Changes in susceptibility of tissues to lipid peroxidation after ingestion of various levels of docosahexaenoic acid and vitamin E

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To examine the effects of dietary docosahexaenoic acid (DHA) on the potential changes in endogenous lipid peroxidation in the liver and kidney, diets containing a fixed amount of vitamin E (VE; *RRR*-α-tocopherol equivalent; 134 mg/kg diet) and a graded amount of DHA at the levels of 0, 1·0, 3·4 and 8·7 % of total dietary energy were fed to rats for 14 d (Expt 1). In Expt 2, diets containing a fixed amount of DHA (8·7 % of total dietary energy) and a graded amount of VE at the levels of 54, 134 and 402 mg/kg were fed to rats for 15 d. In Expt 1 it was found that endogenous lipid peroxide contents of the liver and kidney, as measured by thiobarbituric acid value and chemiluminescence intensity, were higher, and their α-tocopherol contents lower than those of the controls, with a gradual increase and decrease in values respectively as the dietary DHA level increased (Expt 1). However, the contents of water-soluble antioxidants, i.e. ascorbic acid and non-protein-SH (glutathione), increased with increases in the dietary DHA level, while the Se-dependent glutathione peroxidase (EC 1.11.1.9) activities did not change or tended to be lower. When the graded level of VE was given to rats in Expt 2, lipid peroxide contents in the liver and kidney did not change significantly in response to the increasing levels of dietary VE, although their α-tocopherol contents were higher than control values, increasing with increases in the dietary VE levels. The lipid peroxide scavengers other than α-tocopherol changed similarly to those in Expt 1. The results obtained in Expts 1 and 2 indicate that DHA enhances the susceptibility of the liver and kidney to lipid peroxidation concomitant with higher levels of DHA in these tissues, as shown by the fatty acid composition. In addition, VE is unable to protect membranes of the liver and kidney rich in DHA from lipid peroxidation, even after ingestion of the highest level of VE. However, the liver lipid peroxide content of the group given the highest level of DHA was not as high as expected, based on the peroxidizability index which was calculated from the fatty acid composition of the liver lipid.

Vitamin E: Docosahexaenoic acid: Lipid peroxidation

Fish oils are rich in polyunsaturated fatty acids (PUFA) of *n*-3 type, such as eicosapentaenoic acid (20:5*n*-3; EPA), and docosahexaenoic acid (22:6*n*-3; DHA). Consumption of fish oils is particularly associated with a low incidence of atherosclerosis and cardiovascular diseases, and this prophylactic effect of fish oil ingestion is attributed to *n*-3 polyunsaturated fatty acids (Dyerberg, 1986; Herold & Kinsella, 1986; Narris, 1989;

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Simopoulos, 1991). *n*-3 PUFA have been found to have a hypolipidaemic effect by lowering triacylglycerols and total cholesterol in circulating blood (Herold & Kinsella, 1986; Narris, 1989; Nestel, 1990; Simopoulos, 1991) and to decrease platelet aggregation (Herold & Kinsella, 1986; Simopoulos, 1991; Drevon, 1992; Lands, 1992), thereby diminishing the incidence of atherosclerosis and cardiovascular diseases. More recently, studies with non-human primates and newborn infants have shown that DHA is essential to the normal functional development of the retina and brain, particularly in premature babies (Nestel, 1990; Simopoulos, 1991). Consequently, concentrates of *n*-3 PUFA, such as EPA and DHA from fish oil, have been used as medicinal and/or nutritional supplements (Drevon, 1992; Lands, 1992). These products are sometimes over-used by individuals who expect positive health benefits.

Ingestion of fish oils, however, generally augments susceptibility of cellular membranes to lipid peroxidation (Hammer & Wills, 1978; Kobatake *et al.* 1983; Mouri *et al.* 1984; Hu *et al.* 1989; Kaasgaard *et al.* 1992) and increases the requirement for vitamin E (VE), a lipophilic membrane antioxidant (Mouri *et al.* 1984; Hu *et al.* 1989; Chautan *et al.* 1990; Meydani *et al.* 1991; Kaasgaard *et al.* 1992). This is thought to be attributed to the substitution of membrane fatty acids with potentially-unstable *n*-3 PUFA such as EPA and DHA. It is reported that the relative reaction rate constants of peroxidation were 1, 2, 3, 4 and 5 respectively against unsaturated fatty acids in which the number of the methylene group among double bonds was 1, 2, 3, 4 and 5 (Cosgrove *et al.* 1987). The peroxidizability index (PI), therefore, is calculated according to the following equation (Hu *et al.* 1989): PI = (% dienoic × 1) + (% trienoic × 2) + (% tetra-enoic × 3) + (% penta-enoic × 4) + (% hexaenoic × 5).

On the other hand, Miyashita *et al.* (1993) reported that unsaturated fatty acids dispersed in aqueous solution were resistant to peroxidation as the number of double bonds in fatty acids increases. Kashima *et al.* (1991) observed that the oxidative stability of perilla oil in the presence of tocopherols was increased by the addition of phosphatidylethanolamine. In general, DHA is known to be incorporated preferentially into phosphatidylethanolamine in the tissues (Breckenridge *et al.* 1972; Astorg & Chevaller, 1987). Thus, although it is generally thought that DHA is vulnerable to peroxidation due to the high number of double bonds in its molecular structure, many issues remain to be investigated with respect to the *in vivo* oxidative stability of DHA. Few studies have been carried out on the effect of DHA on tissue lipid peroxidation *in vivo* due mainly to the unavailability of refined DHA in large quantities. In the present study, therefore, we employed a refined DHA to characterize the changes in susceptibility of tissues to lipid peroxidation after ingestion of various levels of DHA and VE.

MATERIALS AND METHODS

Animals and diets

Expt 1. Male Sprague–Dawley rats, 4 weeks of age, weighing 75–85 g, were housed individually in stainless-steel wire-bottomed cages at a constant temperature of 22 ± 1° and humidity of 50–60 % with a 12 h light–dark cycle. The compositions of experimental diets, based on AIN-76 purified diet for rats (American Institute of Nutrition, 1976, 1980), are shown in Table 1. The energy density of all diets was 17·4 MJ/kg (4160 kcal/kg), using Atwater (1910) energy factors (17, 37 and 17 kJ/g for protein, fat and carbohydrate respectively). The VE content of all diets was adjusted to 134 mg *RRR*-α-tocopherol equivalent/kg diet by analysing the VE content of the dietary lipids (Saito *et al.* 1992) and then adding *all-rac*-α-tocopheryl acetate to the diets. The relative biological activities for

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Table 1. *Composition of experimental diets (g/kg diet)**

DHA level (% total energy) ...	0	1·0	3·4	8·7
LA level (% total energy) ...	9·0	2·0	2·0	2·1
Basic components†	900·0	900·0	900·0	900·0
Olive oil	50·0	89·8	73·2	40·0
Safflower oil	50·0	5·0	6·3	8·8
DHA concentrate‡		5·2	20·5	51·2

DHA, docosahexaenoic acid; LA, linoleic acid.

* The energy density of all diets was 17·4 MJ/kg diet (4160 kcal/kg), where the Atwater energy factors were used for the energy calculation.

† The basic components of the diet given to all groups were (g): casein 200·0, DL-methionine 3·0, maize starch 150·0, sucrose 225·0, glucose 225·0, cellulose powder 50·0, AIN-76 vitamin mixture 10·0, AIN-76 mineral mixture 35·0, choline bitartrate 2·0. Vitamin E content of all diets was adjusted to 134 mg RRR-α-tocopherol equivalent/kg diet (for details, see pp. 656–657).

‡ DHA ethyl esters (83% in purity) were used.

RRR-α-, *RRR*-β-, *RRR*-γ- and *RRR*-δ-tocopherols were taken as 100:25:5:0·1 in the calculation (Mino *et al.* 1988). The lipid content of the diet was 100 g/kg and 21·6 % of the total energy (energy %). DHA levels of the diets were adjusted to 0, 1·0, 3·4 and 8·7 energy % respectively, by combining olive oil, safflower oil and DHA concentrate. The DHA concentrate was prepared from fatty acid ethyl esters of sardine oil and the composition of its main fatty acids was as follows (g/kg): 20:5*n*-3 23, 22:1*n*-11 21, 22:3*n*-3 46, 22:6*n*-3 831. The fatty acid composition (g/100 g) of dietary lipids is indicated in Table 2. The control lipid, devoid of DHA, contained 414 g linoleic acid (18:2*n*-6; LA)/kg, which was comparable with the DHA level, 402 g/kg in the 8·7 energy % diet. In addition, each test lipid was prepared to provide at least 2 energy % of LA as essential fatty acid, where the amount of LA was a little more than 9 g/100 g total lipid. The degree of unsaturation of dietary lipids is presented as the double bond index (DBI; Pietrangelo *et al.* 1990), and as peroxidizability index (PI; Hu *et al.* 1989). PI was calculated according to the following equation: PI = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

After being fed on the basal diet containing 50 g olive oil/kg for 3 d, six to seven rats of each group were maintained on the experimental diets for 14 d. Food and water were available *ad libitum*. To prevent autoxidation of DHA concentrate in a diet, the diet was prepared beforehand without adding the concentrate and was stored at −20°. DHA concentrate stored at −80° was mixed with the diet every day, immediately before feeding. Furthermore, the diets were given to rats in the evening and removed the following morning.

Expt 2. Groups of six to seven rats were maintained on the experimental diets for 15 d, as in Expt 1. The composition of the experimental diets was the same as the 0 (control) and 8·7 energy % as DHA diets used in Expt 1, except in the VE content, where the content of the control diet was 54 mg/kg and those of the DHA diets were 54, 134 and 402 mg/kg respectively. The procedures for maintaining the animals were the same as those used in Expt 1.

After fasting overnight, the rats were killed by cardiac puncture and the liver and kidney were promptly excised, washed with isotonic saline (9 g NaCl/l), weighed, and the liver was then perfused with ice-cold isotonic saline via the portal vein. Liver and kidney were stored at −80° until analysed. Serum was separated by centrifugation at 2700 ***g*** for 15 min at 4° and stored at −80°.

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Table 2. *Fatty acid composition (g/100 g) of dietary lipids given to rats*

DHA level (% total energy) ...	0	1·0	3·4	8·7
LA level (% total energy) ...	9·0	2·0	2·0	2·1
16:0	8·8	9·5	8·2	5·2
16:1*n*-7	0·6	1·1	0·8	0·5
18:0	2·8	3·0	2·7	1·6
18:1*n*-9	45·9	72·2	60·4	35·7
18:2*n*-6	41·4	9·2	9·1	9·7
18:3*n*-3	0·5	0·6	0·4	0·4
22:1*n*-11			0·6	1·4
20:5*n*-3			0·4	1·0
22:3*n*-3			0·9	2·2
22:5*n*-3			0·9	2·0
22:6*n*-3		4·4	15·6	40·2
n-6	41·4	9·2	9·1	9·7
n-3	0·5	5·0	18·2	45·8
Double bond index*	1·31	1·20	1·84	3·21
Peroxidizability index†	42·4	32·4	94·9	227·9

DHA, docosahexaenoic acid; LA, linoleic acid.

* Expresses mean double bond number and is the sum of the proportion of each fatty acid × number of double bonds in that acid.

† Calculated as follows: (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

Analyses

Thiobarbituric acid (TBA) assay. Serum TBA value was determined by the method of Yagi (1976) with a minor modification: butylated hydroxytoluene (BHT) was added to the reaction mixture as an antioxidant at a final concentration of 0·18 mM (Expt 1) and 0·36 mM (Expt 2). Liver and kidney TBA values were measured according to the method of Ohkawa *et al.* (1979) with a minor modification: BHT was added to the reaction mixture at a final concentration of 0·45 mM. TBA values are expressed in terms of malondialdehyde (MDA) equivalent.

Chemiluminescence assay. Liver and kidney chemiluminescence intensities of the homogenates were determined according to the method of Miyazawa *et al.* (1984). A synchronous, ultra-high-sensitive single-photon-counting apparatus, Chemiluminescence Analyser OX-7 equipped with a Hamamatsu R878 photomultiplier with spectral response range of 300–650 nm (Tohoku Electronic Industries Co., Sendai, Japan), was used for the detection of light emissions from tissue homogenates. The chemiluminescence intensity is given in terms of average counts per 30 s for the 7 min measurement and corrected for the background counts. The average count of 1 count/10 s for the single photoelectron corresponds to the light intensity of approximately 7 photons/10 s or about 3×10^{-19} W at the wavelength of 500 nm for R878 photomultiplier for which the quantum efficiency of the photocathode is approximately 15 % at this wavelength (Inaba *et al.* 1982). The CV obtained for the chemiluminescence value of homogenate was 6·6 (*n* 15).

Water-soluble fluorescent substance analysis. Serum water-soluble fluorescent substances were determined by the method of Tsuchida *et al.* (1985).

All analyses of serum and tissue lipid peroxide levels described previously were completed within 2 weeks of collection.

Lipofuscin assay. Liver microsomes were prepared (Saito & Yamaguchi, 1988) and microsomal lipofuscin content was determined by the method of Fletcher *et al.* (1973).

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Vitamin E analysis. VE (α-tocopherol) levels in serum, liver and kidney were analysed by HPLC as described previously (Saito *et al.* 1992).

Ascorbic acid and non-protein-SH assays. Ascorbic acid (Roe *et al.* 1948) and non-protein-SH (Beutler *et al.* 1963) levels in liver and kidney were measured. The non-protein-SH component consists mostly of GSH.

*Selenium-dependent glutathione peroxidase (*EC *1.11.1.9; GSHPx) assay.* GSHPx activity was determined according to the method of Noguchi *et al.* (1973) with the minor modification previously described (Saito, 1990). One unit is equivalent to the disappearance of 1 % of the substrate (GSH) per min.

Protein assay. Protein content was measured by the method of Lowry *et al.* (1951).

Fatty acid composition analysis. Liver and kidney lipids were extracted according to the method of Folch *et al.* (1957). The fatty acid composition of dietary, liver and kidney lipids was determined as previously described (Saito *et al.* 1990).

Statistical analysis

After confirming the normality of data and the homogeneity of variance of data for the treatment groups (the latter being tested using the Bartlett test), the statistical significance of differences between mean values was assessed by ANOVA coupled with Duncan's multiple-range test at the 5 % level of significance (Duncan, 1957).

RESULTS

Expt 1

Rats consumed 14·7–15·7 g diet/d and gained 6·0–6·6 g body weight/d over 14 d. There was no significant difference in food intake and body-weight gain of the treatment groups. Relative liver weight was significantly lower ($P < 0·05$) only in the 3·4 energy % DHA group when compared with those of the other DHA groups (46 (SD 3), 50 (SD 5), 45 (SD 3) and 49 (SD 2) g/kg body weight for 0, 1·0, 3·4 and 8·7 energy % DHA groups respectively), although the total liver weight was not significantly different between the groups. Total kidney weight (g) was significantly higher for the 3·4 energy % DHA group only ($P < 0·05$) when compared with that for the controls (1·4 (SD 0·2), 1·6 (SD 0·1), 1·7 (SD 0·1) and 1·6 (SD 0·1) for 0, 1·0, 3·4 and 8·7 energy % DHA groups respectively), although the relative weight per kg body weight was not significantly different among the DHA groups.

As shown in Table 3, the serum TBA value for the 8·7 energy % DHA group was significantly higher, being approximately 100 % greater than those of the other groups. Levels of water-soluble fluorescent substances in the DHA groups did not differ significantly from that of the control group. The liver TBA value for the 3·4 energy % DHA group was significantly greater by about 75 % than that of the control group. The TBA value for the 8·7 energy % DHA group, however, was similar to that of the 3·4 energy % DHA group despite the higher supply of DHA. Liver chemiluminescence intensity became higher with increasing dietary DHA levels, but a significant difference was observed only in the 8·7 energy % DHA group as compared with the controls. Since, as already described (p. 658), the chemiluminescence analyser detects light emissions from the liver homogenates very sensitively, the large deviations in the values appeared to be due to variability between animals, not being due to poor precision and CV in the assay. Liver microsomal lipofuscin contents did not change significantly in response to dietary DHA levels. Kidney TBA values for all DHA groups were slightly, but significantly, higher

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Table 3. *Expt 1. Effects of different levels of dietary docosahexaenoic acid (DHA) on serum, liver and kidney thiobarbituric acid (TBA) values, serum water-soluble fluorescent substance levels, liver and kidney chemiluminescence intensities, and liver microsomal lipofuscin content in rats**

(Mean values and standard deviations)

DHA level (% total energy) ...	0		1·0		3·4		8·7	
LA level (% total energy) ...	9·0		2·0		2·0		2·1	
n ...	6		7		7		7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum								
TBA value (μmol MDA/l)	3·64[a]	0·94	2·96[a]	0·23	4·00[a]	0·49	7·10[b]	1·57
Water-soluble fluorescent substance† (relative fluorescence intensity)	3·97[ab]	0·27	4·40[a]	0·33	3·92[b]	0·35	4·37[a]	0·51
Liver								
TBA value (nmol MDA/g)	91·3[a]	12·3	129·4[ab]	0·4	159·5[b]	25·0	154·2[b]	54·5
Chemiluminescence intensity (counts/30 s)	61·6[a]	22·5	60·1[a]	25·5	89·1[ab]	49·0	114·3[b]	34·8
Microsomal lipofuscin‡ (ng/mg protein)	7·3[a]	3·8	6·2[a]	3·5	6·4[a]	3·9	6·2[a]	2·8
Kidney								
TBA value (nmol MDA/g)	101·3[a]	25·7	118·5[b]	4·5	119·1[b]	12·7	140·4[c]	8·1
Chemiluminescence intensity (counts/30 s)	82·5[ab]	52·0	121·6[ab]	51·8	63·9[a]	52·4	132·9[b]	67·7

MDA, malondialdehyde; LA, linoleic acid.

[a,b,c] Means within the same row with unlike superscript letters were significantly different ($P < 0·05$).

* For details of diets and procedures, see Tables 1 and 2 and pp. 656–658.

† The instrument was calibrated to read 100 relative fluorescence units against a quinine sulfate solution (0·1 μg/ml 0·05 M-H_2SO_4).

‡ Quinine sulfate at a concentration of 0·1 μg/ml 0·05 M-H_2SO_4 was used as a standard for fluorescence intensity and wave-length calibration. The level of lipofuscin was expressed in terms of the quinine sulfate equivalents.

than that for the control group. The highest value (about 40 % greater) was observed in the 8·7 energy % DHA group, but the TBA value was not as high as that of the liver of the same group. The kidney chemiluminescence value was also highest in the 8·7 energy % DHA group, although values for the DHA groups were not different from that of the controls due to considerable scatter in the data.

As shown in Table 4, mean serum α-tocopherol concentrations became progressively lower with increasing dietary supply of DHA. However, liver α-tocopherol content was significantly lower only in the 3·4 and 8·7 energy % DHA groups. Ascorbic acid content in the liver was significantly higher in the 3·4 and 8·7 energy % DHA groups than in the controls but there was no significant difference among the DHA groups. Liver non-protein-SH content was higher only in the 8·7 energy % DHA group. Liver GSHPx activity did not change significantly when the activity was expressed in terms of unit weight of liver, but when expressed as mg protein, the activity was significantly lower in the 8·7 energy % DHA group. In the kidney, on the other hand, α-tocopherol content was significantly lower than the control group but only in the group fed on the highest level of dietary DHA; the ascorbic acid content, on the other hand, was significantly higher at the same dietary DHA level. In the kidney, the non-protein-SH content and GSHPx activity did not vary significantly.

In the fatty acid compositions of total liver lipids (Table 5), *n*-3 DHA, docosapentaenoic acid (22 : 5*n*-3, DPA) and EPA were present at much higher levels

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Table 4. *Expt. 1. Effects of different levels of dietary docosahexaenoic acid (DHA) on serum and tissue levels of lipid peroxide scavengers in rats**

(Mean values and standard deviations)

DHA level (% total energy)...	0		1·0		3·4		8·7	
LA level (% total energy)...	9·0		2·0		2·0		2·1	
n...	6		7		7		7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum								
α-Tocopherol concentration (μmol/l)	29·26[a]	5·81	24·61[b]	3·02	17·88[c]	2·55	13·24[d]	2·55
Liver								
α-Tocopherol content (nmol/g)	149·9[a]	16·4	154·3[a]	25·4	104·6[b]	12·9	64·5[c]	5·8
Ascorbic acid content (μmol/g)	0·97[a]	0·10	1·07[ab]	0·15	1·18[b]	0·13	1·18[b]	0·12
Non-protein-SH content (μmol/g)	5·34[a]	0·88	5·27[a]	0·36	5·50[a]	0·49	6·67[b]	0·55
GSHPx activity:								
unit/g ($\times 10^3$)	29·3[a]	1·5	27·0[a]	2·9	27·4[a]	2·8	27·7[a]	2·9
unit/mg protein	224·7[a]	24·8	205·2[ab]	16·6	205·4[ab]	16·3	189·6[b]	19·7
Kidney								
α-Tocopherol content (nmol/g)	53·4[a]	6·0	49·5[a]	4·8	47·7[a]	6·2	39·8[b]	5·9
Ascorbic acid content (μmol/g)	0·69[ab]	0·05	0·67[a]	0·05	0·77[bc]	0·08	0·81[c]	0·08
Non-protein-SH content (μmol/g)	3·12[a]	0·16	3·12[a]	0·16	3·32[a]	0·39	3·45[a]	0·26
GSHPx activity: unit/g ($\times 10^3$)	25·8[a]	2·2	24·3[a]	2·0	25·5[a]	3·8	23·8[a]	2·0
unit/mg protein	168·0[a]	9·9	157·3[a]	9·0	165·7[a]	22·4	153·7[a]	7·5

GSHPx, Se-dependent glutathione peroxidase (*EC* 1.11.1.9); LA, linoleic acid.

[a,b,c,d] Means within the same row with unlike superscript letters were significantly different ($P < 0·05$).

* For details of diets and procedures, see Tables 1 and 2 and pp. 656–659.

than those in the controls, with values becoming progressively higher as dietary DHA level increased. Total *n*-6 PUFA, on the other hand, were significantly lower when the animals were fed on DHA, but the proportions, particularly of LA and arachidonic acid (20 : 4*n*-6, AA), showed a tendency to be higher in response to the increases in dietary DHA. DBI and PI were found to be higher than those of the controls as the dietary DHA level increased, and the PI of the 8·7 energy % DHA group was 137 % greater than that of the control group. Although *n*-6 PUFA, LA and AA, became markedly lower when DHA was given to animals, both DBI and PI correlated well with the changes in proportions of *n*-3 PUFA, but not with those of *n*-6 PUFA.

The fatty acid composition of total kidney lipids (Table 6) did not reflect the dietary fatty acid composition as closely as that of the total liver lipids. The proportion of AA and LA in the DHA groups became progressively lower with increasing dietary DHA levels. The proportions of DHA, *n*-3 DPA and EPA were all low in the control group, but became progressively higher with increasing dietary DHA levels, although the extent of the change was smaller when compared with that of the total liver lipids. DBI and PI in the DHA groups showed a progressively, but slight, increase with increasing dietary DHA level. These slight increases in DBI and PI over the control levels correlated well with the changes in the proportions of *n*-3 PUFA but not with those of *n*-6 PUFA, similar to that observed in the liver.

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Table 5. *Expt 1. Fatty acid composition (g/100 g) of total liver lipids of rats fed on different levels of docosahexaenoic acid (DHA) for 14 d**

(Mean values and standard deviations)

DHA level (% total energy) ...	0		1·0		3·4		8·7	
LA level (% total energy) ...	9·0		2·0		2·0		2·1	
n ...	6		7		7		7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0·7[a]	0·2	0·6[a]	0·1	0·4[b]	0·0	0·3[b]	0·1
16:0	24·7[a]	2·7	26·2[a]	1·6	21·9[b]	1·5	20·6[b]	0·8
16:1*n*-7	3·3[a]	1·1	3·4[a]	0·4	2·5[b]	0·2	1·8[c]	0·3
18:0	12·3[a]	1·6	10·3[b]	0·9	12·8[ac]	0·9	13·7[c]	0·6
18:1*n*-9	27·4[a]	2·3	39·6[b]	2·0	28·4[a]	2·0	18·4[c]	1·3
18:2*n*-6	12·7[a]	2·0	5·2[b]	0·7	6·0[b]	0·5	6·0[b]	0·4
20:1*n*-9	0·4[a]	0·1	0·4[a]	0·1	0·5[b]	0·1	0·5[b]	0·1
20:3*n*-6	0·4[a]	0·1	0·4[a]	0·1	0·4[a]	0·1	0·4[a]	0·1
20:4*n*-6	12·7[a]	1·9	4·8[b]	0·6	5·3[b]	0·6	6·4[c]	0·5
20:5*n*-3	0[a]		1·7[b]	0·5	5·6[c]	0·7	7·2[d]	0·3
22:5*n*-6	2·2[a]	0·9	0[b]		0·4[bc]	0·1	0·6[c]	0·1
22:5*n*-3	0[a]		0·3[a]	0·2	1·7[b]	0·4	2·7[c]	0·2
22:6*n*-3	2·2[a]	0·3	6·8[b]	1·2	14·2[c]	1·1	21·2[d]	1·4
Saturated	37·9[a]	2·6	37·1[ab]	1·7	35·0[bc]	1·9	34·6[c]	1·1
Monounsaturated	31·1[a]	3·0	43·6[b]	2·3	31·6[a]	2·1	21·0[c]	1·5
Polyunsaturated	31·0[a]	4·3	19·4[b]	2·7	33·4[a]	2·1	44·5[c]	1·8
n-6	28·8[a]	4·1	10·4[b]	1·3	12·0[bc]	0·5	13·3[c]	0·8
n-3	2·2[a]	0·3	8·8[b]	1·5	21·4[c]	1·8	31·1[d]	1·6
Double bond index†	1·4[a]	0·2	1·3[a]	0·1	1·9[b]	0·1	2·4[c]	0·1
Peroxidizability index‡	73·4[a]	11·0	62·7[a]	9·2	123·8[b]	9·1	173·8[c]	8·4

LA, linoleic acid.

[a,b,c,d] Means within the same row with unlike superscript letters were significantly different ($P < 0·05$).

* For details of diets and procedures, see Tables 1 and 2 and pp. 656–659.

† Expresses mean double bond number and is the sum of the proportion of each fatty acid × number of double bonds in that acid.

‡ Calculated as follows (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

Expt 2

Rats consumed 13·7–14·7 g diet/d and gained 5·8–6·6 g body weight/d over the 15 d when graded levels of VE and a fixed level of DHA were administered. There were no significant differences in food intake and body-weight gain between treatment groups. Furthermore, there were no significant differences in the organ weights of the liver and kidney, although a significant difference ($P < 0·05$) was observed in the relative liver weights of the 134 and 402 mg VE groups (48 (SD 2), 48 (SD 3), 49 (SD 2), 46 (SD 2) g/kg body weight for 54 (without DHA), 54 (with DHA), 134 (with DHA) and 402 (with DHA) mg VE groups respectively).

As shown in Table 7, serum TBA values for the DHA groups were approximately 100 % higher than that of the control group. There were no significant differences in serum water-soluble fluorescent substances between the groups. Liver TBA values were significantly higher in the DHA groups (approximately 20 % greater), but the differences in dietary VE levels did not affect the values. Liver chemiluminescence intensity tended to be higher in the DHA groups but the differences were smaller at the higher dietary VE levels. There were no significant differences from the control group, however, due to

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Table 6. *Expt 1. Fatty acid composition (g/100 g) of total kidney lipids of rats fed on different levels of docosahexaenoic acid (DHA) for 14 d*

(Pooled samples from six animals for control group and from seven animals for DHA groups)

DHA level (% total energy) ...	0	1·0	3·4	8·7
LA level (% total energy) ...	9·0	2·0	2·0	2·1
14:0	0·5	0·5	0·5	0·5
16:0	23·1	23·9	22·6	23·6
16:1*n*-7	1·6	1·6	1·6	1·5
18:0	15·2	14·7	15·2	15·7
18:1*n*-9	15·5	18·3	17·1	14·6
18:2*n*-6	10·8	9·3	8·4	7·4
20:1*n*-9	0·3	0·3	0·3	0·3
20:3*n*-6	0·9	0·6	0·4	0·3
20:4*n*-6	26·2	18·5	15·1	14·2
20:5*n*-3		3·7	7·5	9·0
22:5*n*-6	0·8		0·2	
22:5*n*-3		0·4	0·5	0·6
22:6*n*-3	1·5	5·3	7·8	9·5
Saturated	38·8	39·1	38·3	39·8
Monounsaturated	19·3	22·3	21·1	18·5
Polyunsaturated	41·9	38·4	40·5	41·6
n-6	39·7	28·4	24·1	21·9
n-3	1·5	9·4	15·8	19·1
Double bond index†	1·7	1·7	1·9	2·0
Peroxidizability index†	101·9	108·9	126·3	136·5

LA, linoleic acid.

* For details of diets and procedures, see Tables 1 and 2 and pp. 656–659.

† Expresses mean double bond number and is the sum of the proportion of each fatty acid × number of double bonds in that acid.

‡ Calculated as follows: (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

considerable scatter in the values. Liver microsomal lipofuscin content did not differ significantly between the groups. Kidney TBA values for all DHA groups were slightly, but significantly, higher (approximately 20% greater) than those of the controls. Chemiluminescence intensities in the kidney changed similarly to those of the liver and were lowest in the group receiving the highest level of vitamin E.

Changes in serum and tissue lipid peroxide scavengers are shown in Table 8. Serum α-tocopherol concentration of the DHA groups was significantly lower in comparison with that of the control group, but the differences were smaller with the greater dietary VE levels. However, the concentration of α-tocopherol did not reach the level of the control group even in the highest VE group. Liver and liver microsomal α-tocopherol contents showed a similar change, increasing as the dietary supply of VE increased. There was no significant difference between the groups in ascorbic acid content of the liver. Liver non-protein-SH content was significantly higher in the groups given DHA, but did not alter in response to the dietary changes in VE level. On the other hand, liver GSHPx activities of the DHA groups, expressed in terms of specific activity, were significantly lower than those of the controls, but again increasing the dietary VE levels was without effect.

In the kidney, the changes in α-tocopherol contents were similar to those of the liver and liver microsomes, but the extent of variation was less in the kidney. The ascorbic acid content was significantly higher in the highest dietary VE group as compared with that of the control group, but no significant differences were present among the DHA groups. The

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Table 7. *Expt 2. Influences of dietary docosahexaenoic acid (DHA) and different levels of dietary vitamin E (VE) on serum and tissue lipid peroxide levels in rats**

(Mean values and standard deviations)

DHA level (% total energy) ...	0		8·7		8·7		8·7	
LA level (% total energy) ...	9·0		2·1		2·1		2·1	
VE level (mg/kg diet) ...	54		54		134		402	
n ...	6		7		7		6	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum								
TBA value (μmol MDA/l)	3·44[a]	0·83	7·90[b]	4·47	7·51[b]	2·14	6·54[ab]	1·84
Water-soluble fluorescent substance† (relative fluorescence intensity)	3·31[a]	0·17	3·21[a]	0·32	3·42[a]	0·29	3·38[a]	0·42
Liver								
TBA value (nmol MDA/g)	77·6[a]	9·1	105·6[b]	15·5	100·5[b]	9·7	101·1[b]	17·8
Chemiluminescence intensity (counts/30 s)	107·7[a]	59·1	166·6[a]	49·1	146·0[a]	43·6	135·7[a]	55·5
Microsomal lipofuscin† (ng/mg protein)	24·6[a]	10·9	25·8[a]	13·0	23·6[a]	11·3	23·7[a]	12·0
Kidney								
TBA value (nmol MDA/g)	110·7[a]	5·3	128·5[b]	8·6	129·8[b]	10·1	129·9[b]	17·9
Chemiluminescence intensity (counts/30 s)	177·0[ab]	58·8	187·9[ab]	54·6	202·3[a]	48·9	136·3[b]	46·0

TBA, thiobarbituric acid; MDA, malondialdehyde; LA, linoleic acid.

[a,b] Means within the same row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see pp. 656–659.

† The instrument was calibrated to read 100 relative fluorescence units against a quinine sulfate solution (0·1 μg/ml 0·05 M-H_2SO_4).

‡ Quinine sulfate at a concentration of 0·1 μg/ml 0·05 M-H_2SO_4 was used as a standard for fluorescence intensity and wave-length calibration. The level of lipofuscin was expressed in terms of the quinine sulfate equivalent.

non-protein-SH contents of the DHA groups were slightly but significantly higher with increasing dietary VE level. The GSHPx activity did not change significantly when the activity was expressed on the basis of unit wet weight, but it was significantly lower in the higher dietary VE groups when the activity was expressed as specific activity.

With respect to the fatty acid profiles of total lipids for the liver, liver microsomes and kidney, there were no significant effects of increasing the dietary VE levels (data not shown).

DISCUSSION

In a previous paper we showed that ingestion of a high-fish-oil diet increased the susceptibility of the liver to lipid peroxidation, as assessed by TBA value and chemiluminescence intensity, and also increased the requirement for VE (Saito & Nakatsugawa, 1994). Similar results are also obtained in other tissues after ingestion of fish-oil diets containing PUFA (Leibovitz *et al.* 1990; Garrido *et al.* 1993). In the present study (Expt 1) a diet containing refined DHA as a primary source of PUFA was given to rats and lipid peroxide levels, measured by TBA value and chemiluminescence intensity, were higher in the liver and kidney, and their α-tocopherol contents lower with increases in dietary DHA level. Thus, DHA ingestion, as observed with fish oil, appeared to enhance the susceptibility of those tissues to lipid peroxidation as a function of the dietary DHA level. Serum TBA value also increased with increasing dietary DHA level, but the levels of serum water-soluble fluorescent substances and liver microsomal lipofuscin were not

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Table 8. *Expt 2. Influences of dietary docosahexaenoic acid (DHA) and different levels of dietary vitamin E (VE) on serum and tissue lipid peroxide scavengers in rats**

DHA level (% total energy) ...	0		8·7		8·7		8·7	
LA level (% total energy) ...	9·0		2·1		2·1		2·1	
VE level (mg/kg diet) ...	54		54		134		402	
n ...	6		7		7		6	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum								
α-Tocopherol concentration (μmol/l)	23·65[a]	8·64	8·97[b]	0·85	13·83[bc]	4·11	16·98[c]	2·20
Liver								
α-Tocopherol content (nmol/g)	50·1[a]	8·7	29·0[b]	6·5	56·4[a]	9·0	102·9[c]	13·3
Microsomal α-tocopherol content (nmol/mg protein)	0·73[a]	0·17	0·36[b]	0·68	0·72[a]	0·14	1·39[c]	0·12
Ascorbic acid content (μmol/g)	1·08[a]	0·11	1·02[a]	0·12	1·11[a]	0·09	1·05[a]	0·19
Non-protein-SH content (μmol/g)	5·14[a]	0·59	6·80[b]	0·78	6·80[b]	0·55	6·48[b]	0·75
GSHPx activity: (unit/g)($\times 10^3$)	28·7[a]	3·1	26·2[ab]	2·7	24·0[b]	3·7	24·0[b]	3·4
(unit/mg protein)	200·0[a]	27·4	173·6[b]	12·2	168·6[b]	24·7	159·3[b]	19·7
Kidney								
α-Tocopherol content (nmol/g)	32·3[a]	3·3	26·4[b]	3·5	34·5[ac]	5·9	38·7[c]	3·6
Ascorbic acid content (μmol/g)	0·59[a]	0·08	0·68[ab]	0·07	0·69[ab]	0·08	0·70[b]	0·09
Non-protein-SH content (μmol/g)	2·73[a]	0·23	3·03[b]	0·13	3·16[b]	0·10	3·35[c]	0·16
GSHPx activity: (unit/g)($\times 10^3$)	21·0[a]	1·9	21·3[a]	3·6	21·4[a]	2·3	22·4[a]	2·4
(unit/mg protein)	164·3[a]	19·4	153·3[ab]	17·9	141·7[b]	10·8	142·2[b]	13·5

LA, linoleic acid; GSHPx, Se-dependent glutathione peroxidase (*EC* 1.11.1.9).
[a,b,c] Means within the same row with unlike superscript letters were significantly different ($P < 0·05$).
* For details of diets and procedures, see pp. 656–659.

higher even in the highest dietary DHA group. Thus, lipid peroxidation in the tissues may not have been stimulated extensively in the current experimental conditions, since these fluorescent compounds are thought to be produced only after lipid peroxidation in tissue is thoroughly enhanced. The levels of lipid peroxide scavengers also support this conclusion, since they seem to be closely associated with the suppression of dietary DHA-stimulated tissue lipid peroxidation, as discussed later (p. 666). Furthermore, serum aspartate aminotransferase (*EC* 2.6.1.1) and glutamic-pyruvic transaminase (*EC* 2.6.1.2) activities, which are indicators of tissue parenchymal cell damage, did not change significantly in any of the groups in the present experiment (data not shown).

In Expt 1, serum α-tocopherol concentration was significantly lower than the control value, even in the 1·0 energy % DHA group. This could be explained partly by the possibility that VE is degraded in part by its activity in suppressing enhanced tissue lipid peroxidation. Since the tissue lipid peroxide levels and α-tocopherol contents of the DHA groups, especially in the liver, correlated negatively with each other, as shown in Tables 3 and 4, the variations in serum α-tocopherol concentration may be a better indicator of changes in tissue α-tocopherol content.

Ascorbic acid and non-protein-SH, the latter consisting mostly of GSH, were higher in the liver and kidney of DHA-fed animals compared with the controls, which was opposite to that of their α-tocopherol content. It has been suggested that three antioxidants, VE, ascorbic acid and GSH, may be interrelated in the maintenance of tissue VE. The recycling of tocopheroxyl radicals to tocopherol is accomplished by reaction with ascorbic acid (Tappel, 1962). The dehydroascorbic acid formed in this reaction is reduced to ascorbic acid again by a reaction with GSH (Meister, 1992). If recycling of tocopheroxyl radicals to tocopherol is a major mechanism to maintain tissue VE levels, a decrease in ascorbic acid

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might be expected to promote a reduction in tissue VE. However, the ascorbic acid and GSH levels were higher in the liver and kidney in the present experiment, particularly in the highest dietary DHA group, which may be to compensate for the losses of VE. This phenomenon could be accounted for as follows: as the requirement for VE is raised to suppress tissue lipid peroxidation with increasing DHA intake, the requirements for ascorbic acid and GSH are also raised in an attempt to maintain normal tissue α-tocopherol levels, and this results in an enhancement in the biosynthesis of ascorbic acid and GSH. In spite of this potent compensating mechanism, the tissue α-tocopherol levels were significantly lower in the two higher-DHA groups. Thus, tissue α-tocopherol may be lost steadily as the supply of plausible substrate, i.e. DHA, and the risk of tissue lipid peroxidation increase.

Since GSHPx activities in the liver and kidney barely changed or tended to be lower in the DHA-fed rats, it is possible that the enzyme does not efficiently degrade tissue lipid peroxides formed after DHA intake. The GSHPx assayed in the present experiment is a Se-dependent enzyme, commonly found in cytosol (Flohé & Schlegel, 1971). When membrane PUFA, which are primarily present in the substitution nucleophilic 2-molecular (*sn*-2) position of membrane phospholipids, are oxidized to their hydroperoxides, the GSHPx can reduce the fatty acid hydroperoxides to the corresponding monohydroxyl fatty acids utilizing GSH. However, the fatty acid hydroperoxides in membrane phospholipids must be hydrolysed first by phospholipase A_2 (*EC* 3.1.1.4) before the resultant hydrolysed fatty acid hydroperoxides can move to cytoplasm (Yasuda & Fujita, 1977; van Kuijk *et al.* 1987; Salgo *et al.* 1992). It is known that membrane DHA, probably including DHA hydroperoxides present in the *sn*-2 position of phospholipids, is resistant to hydrolysis by phospholipase A_2 because DHA supplementation appeared to attenuate the phospholipase A_2 reaction (Shikano *et al.* 1993). Thus, the GSHPx activities may be unaltered, irrespective of the fact that the tissue lipid peroxide levels were higher in the DHA-fed rats.

The findings relating to tissue-lipid peroxide formation in Expt 1 suggest that the protective mechanisms against lipid peroxidation might be different in the liver and kidney. The TBA values and chemiluminescence intensity in the control group tended to be higher in the kidney than in the liver tissue. Levels of antioxidants measured in the present study (Table 4) were also lower in the kidney, suggesting that the susceptibility of the kidney to lipid peroxidation may be greater than that of the liver. However, the extent of the increase in lipid peroxide levels in response to increasing dietary DHA level is lower in the kidney than in the liver. This finding could be explained partially by the changes in the profiles of PUFA which serve as substrates for lipid peroxide formation in these tissues. In the kidney, the proportions of *n*-3 PUFA, particularly DHA, were lower than those of the liver in response to increasing dietary DHA intake and, as a result, the increases in PI may be less in the kidney. Consequently, the kidney may be protected from lipid peroxidation because its lipid composition is more resistant to dietary influences.

When the vulnerability of tissue to lipid peroxidation is assessed by PI calculated from the tissue fatty acid composition, the kidney of the 8·7 energy % DHA group is approximately 34 % more susceptible to lipid peroxidation than the kidney of the control group (Table 6). The kidney TBA value for the DHA-fed rats was approximately 20–40 % more than that of the controls, and, thus, the degree of increase in the kidney TBA value almost coincided with that of PI in this tissue. On the other hand, the liver of the 8·7 energy % DHA group was about 137 % more susceptible to lipid peroxidation (Table 5). However, the increase in liver TBA value of the DHA-fed rats was at most 40–75 % greater than that of the control (Table 3). Similar results were also obtained for the chemiluminescence intensity. Thus, dietary DHA might not increase liver lipid peroxidation to the extent

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expected from the PI. In VE-deficient animals, PI correlated well with urinary creatine excretion (Witting & Horwitt, 1964). But in a VE-sufficient condition, as observed in the present study, PI might not correlate with liver lipid peroxide levels, as measured by TBA value and chemiluminescence intensity. Further investigation is now in progress to focus on the susceptibility of membrane DHA to lipid peroxidation.

In Expt 2, the fatty acid profiles of total lipids for the liver, liver microsomes and kidney did not change significantly in response to increasing dietary VE level, and almost the same findings were obtained in the DHA groups as were found in Expt 1 (data not shown). The liver and kidney α-tocopherol content was higher in the highest-VE-fed animals than in the controls, while other antioxidants, ascorbic acid, GSH and GSHPx, produced variable results. Furthermore, dietary DHA-stimulated lipid peroxide formation, as assessed by TBA values in those tissues, did not decrease significantly. It appears, also, that the dietary DHA-stimulated lipid peroxidation was not suppressed even at the highest level of VE supplementation. Thus, the supply of VE may be inadequate and/or the antioxidative function of VE was inefficient in the presence of the highly-unsaturated fatty acid. Farwer *et al.* (1994) observed similar results in rats fed on diets high in fish oil. Thus, VE may not protect membranes rich in *n*-3 fatty acids, especially those with five or six double bonds, from lipid peroxidation as efficiently as membranes rich in *n*-6 fatty acids, as suggested by Kaasgaard *et al.* (1992). This has also been observed in rat and guinea-pig tissues (Kivits *et al.* 1981; Kunert & Tappel, 1983; Gavino *et al.* 1984; Leibovitz *et al.* 1990).

In conclusion, ingestion of DHA enhanced the susceptibility of the liver and kidney to lipid peroxidation concomitant with higher levels of DHA in these tissues. In addition, VE was unable to protect membranes of these tissues from lipid peroxidation even after ingestion of high levels of VE.

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