mitochondrial and microsomal fractions of rats given all the diets (shown as the zero time values in Tables 3 and 4 respectively). MDA was generated during incubation with Fe^{2+}/ADP at strikingly different rates in the mitochondrial as compared to the microsomal fraction, irrespective of the dietary treatment of the rats from which the liver fractions were derived. Despite the fact that there were differences in the initial α-T concentration in the fractions (Tables 1 and 2), the rate of generation of MDA was not simply related to the concentration of this lipid antioxidant. Thus for example there was fifteen times more α-T in the

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TABLE 3
Malonaldehyde content (μg/mg protein) following exposure *in vitro* to Fe^{2+}/ADP of liver mitochondrial fractions of rats given four dietary treatments

Time of incubation (min)	Diet [A] Vitamin E and selenium-deficient	Diet [B] Selenium-deficient	Diet [C] Vitamin E-deficient	Diet [D] Adequate in vitamin E and selenium
0	ND	ND	ND	ND
0.5	0.06 ± 0.01	0.04 ± 0.01	0.14 ± 0.03§	0.06 ± 0.02
1.0	0.23 ± 0.11*	0.06 ± 0.03	0.19 ± 0.09*	0.10 ± 0.06
2.0	0.28 ± 0.13*	0.13 ± 0.17	0.26 ± 0.12§	0.16 ± 0.10
5.0	0.57 ± 0.21*	0.25 ± 0.12	0.54 ± 0.20*	0.37 ± 0.12
20.0	1.46 ± 0.52*	0.75 ± 0.16	1.43 ± 0.54	1.11 ± 0.50
30.0	1.84 ± 0.54	1.26 ± 0.56	1.86 ± 0.63	1.63 ± 0.56

Details of the composition of the diets and of the methods used are given in the text.
Values given are means ±SD of duplicate measurements on at least 6 individual livers
ND = not detected (i.e. below limit of detection)
*Significantly different from diet [D] value, $p < 0.05$
§Significantly different from diet [D] value, $p < 0.001$

TABLE 4
Malonaldehyde content (μg/mg protein) following exposure *in vitro* to Fe^{2+}/ADP of liver microsomal fractions of rats given four dietary treatments

Time of incubation (min)	Diet [A] Vitamin E and selenium-deficient	Diet [B] Selenium-deficient	Diet [C] Vitamin E-deficient	Diet [D] Adequate in vitamin E and selenium
0	ND	ND	ND	ND
0.5	0.47 ± 0.17*	0.29 ± 0.20	0.69 ± 0.24§	0.28 ± 0.12
1.0	0.76 ± 0.26*	0.55 ± 0.27	0.99 ± 0.26§	0.50 ± 0.16
2.0	1.26 ± 0.27*	1.04 ± 0.47	1.59 ± 0.37§	0.96 ± 0.31
5.0	2.45 ± 0.69	2.30 ± 0.94	3.13 ± 1.04	1.66 ± 0.82
20.0	5.91 ± 1.04*	6.89 ± 2.66	7.07 ± 2.20	4.52 ± 1.20
30.0	8.06 ± 1.94	8.99 ± 3.65	9.26 ± 2.21	7.92 ± 1.92

Details of the composition of the diets and of the methods used are given in the text.
Values given are means ±SD of duplicate measurements on at least 6 individual livers
ND = not detected (i.e. below limit of detection)
*Significantly different from diet [D] value, $p < 0.05$
§Significantly different from diet [D] value, $p < 0.001$

microsomal fraction of the doubly-supplemented rats (Diet D) (Table 2) compared to the doubly-deficient microsomal fraction, yet the rate of generation of MDA was only slightly slower in the doubly supplemented rat microsomal fraction (Table 4) compared to the doubly deficient group. Similarly, there was a nine-fold difference in the mitochondrial α-T content of the two groups (Table 1) with only a small difference in the MDA generation in the two groups (Table 3). When the rate of destruction of α-T was studied in the two vitamin E-supplemented dietary groups (B and D) it was found (Table 5) that there were also differences between the rates for the mitochondrial and microsomal fractions; (it was not possible to study this parameter in the vitamin E-deficient groups because of the very low initial levels of α-T which were below the detection limit of our method). Thus in the selenium-deficient

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TABLE 5
α-Tocopherol content (μg/mg protein) following exposure *in vitro* to Fe^{2+}/ADP of liver mitochondrial and microsomal fractions of rats given four dietary treatments

Time of incubation (min)	Diet [B] Selenium-deficient		Diet [D] Adequate in vitamin E and selenium	
	Mitochondrial fraction	Microsomal fraction	Mitochondrial fraction	Microsomal fraction
0.0	0.236 ± 0.047*	0.488 ± 0.052*	0.160 ± 0.043	0.334 ± 0.023
0.5	0.186 ± 0.039	0.424 ± 0.048*	0.151 ± 0.027	0.287 ± 0.041
1.0	0.175 ± 0.044	0.405 ± 0.046	0.115 ● 0.030	0.280 ± 0.036
2.0	0.132 ± 0.075	0.395 ± 0.047	0.093 ± 0.019	0.273 ± 0.031
5.0	0.106 ± 0.033	0.336 ± 0.039	0.081 ± 0.016	0.257 ± 0.037
20.0	0.059 ± 0.018	0.283 ± 0.033	0.056 ± 0.013	0.207 ± 0.026
30.0	0.043 ± 0.011	0.271 ± 0.035	0.048 ± 0.009	0.201 ± 0.021

Details of the composition of the diets and of the methods used are given in the text.
Values given are means ±SD of duplicate measurements on at least 6 individual livers
*Significantly different from diet [D] value, $p < 0.05$

mitochondrial fraction (Diet B) (Table 5) 82% of the initial α-T was destroyed in 30 minutes' incubation whereas only 70% was destroyed in the selenium- and vitamin E-supplemented situation (Diet D); corresponding figures for the microsomal fraction were 44% and 40%. Thus, although there was not a very large difference in the initial α-T levels in the mitochondrial and microsomal fractions, the amount of α-T destroyed in the mitochondrial fraction was considerably greater than the rate in the microsomal fraction.

In earlier experiments it was found[4] that there was a small but consistent difference in the total polyunsaturated fatty acid (PUFA) content of microsomal and mitochondrial fractions with a greater content of PUFA in the microsomal fraction. In the experiments reported here the proportions of individual fatty acids in the PUFAs were measured by the techniques used earlier[4] and the total PUFA calculated. It was found that the total PUFA was higher than that reported earlier,[4] being about 37% of the total phospholipids in the mitochondrial fraction and about 39% in the microsomal fraction. It is therefore unlikely that the differences in the unsaturation of the substrate, or in the content of PUFA, was the explanation of the differences found in the peroxidisability of the two fractions studied.

In order to attempt to throw further light on the relationship between the α-T levels and the generation of MDA during incubation with Fe^{2+}/ADP, plots were constructed of the corresponding values of α-T and MDA at the seven different time intervals studied and these are given in Fig. 1. In the mitochondrial fraction (Fig. 1) the kinetics were found to be exponential with a statistically significant correlation ($R^2 = 0.945$ for Diet B and $R^2 = 0.934$ for Diet D). In the microsomal fraction there was no such inter-relationship between the two parameters and further analysis revealed a linear relationship when the first data point was excluded ($R^2 = 0.921$ for Diet B and $R^2 = 0935$ for Diet D).

DISCUSSION

Vitamin E was given in these experiments at the same level as that described before;[6] selenium was however given at a level of 0.1 mg/litre in the drinking water

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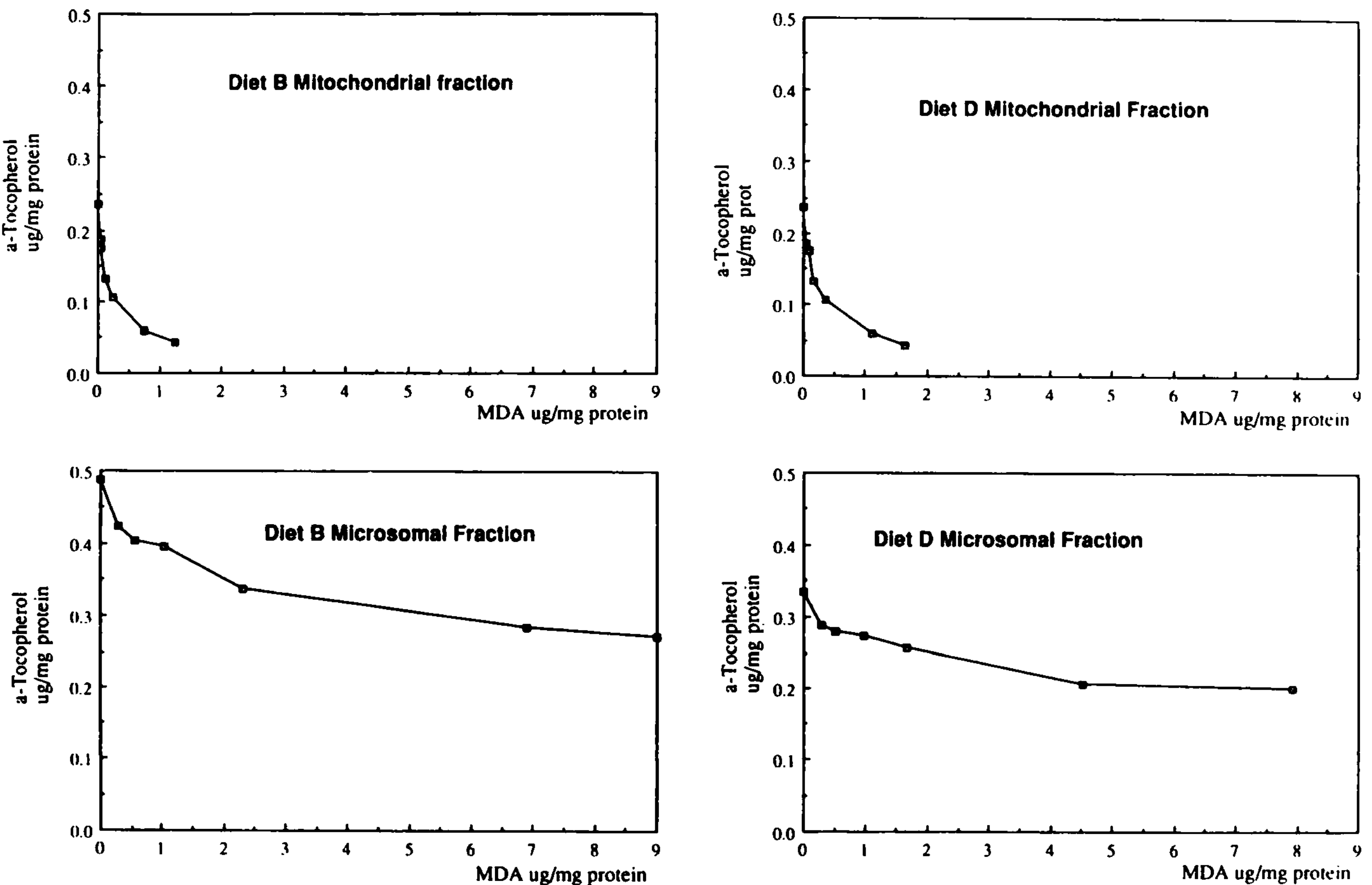

FIGURE 1 The relationship between MDA levels and α-tocopherol content during FE^{2+}/ADP-induced peroxidation *in vitro* of mitochondrial and microsomal fractions from rats given diets adequate or deficient in selenium. Diet B was the selenium-deficient diet adequate with respect to vitamin E. Diet D was the adequate diet supplemented with both selenium and vitamin E. Details of the composition of the diets of the methods used are given in the text. The graphs were plotted, and the statistics done, using Cricket Graph software version 1.3.2. on an Apple Macintosh computer. Error bars have been omitted for the sake of clarity; the mean ±S.D. values are given in Tables 3, 4 and 5 respectively for MDA and α-T.

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approximating to the dietary level of intake used previously. This level of selenium in the drinking water was sufficient to prevent hepatic necrosis; the advantage is that only two diets had to be made instead of four.

The elevated level of α-T in the liver mitochondrial and microsomal fractions of rats given adequate vitamin E but deprived of selenium has been reported before[4] probably reflecting a response to increased oxidative stress in selenium depletion. The extra α-T needed to provide this is likely to come from the diet since there is no known storage site for vitamin E. We have also reported previously the higher level of α-T in the microsomal as compared to the mitochondrial fraction.[4] The extent of depletion of α-T and selenium was greater in the microsomal than in the mitochondrial fraction when vitamin E and selenium were withdrawn together from the diet.

The absence of detectable levels of MDA in the liver fractions of rats deprived of both vitamin E and selenium, which might be expected to be undergoing severe free radical-initiated oxidative stress, is surprising. It is clear that MDA is a secondary product of peroxidation of membrane polyunsaturated fatty acids during peroxidative stress[15,16] and MDA may be the product of several metabolic pathways. MDA is not an end product and further metabolism undoubtedly occurs.[17] It is not known what factors, enzymic or otherwise, influence the rapid metabolism of MDA. Thus, in the intact liver, MDA metabolism to further products may explain the absence of detectable levels of MDA in doubly deficient rats. Metabolism of MDA must be a very efficient process since the level of lipid peroxidation will be high under these conditions. MDA measurement was used here as an index of short-term oxdative stress-induced lipid peroxidation. Although some metabolism of MDA will have been taking place during our experiments, it can be regarded as a genuine index of the quite rapid oxidative damage to membrane polyunsaturated fatty àcids following stress with Fe^{2+}/ADP.

It is clear from Tables 3 and 4 and from Table 5 that the rise in peroxidation following exposure of both cellular organelles to oxidative stress is accompanied by destruction of α-T. However the relationship between these two parameters is a complex one. Peroxidation of a polyunsaturated fatty acid in solution in an organic solvent is exponential;[18,19] there is an initial lag phase during which there is rapid destruction of any lipid-soluble antioxidants present but little peroxidation taking place. This is followed by an almost linear rapid rate of peroxidation during which the remaining antioxidant is destroyed and the fatty acids become severely peroxidised. It has also been shown[20] that in rat liver microsomal membrane fractions stressed by exogenous NADPH/Fe/ADP, the lag phase preceeding the onset of lipid peroxidation is much shortened in preparations from vitamin E-deficient rats. The exponential relationship found here in the mitochondrial fraction (Fig. 1) is closely similar to the situation in a peroxidising lipid in solution. There is an initial lag phase (Fig. 1) that involves rapid destruction of α-T accompanied by low levels of peroxidation; when the level of α-T fell below about 0.1 μg/mg protein there was a much slower rate of destruction of the remaining α-T which was accompanied by a rapid rise in the rate of peroxidation. The rate of peroxidation in the selenium deficiency was slightly greater than in the adequately fed rats, but this difference was not found to be statistically significant because the small number of values available limited the statistical evaluation. In the microsomal fraction the situation was quite different; the relationship between MDA level and α-T level was found (Fig. 1) to be linear, with a highly significant correlation coefficient ($P < 0.005$), throughout the period of the experiment following the first incubation time. Close examination of the curves (Fig. 1) suggests that there may be a very short initial lag period which was over within the first minute of the incubation.

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It was shown[18] that, in a peroxidising lipid in solution the addition of secondary antioxidants such as ascorbate to the reaction mixture would delay the onset of peroxidation by delaying the destruction of the primary lipid antioxidant. In the mitochondrial and microsomal fractions studied here it is apparent that the mitochondrial fraction is better able to meet the added oxidative stress, as demonstrated by lower levels of peroxidation despite the fact that nearly all the α-T was consumed during the experimental period. It is particularly remarkable, in view of the higher initial levels of α-T in the microsomal fraction, that this fraction was apparently very susceptible to peroxidation even though the level of α-T remained higher throughout the experiment than it was in the mitochondrial fraction. It is difficult to see how this could be due to differences in the capacity for regeneration of α-T from its radical in the two tissue fractions but the mechanism of regeneration of α-T *in vitro* or *in vivo* is not fully understood. Although it has been thought to be due to the interaction demonstrated to occur in solution with ascorbate,[21] this has been questioned.[22] It is also now pertinent to note that β-carotene may be able to cause the regeneration of α-tocopherol from its radical.[23] Furthermore, ubiquinone, in the reduced form as ubiquinol, is capable of acting as an antioxidant and it is noteworthy that experiments with rats given identical diets to those used in the present experiments showed[24] that there was less ubiquinone in the livers of rats deprived of vitamin E and selenium. The presence of quite large amounts of ubiquinol in the inner mitochondrial membrane, in contrast to the microsomal membrane, may have been a decisive factor in our experiments.

The MDA that was measured represents the steady state level between its formation and destruction; it may be that the microsomal fraction is less able to metabolise MDA than the mitochondrial fraction so that in the mitochondria the low level of MDA that was found despite the almost complete destruction of α-T was due to rapid destruction of MDA. In this connexion it should be noted that recent measurements of phospholipid hydroperoxide GSH peroxidase[25] (PHGPx) in fractions similar to those discussed here showed no differences in the amount of this enzyme in the two fractions studied although the enzyme was severely depleted in selenium deficiency. The possibility must also be considered that iron-driven re-formation of lipid peroxyl and alkoxyl radicals from lipid hydroperoxides may alter the availability of substrate for MDA formation. The two tissue fractions studied here were clearly quite different with respect to their susceptibility to pro-oxidative challenge and this was unrelated to the level of α-T in them. The ability of the fractions to regenerate α-T from its radical, by whatever mechanism this is done, is considered to be the key factor that determines the ability of a tissue or subcellular organelle to withstand peroxidation.

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