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The Role of Lipid Components of the Diet in the Regulation of the Fatty Acid Composition of the Rat Liver Endoplasmic Reticulum and Lipid Peroxidation

By CATHERINE T. HAMMER and ERIC D. WILLS
Department of Biochemistry and Chemistry, Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ, U.K.

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The fatty acid compositions of the lipids and the lipid peroxide concentrations and rates of lipid peroxidation were determined in suspensions of liver endoplasmic reticulum isolated from rats fed on synthetic diets in which the fatty acid composition had been varied but the remaining constituents (protein, carbohydrate, vitamins and minerals) kept constant. Stock diet and synthetic diets containing no fat, 10% corn oil, herring oil, coconut oil or lard were used. The fatty acid composition of the liver endoplasmic-reticulum lipid was markedly dependent on the fatty acid composition of the dietary lipid. Feeding a herring-oil diet caused incorporation of 8.7% eicosapentaenoic acid ($C_{20:5}$) and 17% docosahexaenoic acid ($C_{22:6}$), but only 5.1% linoleic acid ($C_{18:2}$) and 6.4% arachidonic acid ($C_{20:4}$), feeding a corn-oil diet caused incorporation of 25.1% $C_{18:2}$, 17.8% $C_{20:4}$ and 2.5% $C_{22:6}$ fatty acids, and feeding a lard diet caused incorporation of 10.3% $C_{18:2}$, 13.5% $C_{20:4}$ and 4.3% $C_{22:6}$ fatty acids into the liver endoplasmic-reticulum lipids. Phenobarbitone injection (100mg/kg) decreased the incorporation of $C_{20:4}$ and $C_{22:6}$ fatty acids into the liver endoplasmic reticulum of rats fed on a lard, corn-oil or herring-oil diet. Microsomal lipid peroxide concentrations and rates of peroxidation in the presence of ascorbate depended on the nature and quantity of the polyunsaturated fatty acids in the diet. The lipid peroxide content was 1.82 ± 0.30nmol of malonaldehyde/mg of protein and the rate of peroxidation was 0.60 ± 0.08nmol of malonaldehyde/min per mg of protein after feeding a fat-free diet, and the values were increased to 20.80nmol of malonaldehyde/mg of protein and 3.73nmol of malonaldehyde/min per mg of protein after feeding a 10% herring-oil diet in which polyunsaturated fatty acids formed 24% of the total fatty acids. Addition of α-tocopherol to the diets (120mg/kg of diet) caused a very large decrease in the lipid peroxide concentration and rate of lipid peroxidation in the endoplasmic reticulum, but addition of the synthetic anti-oxidant 2,6-di-t-butyl-4-methylphenol to the diet (100mg/kg of diet) was ineffective. Treatment of the animals with phenobarbitone (1mg/ml of drinking water) caused a sharp fall in the rate of lipid peroxidation. It is concluded that the polyunsaturated fatty acid composition of the diet regulates the fatty acid composition of the liver endoplasmic reticulum, and this in turn is an important factor controlling the rate and extent of lipid peroxidation *in vitro* and possibly *in vivo*.

The oxidative metabolism of drugs and carcinogens in the liver endoplasmic reticulum is markedly dependent on the composition of the dietary lipid (Marshall & McLean, 1971; Rowe & Wills, 1976; Lambert & Wills, 1977*a*) and modification of the fatty acid composition of the endoplasmic reticulum may be of major importance in the regulation of this metabolism.

Lipid peroxidation occurs in suspensions of the microsomal fractions of liver when incubated *in vitro*, and the rate of peroxidation is strongly stimulated by addition of NADPH or ascorbate (Wills, 1969). If no substrates are added to the microsomal fraction, the endogenous fatty acids in the membrane phospholipids are the substrates of lipid peroxidation (May & McCay, 1968*a*). It is therefore apparent that the nature and relative quantities of polyunsaturated fatty acids in the endoplasmic reticulum play an important role in the regulation of the rate and extent of lipid peroxidation.

In the present investigation the effect of varying the nature of the dietary lipid on the fatty acid composition of the liver endoplasmic reticulum has been studied, and the role of microsomal fatty acids in the regulation of lipid peroxidation has been investigated.

Materials and Methods

Animals

Male albino Wistar rats 6–7 weeks old and weighing 120–150 g were used in all experiments.

Diets

Some animals were fed on stock diet (Spratts no. 1, Central House, Barking, Essex, U.K.), but most were fed on diets specially prepared as described by Diplock *et al.* (1961). They contained, by weight, 25% casein (Unigate, Trowbridge, Wilts., U.K.), 30% sucrose, 20% wheat starch (Adcol, Ashford, Kent, U.K.), 10% dried yeast (Bovril, Kings Lynn, Norfolk, U.K.), 5% Cox's salt mix (Diplock *et al.*, 1961) and, normally, 10% lipid. For fat-free diets the starch content was increased to 30% and the lipid was omitted. All diets were supplemented with Rovimix AD_3 (Roche, Welwyn Garden City, Herts., U.K.) supplying 5000 i.u. of retinol and 1000 i.u. of cholecalciferol (D_3)/kg of diet.

Lipid components

Lard (anti-oxidant-free) was obtained from Unigate, refined herring oil from Marfleet Refining Co., Hull, Yorks, U.K., corn oil from Mazola, Brown and Polson, C.P.C., Esher, Surrey, U.K., and coconut oil was a gift from the Tropical Products Institute, London E.C.1, U.K.

2,6-Di-t-butyl-4-methylphenol was obtained from British Drug Houses, Poole, Dorset, U.K., as were all other chemicals used in the investigation.

Determination of fatty acid composition of the dietary lipids

The fatty acid composition of the dietary lipids was determined by g.l.c. The lipids were methylated with BF_3–methanol complex as described by Morrison & Smith (1964) and the percentage methylation and recovery checked by addition of arachidic acid methyl ester as an internal standard. The fatty acid methyl esters were separated on a column of 10% poly(ethylene glycol) adipate on Chromosorb W 80–100 mesh by using a temperature of 180°C and nitrogen carrier at a flow rate of 60 ml/min in a Perkin–Elmer F11 chromatograph with a flame ionization detector.

Determination of lipid peroxide contents of diets

The lipid peroxide content of the diets was determined by the thiobarbituric acid method (Wills, 1964), which gives a measure of the aldehyde breakdown products of lipid peroxidation: 10–50 mg of the diet or 0.2 ml of an ethanolic extract (100 mg/2 ml of ethanol) was used.

Preparation of the liver microsomal fraction

This was prepared by the calcium precipitation method described by Kamath & Rubin (1972). The fraction was suspended in 125 mM-KCl (2 ml/g of liver used in the preparation). This is described as a '50% microsomal suspension'.

Extraction of lipids from the microsomal fraction

The unsaturated fatty acids present in membrane phospholipids are particularly vulnerable to peroxidation and, to prevent peroxidation occurring during preparation, the anti-oxidant 2,6-di-t-butyl-4-methylphenol was added to all solvents used to extract the lipids and all solvents used subsequently. Lipids were extracted from microsomal fractions with chloroform/methanol as described by Recknagel & Ghoshal (1966). The lipids were redissolved in a small quantity of hexane containing 2,6-di-t-butyl-4-methylphenol (5 mg/100 ml) and stored in sealed tubes under nitrogen at −20°C.

Determination of fatty acid composition of microsomal lipids

The microsomal lipids were methylated with BF_3–methanol complex as described by Morrison & Smith (1964). The fatty acid methyl esters were separated and identified by g.l.c. as described for the diet samples.

Determination of lipid peroxide content of the microsomal fraction

This was measured by determination of the malonaldehyde equivalent in the tissue by the thiobarbituric acid method (Wills, 1966). This method determines the aldehyde breakdown products formed as a consequence of peroxidation in the microsomal fraction.

Determination of the rate of lipid peroxidation in microsomal suspensions

The method used was that of Wills (1969) with minor modifications. Microsomal suspension (50%) in 125 mM-KCl (0.5 ml), 0.2 M-sodium phosphate buffer, pH 7.0 (2.0 ml), 2 mM-ascorbic acid, pH 7.0 (2.0 ml), and 125 mM-KCl (1.5 ml) were mixed and incubated at 37°C. Samples (0.5 ml) were taken immediately after mixing of the microsomal suspension and then again after 10 and 20 min. The thiobarbituric acid assay was carried out as described (Wills, 1969).

Experimental plan for feeding experiments

Groups of eight to twelve rats were fed on a fat-free diet or on a diet to which 10% lard (anti-oxidant-free), 10% coconut oil, 10% herring oil, 10% corn oil or 10% heated corn oil had been added. In most experiments, pairs of rats were killed after

Table 1. *Fatty acid composition of dietary fats and of stock diet*
Lipids were extracted from prepared diets containing 10% fat or from stock diet for analysis. Mean values ± S.E.M. for 4–10 determinations are shown, and expressed as % by weight of total fatty acids.

Fatty acid	Herring oil	Lard	Corn oil	Corn oil (boiled)	Coconut oil	Stock diet
$C_{8:0}$	—	—	—	—	5.8 ± 0.7	—
$C_{10:0}$	—	—	—	—	5.6 ± 0.2	—
$C_{12:0}$	—	—	—	—	48.7 ± 0.6	—
$C_{14:0}$	6.5 ± 0.3	1.7 ± 0.1	—	—	20.9 ± 0.3	2.9 ± 0.2
$C_{16:0}$	14.0 ± 0.6	27.4 ± 0.5	13.7 ± 0.2	39.7 ± 0.6	8.9 ± 0.2	24.1 ± 1.0
$C_{16:1}$	7.0 ± 0.4	3.4 ± 0.4	—	—	—	3.6 ± 0.4
$C_{18:0}$	2.4 ± 0.1	18.3 ± 0.5	2.1 ± 0.1	10.4 ± 1.2	1.8 ± 0.2	18.9 ± 1.0
$C_{18:1}$	17.8 ± 0.4	41.9 ± 0.7	28.7 ± 0.2	41.2 ± 0.4	7.2 ± 0.3	36.2 ± 0.8
$C_{18:2}$	1.9 ± 0.1	7.4 ± 0.9	55.2 ± 0.1	8.7 ± 0.3	1.2 ± 0.1	14.4 ± 0.2
$C_{18:3}$	1.3 ± 0.1	—	0.3 ± 0.06	—	—	—
$C_{18:4}$	2.6 ± 0.3	—	—	—	—	—
$C_{20:1}$	9.5 ± 0.5	—	—	—	—	—
$C_{20:5}$	7.6 ± 0.8	—	—	—	—	—
$C_{22:1}$	15.8 ± 0.4	—	—	—	—	—
$C_{22:5}$	0.7 ± 0.1	—	—	—	—	—
$C_{22:6}$	9.8 ± 0.9	—	—	—	—	—

Table 2. *Lipid peroxide contents of diets*
Lipid peroxide contents were measured by the thiobarbituric acid method and expressed as nmol of malonaldehyde/g of diet. Mean values ± S.E.M. for 4–10 determinations are shown.

Diet	Lipid peroxide (nmol of malonaldehyde/g of diet)
10% Herring oil	2090 ± 195
10% Herring oil + 0.01% 2,6-di-t-butyl-4-methylphenol	530 ± 120
10% Lard	114 ± 13
10% Lard + 0.01% 2,6-di-t-butyl-4-methylphenol	20 ± 0.8
10% Corn oil	137 ± 27
10% Corn oil (boiled)	22 ± 1.2
10% Coconut oil	60 ± 10

eating the diet for 10 days and then at intervals of 2 days until the 25th day. Livers were removed and the microsomal fraction was prepared as described. For the analysis of microsomal fatty acid composition, lipids were extracted with chloroform/methanol, methylated and separated by g.l.c. For the measurement of the rate of lipid peroxidation the suspensions were incubated in the presence of ascorbate by the methods described.

Results

Analysis of dietary lipids and of diets

The percentage fatty acid compositions of the fats used in the investigation are shown in Table 1. Dietary linoleic acid is of major importance for efficient oxidative drug and carcinogen metabolism (Rowe & Wills, 1976; Lambert & Wills, 1977*b*). Linoleic acid comprised 14.4% of the fatty acids in the stock diet, lard contained 7.4% linoleic acid and coconut oil contained only 1.2% linoleic acid and a large proportion of saturated fatty acids (Table 1). Corn oil contained 55.2% linoleic acid, but treatment of corn oil at 120°C for 30min decreased the linoleic acid content to 8.7%. Herring oil contained only 1.9% linoleic acid, but also contained large quantities of highly unsaturated acids such as $C_{18:4}$, $C_{20:5}$ and $C_{22:6}$.

Lipid peroxides, formed from unsaturated fatty acids, are always present in diets containing unsaturated fatty acids in quantities partly dependent on the preparation and period of storage of the diet. Polyunsaturated fatty acids are particularly vulnerable to peroxidation (Holman, 1954), and diets containing a large proportion of highly unsaturated fatty acids also contain large amounts of lipid peroxide breakdown products (Table 2). In addition to the polysaturated fatty acid composition, the anti-oxidant content of the diet is of major importance. A herring-oil diet contains a low concentration of vitamin E (12mg/kg of diet) (Lambert & Wills, 1977*a*), but a corn-oil diet contains a high concentration (100mg/kg) (Rowe & Wills, 1976), which prevents peroxidation and accounts for the low peroxide content of a corn-oil diet (Table 2).

Effects of dietary lipids on the fatty acid composition of microsomal lipids

The total amount of lipid extracted from the liver endoplasmic reticulum did not vary whichever diet was fed. The proportions of saturated fatty acids

(palmitic, $C_{16:0}$; stearic, $C_{18:0}$) in the endoplasmic reticulum of rats given diets containing different lipids did not vary widely (Table 3), although the quantity taken in was different depending on the diet given (Table 1). However, a coconut-oil diet (91.6% saturated fatty acids) caused rapid incorporation of short-chain saturated fatty acids, such as $C_{12:0}$ and $C_{14:0}$, into the liver endoplasmic reticulum (Table 3).

The proportions of polyunsaturated fatty acids in the liver endoplasmic reticulum reflected the unsaturated fatty acid composition of the diets. A saturated coconut-oil or lard diet caused incorporation of smaller amounts of $(n-6)$ ($\omega 6$) polyunsaturated fatty acids (linoleic, $C_{18:2}$ and arachidonic, $C_{20:4}$) than after eating the stock diet or a corn-oil diet which contained larger proportions of linoleic acid (Table 3). After a highly unsaturated herring-oil diet, a large proportion of $(n-3)$ ($\omega 3$) polyunsaturated fatty acids, such as eicosapentaenoic ($C_{20:5}$) acid and docosahexaenoic ($C_{22:6}$) acid, was incorporated into the liver endoplasmic reticulum (Table 3).

Effects of dietary lipids on the lipid peroxide content of the microsomal fractions

The lipid peroxide content of the microsomal fraction was measured, immediately after preparation, by the thiobarbituric acid method, which gives an indication of the aldehyde breakdown products formed by peroxidation. The lipid peroxide content was lowest in the microsomal fraction of rats given the fat-free diet. Very small changes in the lipid peroxide content were caused by the addition of 10% lard or 10% coconut oil to the diet, but very large quantities were observed after adding 10% herring oil or 10% corn oil (Table 4).

The lipid peroxide content measured by the thiobarbituric acid method immediately after preparation of the microsomal fraction could be formed during the preparation. To test this possibility the anti-oxidant 2,6-di-t-butyl-4-methylphenol was added at a concentration of 1 mg/ml to the original medium used for homogenization and to all other solutions used in the preparation of the microsomal fraction. Addition of 2,6-di-t-butyl-4-methylphenol caused a

Table 3. *Fatty acid composition of liver microsomal lipids after giving various diets*
Diets were given for 10–25 days. Lipids were extracted from the microsomal fraction and fatty acid compositions analysed by g.l.c. Mean values ± S.E.M. are shown, and expressed as % by weight of total fatty acids.

Fatty acid	Herring oil	Lard	Corn oil	Coconut oil	Stock diet
$C_{12:0}$	—	—	—	1.1 ± 0.08	—
$C_{14:0}$	—	—	—	4.3 ± 0.2	—
$C_{16:0}$	22.3 ± 0.9	22.8 ± 1.1	21.2 ± 0.9	28.5 ± 0.4	26.1 ± 0.8
$C_{16:1}$	3.4 ± 0.1	4.8 ± 0.8	2.2 ± 0.2	9.2 ± 0.4	4.0 ± 0.3
$C_{18:0}$	16.0 ± 0.5	17.9 ± 0.9	15.9 ± 0.9	14.5 ± 0.4	16.4 ± 0.6
$C_{18:1}$	17.8 ± 0.5	26.5 ± 1.3	15.3 ± 0.9	23.5 ± 0.3	21.1 ± 0.6
$C_{18:2}$	5.1 ± 0.3	10.3 ± 0.6	25.1 ± 1.1	7.6 ± 0.2	14.9 ± 0.4
$C_{20:1}$	1.6 ± 0.2	—	—	—	—
$C_{20:4}$	6.4 ± 0.2	13.5 ± 0.7	17.8 ± 0.9	8.8 ± 0.4	11.1 ± 0.3
$C_{20:5}$	8.7 ± 0.5	—	—	—	0.7 ± 0.1
$C_{22:5}$	1.5 ± 0.3	—	—	—	—
$C_{22:6}$	17.0 ± 0.6	4.3 ± 0.2	2.5 ± 0.3	2.6 ± 0.3	7.7 ± 0.5
No. of rats	12	10	10	12	6

Table 4. *Lipid peroxide contents and rates of lipid peroxidation in liver microsomal fractions after giving various diets*
Diets were given to groups of 12 rats for 20 days. Values shown are mean results ± S.E.M. obtained from rats killed 10–20 days after commencing feeding. Lipid peroxide contents of microsomal fractions and rates of lipid peroxidation after addition of ascorbate were determined by the thiobarbituric acid method (Wills, 1969).

Diet	Lipid peroxide content (nmol of malonaldehyde/mg of protein)	Rate of peroxidation (nmol of malonaldehyde/min per mg of protein)
Fat-free	1.82 ± 0.3	0.60 ± 0.08
10% Coconut oil	2.60 ± 0.2	1.22 ± 0.17
10% Lard	2.26 ± 0.3	1.02 ± 0.05
10% Corn oil	13.60 ± 3.4	1.42 ± 0.15
10% Corn oil (boiled)	2.20 ± 0.4	0.78 ± 0.06
10% Herring oil	20.80 ± 4.6	3.73 ± 0.34
Stock	8.76 ± 0.8	1.98 ± 0.09

Table 5. *Fatty acid composition of liver microsomal lipids after phenobarbitone treatment of rats given various diets*
Diets were given for 10–25 days. Rats received a single injection of phenobarbitone (100mg/kg) after at least 10 days on the diets and were killed 24h later. Other experimental details were as described in Table 3. Mean values ± S.E.M. are shown, and expressed as % by weight of total fatty acids. Results were compared with those in Table 3 by means of Student's t test.

Fatty acid	Herring oil	Lard	Corn oil
$C_{16:0}$	22.4 ± 0.6	23.8 ± 1.0	20.1 ± 0.7
$C_{16:1}$	4.1 ± 0.2	4.9 ± 0.4	2.4 ± 0.3
$C_{18:0}$	15.0 ± 0.4	16.1 ± 0.6	13.8 ± 0.6
$C_{18:1}$	19.5 ± 0.4	31.2 ± 0.9	18.3 ± 0.7
$C_{18:2}$	4.9 ± 0.2	10.1 ± 0.3	29.4 ± 0.9*
$C_{20:1}$	2.1 ± 0.2	—	—
$C_{20:4}$	5.7 ± 0.3†	10.7 ± 0.4*	14.3 ± 0.4*
$C_{20:5}$	10.3 ± 0.4†	—	—
$C_{22:5}$	1.3 ± 0.3	—	—
$C_{22:6}$	14.7 ± 0.3*	3.3 ± 0.4†	1.7 ± 0.2†
No. of rats	12	10	10

* $P < 0.01$ compared with results shown in Table 3.
† $P < 0.05$ compared with results shown in Table 3.

large fall in the peroxide content after a herring-oil diet, from 20.8 ± 4.6 to 9.82 ± 1.8nmol of malonaldehyde/mg of protein, and a smaller fall after a lard diet, from 2.26 ± 0.33 to 1.02 ± 0.25nmol of malonaldehyde/mg of protein. Thus part of the large values of lipid peroxide content after feeding herring oil must be caused by peroxidation occurring during the preparation, despite the taking of all normal precautions to obviate oxidation by carrying out the preparation at a low temperature.

Effects of dietary lipids on the rate of lipid peroxidation

The rate of lipid peroxidation in the microsomal fraction of rats fed on different diets was measured, immediately after preparation, by incubating the fractions in the presence of ascorbate as described in the Materials and Methods section and determination of aldehyde breakdown products by the thiobarbituric acid method.

The peroxidation rate was higher in the microsomal fraction of rats fed on the stock diet or a corn-oil diet, in which relatively high proportions of polyunsaturated fatty acids were incorporated, than in rats fed on a fat-free diet or a highly saturated coconut-oil or lard diet (Table 4). The peroxidation rate was highest in the microsomal fraction of rats fed on a herring-oil diet in which a large proportion of highly unsaturated fatty acids, such as $C_{20:5}$ and $C_{22:6}$, which are particularly prone to peroxidation, were incorporated (Table 4).

Effects of phenobarbitone on the fatty acid composition of the microsomal lipids

It is well established that injecting or feeding drugs such as phenobarbitone causes induction of the enzymes involved in oxidative drug metabolism in the liver and extensive new synthesis and proliferation of membranes of the endoplasmic reticulum (Remmer & Merker, 1963; Fouts & Rogers, 1965). The effect of phenobarbitone on the fatty acid composition of the endoplasmic reticulum was studied

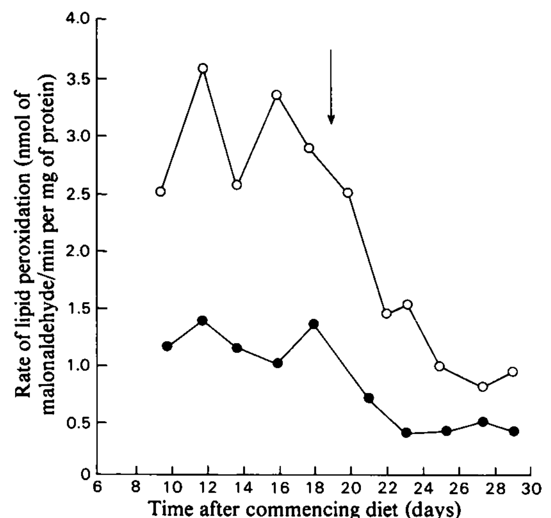

Fig. 1. *Effect of phenobarbitone treatment on the rate of lipid peroxidation in the liver microsomal fraction*
Two groups of 20 rats were used. One group was fed on a 10% lard diet (●) and one on a 10% herring-oil diet (○). After 9 days on the diets, pairs of rats receiving each diet were killed and the microsomal fractions of the livers prepared for measurement of the rate of lipid peroxidation. After 18 days on the diets, the remaining rats had their drinking water supplemented with phenobarbitone (1mg/ml; arrow), which was continued until the end of the experiment. Determinations of the rate of lipid peroxidation were continued for a further 18 days. Each point is the mean of determinations on two animals.

after giving different diets to ascertain whether the composition of the 'induced' membrane is different from that of the uninduced membrane.

Rats were fed on a 10% lard, 10% corn-oil or 10% herring-oil diet for up to 25 days. Rats selected for induction experiments were given a single injection of phenobarbitone (100mg/kg) after at least 10 days' feeding and killed 24h later.

Phenobarbitone increased the proportion of oleic acid ($C_{18:1}$) in the liver endoplasmic reticulum of rats fed on a lard diet ($P<0.01$), corn-oil diet ($P<0.02$) or herring-oil diet ($P<0.05$) (Tables 3 and 5). Phenobarbitone increased the proportion of linoleic acid ($C_{18:2}$) ($P<0.01$) in rats fed on a corn-oil diet and increased the proportion of eicosapentaenoic acid ($C_{20:5}$) ($P<0.05$) in rats fed on a herring-oil diet (Tables 3 and 5). Incorporation of arachidonic acid ($C_{20:4}$) and docosahexaenoic acid ($C_{22:6}$) into the liver endoplasmic reticulum was decreased by phenobarbitone injection (Tables 3 and 5).

Effects of inducing agents on the peroxidation rates

In view of the importance of synthesis of new membranes during the induction process, it was thus decided to examine the effects of inducing agents on the rate of peroxidation in the microsomal fraction.

The experimental procedure was as previously described, but the animals selected for induction experiments were given phenobarbitone in their drinking water (1 mg/ml) after 18–20 days on the diets. The animals were then killed at intervals 4–11 days after starting on the phenobarbitone. After a 10% lard diet, phenobarbitone induces the rate of oxidative demethylation of aminopyrine (Rowe & Wills, 1976), but it caused a very marked decrease in the rate of lipid peroxidation (Fig. 1). If the diet contained 10% herring oil, the effect of phenobarbitone was even more marked, because the initial rate of peroxidation was much greater (Fig. 1). Phenobarbitone caused an increase in total protein content of the microsomal fraction, from 15.6 to 21.3 mg of protein/g of liver after a herring-oil diet and from 14.8 to 19.5 mg/g of liver after a lard diet. The total lipid content of the microsomal fraction also increased after phenobarbitone treatment, from 10.8 ± 0.8 to 18.8 ± 1.1 mg/g of liver after a herring-oil diet and from 9.8 ± 1.4 to 18.7 ± 1.1 mg/g of liver after a lard diet. The protein/lipid ratio in the microsomal fraction was thus decreased by phenobarbitone from 1.44:1 to 1.13:1 after a herring-oil diet and from 1.51:1 to 1.04:1 after a lard diet.

As an alternative to phenobarbitone, groups of animals fed on 10% herring-oil diets were injected with methylcholanthrene (20mg/kg) in arachis oil.

Table 6. *Effect of methylcholanthrene on lipid peroxide content and rate of lipid peroxidation*

Two groups of 10 rats were fed for 10 days on a 10% herring-oil diet. Each member of one group was then injected with methylcholanthrene (20mg/kg). Liver microsomal fractions were prepared 1 day after injection and then at intervals of 2 days. Lipid peroxide contents and rates of peroxidation were determined by the thiobarbituric acid method as described for Table 4. Mean values ± S.E.M. are shown.

	Lipid peroxide content (nmol of malonaldehyde/mg of protein)	Rate of lipid peroxidation (nmol of malonaldehyde/min per mg of protein)
Control	18.37 ± 3.72	3.17 ± 0.21
Methylcholanthrene	13.81 ± 3.56	3.56 ± 0.32

Table 7. *Effects of dietary antioxidants on lipid peroxide content and rate of peroxidation in the liver microsomal fraction*

Six groups of 12 rats were used; three groups were fed on a 10% lard diet and three groups on a 10% herring-oil diet. The lard diet given to one group was supplemented with vitamin E (120mg/kg) and that given to another group with 2,6-di-t-butyl-4-methylphenol (100mg/kg). Similar supplements were provided for two groups given herring-oil diet. Two rats from each group were killed 10 days after starting the diet and peroxide contents and peroxidation rates were determined in the microsomal fractions as described for Table 4. Additional pairs of rats were killed at intervals of 2 days for a further 10 days. Mean values ± S.E.M. are shown.

Diet	Addition to diet	Lipid peroxide content (nmol of malonaldehyde/mg of protein)	Rate of lipid peroxidation (nmol of malonaldehyde/min per mg of protein)
10% lard	None	2.26 ± 0.33	1.02 ± 0.05
	Vitamin E	1.70 ± 0.48	0.39 ± 0.05
	2,6-Di-t-butyl-4-methylphenol	3.00 ± 0.42	1.10 ± 0.07
10% herring oil	None	20.80 ± 4.60	3.73 ± 0.34
	Vitamin E	5.83 ± 0.59	0.87 ± 0.02
	2,6-Di-t-butyl-4-methylphenol	24.30 ± 5.70	3.22 ± 0.61

Pairs of animals were killed 1–10 days after injection and the rate of peroxidation in the microsomal fraction was measured. Methylcholanthrene, unlike phenobarbitone, does not cause proliferation of the smooth-endoplasmic-reticulum membranes, but induces the specialized enzyme system, which metabolizes biphenyl and carcinogenic hydrocarbons (Fouts & Rogers, 1965). Methylcholanthrene did not decrease the rate of peroxidation, but caused a decrease in the peroxide content of the microsomal fraction (Table 6).

Effects of feeding anti-oxidants with dietary lipids

The question whether dietary anti-oxidants, such as the naturally occurring vitamin E, or artificial anti-oxidants, such as 2,6-di-t-butyl-4-methylphenol, function *in vivo* by acting as anti-oxidants is still unsolved (Wasserman & Taylor, 1972), and studies on lipid peroxidation in the microsomal fraction could help to elucidate this problem.

Corn oil naturally contains large quantities of vitamin E, but lard and herring oil both contain small quantities of this anti-oxidant and these dietary lipids were therefore suitable for studying the effect of vitamin E. Vitamin E was added to diets containing 10% lard or 10% herring oil so that the final vitamin E concentration was 120mg/kg of diet, and the lipid peroxide content and rate of peroxidation were determined in the microsomal fraction as described. Vitamin E caused a decrease in the peroxide content of the microsomal fraction after a lard or herring-oil diet and a very large decrease in the rate of lipid peroxidation (Table 7). 2,6-Di-t-butyl-4-methylphenol, however, mixed with a 10% lard diet or 10% herring-oil diet to give a final concentration of 100mg of 2,6-di-t-butyl-4-methylphenol/kg of diet caused no significant decrease in the rate of peroxidation. After addition of 2,6-di-t-butyl-4-methylphenol to either diet a small increase in the peroxide content of the microsomal fraction was observed (Table 7).

Discussion

It has been generally assumed for many years that, although the fatty acid composition of adipose tissue and other stored triacylglycerols can fluctuate according to changes in the fatty acid composition of the dietary lipid, the fatty acid composition of the phospholipids of cell membranes, vital to their function, does not change to any significant extent as the fatty acid composition of the diet is altered (Horwitt, 1962; Cook *et al.*, 1970). However, it has been demonstrated that the nature of the dietary lipid exerts a marked effect on the endoplasmic-reticulum membranes of the liver, on the cytochrome *P*-450 content and on the rate of oxidative drug metabolism (Rowe & Wills, 1976). It has been suggested that the effects of dietary lipids were exerted through changes brought about in the fatty acid composition of the membrane phospholipids (Norred & Wade, 1972). An adequate dietary supply of linoleic acid causes a rapid rate of oxidative drug and carcinogen metabolism, but fats containing other polyunsaturated fatty acids were equally effective (Lambert & Wills, 1977*a*). Linoleic acid and some $(n-3)(\omega 3)$ polyunsaturated fatty acids cannot be synthesized by the rat and are essential in the diet (Alfin-Slater & Aftergood, 1968).

We have now analysed the fatty acid composition of preparations of liver endoplasmic reticulum after giving several diets containing saturated and unsaturated fats and have provided strong support for a dietary regulation of membrane phospholipid composition.

Phospholipids comprise the majority of the total lipids of the liver endoplasmic reticulum (Victoria & Barber, 1969) and we have thus concluded that the changes in fatty acid composition that we observed must be occurring mainly in the phospholipids of the membrane. When rats are transferred from the stock diet to a 10% coconut-oil or 10% lard diet there is a marked fall in the linoleic acid and docosahexaenoic ($C_{22:6}$) acid contents of the endoplasmic reticulum, accompanied by increased incorporation of saturated and monounsaturated fatty acids into the membrane lipids (Table 3). After coconut oil was given, 1.1% of $C_{12:0}$ and 4.3% of $C_{14:0}$ fatty acids were incorporated into the endoplasmic reticulum. These fatty acids are not found in the endoplasmic reticulum after giving any of the other diets tested (Table 3).

The rate of oxidative demethylation is much decreased by giving a lard or coconut-oil diet (Norred & Wade, 1973; Rowe & Wills, 1976), and thus linoleic acid and docosahexaenoic acid ($C_{22:6}$) appear to be especially important for maximum efficiency of the enzyme system. The importance of linoleic acid in the membrane phospholipids was further substantiated by the results obtained after giving a corn-oil diet. This causes a very rapid rate of oxidative demethylation (Rowe & Wills, 1976) and also a very marked incorporation of linoleic acid (Table 3). Induction experiments using phenobarbitone (Davison & Wills, 1974) had also shown that linoleic acid was of major importance in the structure of membrane phospholipids required for oxidative demethylation.

However, linoleic acid is unlikely to be an essential component of the diet or of the phospholipids of the endoplasmic reticulum, because the rate of oxidative demethylation is very high when a diet containing 10% herring oil is given (Lambert & Wills, 1977*a*). This oil contains less than 2% linoleic acid (Table 1) and, as a consequence, the linoleic acid in the endo-

plasmic reticulum falls from 15 to 5% (Table 3). Herring oil does, however, possess relatively large quantities of highly unsaturated acids, eicosapentaenoic ($C_{20:5}$), docosapentaenoic ($C_{22:5}$) and docosahexaenoic ($C_{22:6}$), which are readily incorporated into the membrane. Phospholipids containing these ($n-3$) ($\omega 3$) acids must be able to perform the functions of ($18-6$) ($\omega 6$) linoleic acid, because the rate of oxidative demethylation of aminopyrine is greater after giving a herring-oil diet than after a corn-oil diet (Lambert & Wills, 1977*a*).

The fatty acids incorporated into the membrane phospholipids can also affect the lipid peroxide contents of the endoplasmic-reticulum preparations and the rates of peroxidation, although a small proportion of microsomal lipid peroxides may originate from triacylglycerols. Despite the taking of all normal precautions to minimize deteriorative changes, lipid peroxide was always measured in the preparations. The peroxide contents were greatest in the group of animals fed on herring oil and less in those fed on corn oil or lard (Table 4). This is clearly due to the high concentration of highly unsaturated fatty acids, incorporated into the endoplasmic reticulum after giving herring oil, which are peroxidized very readily. Addition of an anti-oxidant, 2,6-di-t-butyl-4-methylphenol, at the start of the preparation decreased the lipid peroxide to about 50% of that with no 2,6-di-t-butyl-4-methylphenol, but did not abolish it completely. These results may be interpreted to mean that some degradation of the membrane fatty acids occurs normally *in vivo* or that peroxidation must be very rapid after killing and before preparation. In either case, the extent of peroxidation is clearly dependent on the type of diet given.

Peroxidation leads to degradation of fatty acids into many short-chain aldehydes and other small molecules (King, 1956), and from the determination of malonaldehyde it is possible to calculate the extent of fatty acid loss by using the data of Tables 3 and 4. May & McCay (1968*b*), using incubated suspensions of endoplasmic reticulum prepared from animals fed on a stock diet, have shown that for every 100 nmol of malonaldehyde produced, approx. 890 nmol of fatty acids in the endoplasmic reticulum was utilized. The malonaldehyde content of the endoplasmic reticulum was 20.8 nmol/mg of protein or 14.4 nmol/mg of lipid after feeding herring oil, and 2.26 nmol/mg of protein or 1.50 nmol/mg of lipid after feeding lard (Table 4). Therefore 14.4 nmol of malonaldehyde produced after feeding a herring-oil diet results from the degradation of approx. 128 nmol of microsomal fatty acid/mg of lipid, and 1.50 nmol of malonaldehyde produced after feeding a lard diet results from the degradation of 13.4 nmol of fatty acid/mg of lipid. The polyunsaturated fatty acids in the endoplasmic reticulum are most easily peroxidized and account for 38.7% of the total fatty acids in rats fed on herring oil and 28.1% of the total fatty acids in rats fed on lard (Table 3). Assuming an average molecular weight of the fatty acids destroyed as 300, then there are approx. 1270 nmol of microsomal fatty acid/mg of lipid in rats fed on herring oil and 940 nmol of these fatty acids/mg of lipid in rats fed on lard. We can thus calculate that destruction of approx. 10% of the polyunsaturated fatty acids can occur during the preparation of the microsomal fraction of rats fed on a herring-oil diet, but if a lard diet is given, only 1.5% of the polyunsaturated fatty acids are destroyed.

It is possible that a proportion of the high peroxide contents of the endoplasmic reticulum in rats fed on herring oil could be caused by direct ingestion of peroxides. The lipid peroxide contents of the diets varied with the degree of unsaturation of the dietary fatty acids and were high in herring-oil diets and low in lard and coconut-oil diets (Table 2). It is unlikely, however, that ingestion of peroxides is a major factor for two reasons; firstly there is normally a very low percentage of lipid peroxides, if any, absorbed from the diet (Andrews *et al.*, 1960; Rowe, 1975), and secondly, the addition of the anti-oxidant 2,6-di-t-butyl-4-methylphenol to the herring-oil diet caused a large decrease in the lipid peroxide content of the diet, but did not cause a decrease in the lipid peroxide content of the endoplasmic reticulum preparations (Table 4). It may therefore be concluded that most, if not all, of the peroxide contents of the endoplasmic reticulum are formed *in situ*.

When preparations of endoplasmic reticulum were incubated with ascorbate, the rate of lipid peroxidation was, like the peroxide content, dependent on the diet. It was highest after giving a herring-oil diet, a fact that supports the view that it is the polyunsaturated fatty acids, such as $C_{20:5}$ and $C_{22:6}$, supplied by the herring oil which are most prone to peroxidation rather than the less unsaturated linoleic acid ($C_{18:2}$) (Table 4).

Vitamin E, unlike 2,6-di-t-butyl-4-methylphenol which when included in the diet decreased the dietary peroxide but did not affect the microsomal peroxide content or rate of peroxidation (Table 7), caused a large decrease in both the peroxide content and rate of peroxidation in the endoplasmic reticulum. It thus appears that vitamin E is incorporated into the endoplasmic reticulum where it can exert its anti-oxidant action. On the other hand, as 2,6-di-t-butyl-4-methylphenol is ineffective, it must be inadequately absorbed, or not incorporated into the endoplasmic reticulum as such, or incorporated into the endoplasmic reticulum at a location which is ineffective in protecting the polyunsaturated fatty acids against peroxidation.

The reason for the dramatic fall in lipid-peroxidation rate after phenobarbitone induction (Fig. 1) is

difficult to explain. It is not due to a large increase in protein content of the microsomal fraction in relation to lipid, because exactly the reverse occurred, and the lipid/protein ratio increased after induction. Phenobarbitone did not cause a decrease in the polyunsaturated fatty acids constituting the membrane phospholipids (Table 5). After a lard diet a small decrease in arachidonic acid ($C_{20:4}$) from 13.5 to 10.5% occurred, but this does not appear sufficient to explain the large decrease in peroxidation rate. Similarly, after a herring-oil diet a small decrease in docosahexaenoic acid ($C_{22:6}$) from 17.0 to 14.7% was observed, but this was accompanied by an increase in the concentration of eicosapentaenoic acid ($C_{20:5}$) from 8.7% to 10.3%, and the total percentage of these highly unsaturated fatty acids therefore changes to a very small extent. If changes in the fatty acid composition of the membrane phospholipids cannot explain the dramatic fall in peroxidation rate then it is possible that phenobarbitone or a metabolite is acting as an anti-oxidant. Phenobarbitone itself is not an active anti-oxidant when added to microsomal suspensions *in vitro* (Wills, 1969) and thus it is more likely that a metabolite of phenobarbitone would be the active anti-oxidant. Alternatively, during membrane proliferation the configuration of the membrane lipids could be altered, so that peroxidation is less likely, or dilution of catalysts of peroxidation, such as iron proteins, could occur during membrane proliferation.

It is therefore clear that the fatty acid composition of the dietary lipid exerts a marked influence on the fatty acid composition of the liver endoplasmic reticulum, which in turn regulates lipid peroxidation in the microsomal fraction.

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