Effect of fatty acid saturation on NADPH-dependent lipid peroxidation in rat liver microsomes

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Abstract NADPH-dependent lipid peroxidation in liver microsomes, as measured by malondialdehyde formation, decreased by 90% when rats were fed a diet containing 16% coconut oil. This reduction occurred within 1 to 3 days after the rats were placed on this highly saturated diet. The decrease in peroxidation activity was associated with a reduction in the polyunsaturated fatty acid content of the microsomal phospholipids, particularly arachidonic acid. When the rats were transferred to a highly polyunsaturated diet containing 16% sunflower seed oil, microsomal lipid peroxidation and arachidonic acid content were restored to normal values within 10 days. Arachidonic acid contained in the microsomal choline and ethanolamine phosphoglycerides was the main substrate for peroxidation. Addition of diarachidonyl phosphatidylcholine, but not free arachidonic acid, to the assay system restored peroxidation activity in the microsomes prepared from the livers of the rats fed saturated fat. Likewise, prior incubation of these microsomes with a mixture of phospholipid exchange protein and liposomes containing diarachidonyl phosphatidylcholine restored peroxidation activity. These results indicate that diets rich in saturated fat reduce microsomal lipid peroxidation by decreasing the availability of polyunsaturated fatty acids in substrate phospholipids.—**Lokesh, B. R., S. N. Mathur, and A. A. Spector.** Effect of fatty acid saturation on NADPH-dependent lipid peroxidation in rat liver microsomes. *J. Lipid Res.* 1981. **22:** 905–915.

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The fatty acid composition of rat liver microsomes can be altered considerably by changes in the saturation of the dietary fat (1–3). This leads to a change in the activity of several microsomal enzymes (4–7), including the ascorbate-induced lipid peroxidation system (1). Peroxidation is reduced when the microsomes are prepared from the livers of rats fed a highly saturated diet containing lard or coconut oil. Conversely, the activity of this peroxidation system is enhanced when a polyunsaturated fat-rich diet containing herring oil is fed. The microsomal peroxidation system utilizes the polyunsaturated fatty acid components of phospholipids, especially arachidonic acid, as substrate (8–10). Therefore, feeding saturated fat probably reduces peroxidation by decreasing the availability of arachidonate or other polyunsaturates in the microsomal phospholipid substrates. On the other hand, the possibility that the peroxidation effect was not a direct consequence of the changes in microsomal fatty acid composition could not be excluded since the lipid modifications were produced by diet in the intact animal.

In the present study, we have observed that, like the ascorbate-dependent system (1), NADPH-dependent lipid peroxidation in rat liver microsomes is reduced considerably by feeding a diet rich in saturated fat. Furthermore, the peroxidation activity of these microsomes was restored by either adding diarachidonyl phosphatidylcholine to the assay medium or incubating the microsomes prior to assay with a mixture of phospholipid exchange protein and liposomes containing diarachidonyl phosphatidylcholine. Based upon these findings, it appears that the diet-induced changes in lipid peroxidation can be explained entirely by the modifications that are produced in the fatty acid composition of the microsomes.

MATERIALS AND METHODS

Materials

NADPH, ADP, ATP, coenzyme A, cytochrome c, glucose 6-phosphate and the phospholipids used for liposome preparations were purchased from Sigma Chemical Company (St. Louis, MO). The purity of the synthetic phospholipids was checked by thin-layer chromatography, and their fatty acid compositions were determined by gas–liquid chromatography. All of the phospholipids used were >99% pure. Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN) and was also found to be 99.5% pure

Abbreviations: M_p, microsomes prepared from the livers of rats fed the diet supplemented with sunflower seed oil (polyunsaturated-enriched); M_s, microsomes prepared from the livers of rats fed the diet supplemented with coconut oil (saturated-enriched).

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by gas–liquid and thin-layer chromatography. 2-Thiobarbituric acid was obtained from Eastman Kodak Co. (Rochester, NY). Weanling male Sprague-Dawley rats weighing between 50–65 g were employed for all of these studies. They were maintained on a semi-synthetic diet consisting of 54% sucrose, 27% casein, 1% vitamin mix, and 4% mineral mix (Teklad Mills, Madison, WI) supplemented with either 16% sunflower oil (Cargill, Inc., Minneapolis, MN) or 16% coconut oil (Ruger Chemical Co., Irvington, NJ). The preparation and fatty acid compositions of these diets have been reported (11). Fresh diets were prepared every week, and they were analyzed every 3–4 days for fatty acid composition and for autoxidation. When initially prepared, the coconut oil diet contained 7% unsaturated fatty acids as measured by gas–liquid chromatography and 15 to 30 nmol/g of malondialdehyde as determined by the thiobarbituric acid method. The sunflower oil diet contained 89% unsaturated fatty acid and 20 to 55 nmol/g of malondialdehyde. If either the unsaturated fatty acid content decreased by more than 5% or the malondialdehyde value increased by more than 50%, the batch of diet was discarded and replaced with a fresh preparation. The animals were housed in a room maintained at 20–22°C with lights on from 0700 to 1900.

Preparation of microsomes

Rat liver microsomes were prepared as described by Kamp and Wirtz (12). Unless otherwise stated, two groups of rats from the same batch were maintained on the special diet for 40 days. The animals had free access to food and water until they were decapitated. Immediately after decapitation, the liver was removed and placed in cold Krebs Ringer phosphate buffer, pH 7.4. All the subsequent operations were carried out at 4°C. The livers were perfused with cold Krebs Ringer phosphate buffer, weighed, minced, and homogenized in 0.25 M sucrose–10 mM Tris-1 mM EDTA buffer, pH 7.4 (10% w/v) with a motor-driven Teflon pestle. Fractions of the homogenate were isolated by sedimentation at 600 *g* for 15 min, 15,000 *g* for 20 min, and 100,000 *g* for 60 min. The 100,000 *g* pellet was washed with 10 mM Tris HCl buffer, pH 8.6, followed by 1 mM Tris HCl buffer, pH 8.6. The washed microsomal pellet was suspended (4–6 μg/ ml) either in 0.154 M KCl for immediate use or in the sucrose buffer for storage in liquid nitrogen. No thiobarbituric acid reactive material was detected in any of the microsomal preparations that were used for this study.

Microsomal enzyme assays

Whenever possible, the freshly prepared microsomes suspended in 0.154 M KCl were tested immediately after preparation. If storage in liquid nitrogen was required, the microsomes were sedimented from the sucrose buffer, dispersed in 0.154 M KCl, resedimented, and then suspended in fresh 0.154 M KCl to give a protein concentration of 4–6 mg/ml. In order to measure peroxidase activity, microsomal suspensions (between 400 to 600 μg of protein), 0.025 M Tris HCl buffer, pH 7.5, 1 mM ADP, and 10 μM $FeCl_3$ were mixed, and the volume was made up to 3 ml with 0.154 M KCl. After incubation at 37°C for 5 min, the reactions were initiated by adding 50 μM NADPH. Appropriate incubations containing no enzyme or cofactors were included, and any peroxide formed in these controls was subtracted from the experimental values. The highest malondialdehyde value obtained in these control incubations was 0.20 to 0.45 nmol/mg protein, and the value for the control never exceeded 2.5% of the experimental value. After 10 min, the reaction was terminated with 0.1 ml trichloroacetic acid and malondialdehyde was measured spectrophotometrically with thiobarbituric acid (13) using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. In certain cases where sonicated phospholipids were contained in the assay, slightly elevated blank malondialdehyde values were obtained. These values were 0.32, 1.02, and 1.61 nmol of malondialdehyde per assay for dioleoyl-, dilinoleoyl- and diarachidonyl phosphatidylcholine, respectively. No increase in the blank value occurred with dipalmitoyl phosphatidylcholine. The blank malondialdehyde values were subtracted from the corresponding experimental values in each case.

Glucose 6-phosphatase activity was determined by measuring inorganic phosphate release (14). NADPH-cytochrome c reductase activity was determined spectrophotometrically by measuring the reduction of cytochrome c at 550 nm (15). Protein was estimated by the method of Lowry et al. (16) using bovine serum albumin as reference standard.

Lipid analysis

Lipids were extracted from the microsomes with 20 ml chloroform–methanol 2:1 (v/v) (17), and the washed chloroform phase was taken for analysis. Phospholipids were estimated by the method of Reheja et al. (18) using dipalmitoyl phosphatidylcholine as reference standard. Cholesterol was estimated by the cholesterol oxidase method (19). Vitamin E was measured by the fluorometric method of Taylor, Lamden, and Tappel (20). Phospholipid classes were separated by thin-layer chromatography on silica gel H with chloroform–methanol–acetic acid–water 100:60:16:8 (21). Phospholipids were eluted and measured by the method of Reheja et al. (18) with dipalmitoyl phosphatidylcholine as the standard.

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Fatty acid composition was determined by gas–liquid chromatography after the lipid samples were saponified with 0.5 N NaOH in methanol and methylated with 14% BF_3 in methanol (3). Fatty acid methyl esters were separated with a Hewlett-Packard 5710A gas chromatograph equipped with flame ionization detector and glass column (6 feet × 4 mm inside diameter) packed with 10% SP2330 on Chromosorb WAW. Peak areas were measured with Hewlett-Packard 3380A integrator. N_2 (40 ml/min) was used as the carrier gas. The individual fatty acids were identified by comparing their retention time with standards obtained from either Supelco, Inc., (Bellefonte, PA) or Nu-Chek Prep (Elysian, MN).

The fatty acid composition results are expressed in terms of nmol/mg microsomal protein. For conversion to molar values, the fatty acid percentages obtained from the chromatograms were multiplied by twice the amount of phospholipid measured as nmol/mg microsomal protein. This calculation assumes that there are two moles of fatty acid per mole of phospholipid. A similar procedure was used to convert the fatty acid percentages obtained from the chromatograms of the choline and ethanolamine phosphoglyceride fractions into molar quantities. First, the percentage that these two fractions contributed to the total microsomal phospholipid content was determined following separation by thin-layer chromatography (18, 21). From these percentages and the total microsomal phospholipid content, the amount of choline and ethanolamine phosphoglycerides present was calculated in nmol/mg microsomal protein. Finally, these values were multiplied by the fatty acid percentages obtained from the gas–liquid chromatograms to give values for each fatty acid in the two phospholipid fractions in terms of nmol/mg microsomal protein.

Membrane modification using phospholipid exchange proteins

Phospholipids dissolved in organic solvent were evaporated to dryness under a stream of N_2 and 0.154 M KCl was added to give a concentration of 2.5 mM. After vortex mixing, the suspension was sonicated for 1 min in ice under a stream of N_2. This procedure produced a clear suspension of phospholipid vesicles. When dipalmitoyl phosphatidylcholine vesicles were prepared, the sonication was done at 45°C.

Phospholipid exchange protein was purified from beef liver (22). Rat liver microsomes (500–600 μg protein) in the sucrose buffer, pH 7.4, were incubated with diarachidonyl phosphatidylcholine liposomes (556 μg) in the presence or absence of 150 μl of purified phospholipid exchange protein (1975 units) at 37°C for 60 min. The mixture was layered over 7 ml of 20% sucrose in 10 mM Tris HCl buffer, pH 7.4, and centrifuged at 100,000 *g* for 30 min. The microsomal pellet was washed and suspended in 0.154 M KCl. These microsomes were used for lipid analysis and enzyme assay.

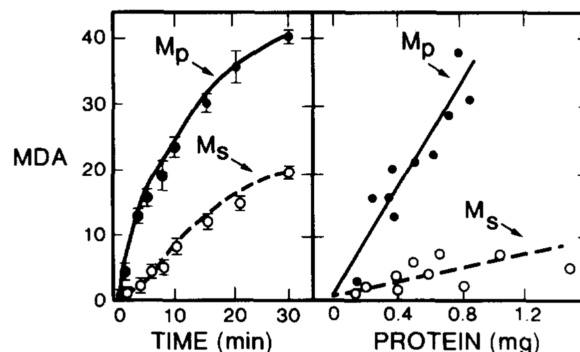

Fig. 1. Effect of time and protein concentration on microsomal lipid peroxidation. The reaction mixture contained 400–600 μg of microsomal protein in 0.154 M KCl, 0.025 M Tris HCl buffer, pH 7.5, 1 mM ADP, and 10 μM $FeCl_3$. After equilibrating at 37°C for 5 min, the reaction was initiated by adding 50 μM NADPH. The incubations were terminated at the times indicated on the left by adding 0.1 ml of trichloroacetic acid, and the malondialdehyde formed was measured. Each value is the mean ± SE of four separate microsomal preparations. The experimental conditions were the same in the experiment shown on the right except that the microsomal protein concentration was varied. All of the incubations were for 10 min. The data points were obtained from three different microsomal preparations. Peroxidation is expressed as malondialdehyde formed (MDA) in nmol/mg protein on the left side and nmol/10 min on the right side.

RESULTS

Differences in peroxidation

Incubation of rat liver microsomes at 37°C in the presence of NADPH, ADP, and $FeCl_3$ resulted in the formation of malondialdehyde as detected by the thiobarbituric acid assay. As seen in **Fig. 1** (Left side), the amount of malondialdehyde formed at each time point was considerably greater with the microsomes prepared from the livers of rats fed the sunflower oil diet (M_p) as compared with those from the livers of rats fed the coconut oil diet (M_s). The rate with M_p was linear for approximately 10 min, after which time it decreased somewhat. With M_s, the reaction rate was quite slow for the first 4 min. After this initial lag period, the rate increased and was approximately linear for the next 6 to 8 min before it decreased. The slope of the linear increase in malondialdehyde formation was 2.4 nmol/mg protein per min for M_p, whereas it was only 0.6 nmol/mg protein per min for the linear segment of the M_s curve. At the end of the 30-min incubation, the amount of malondialdehyde formed by M_p was twice as much as that formed by M_s.

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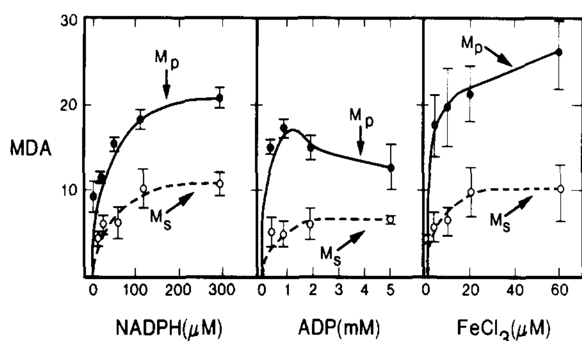

Fig. 2. Effect of cofactors on microsomal lipid peroxidation. The experimental conditions are the same as in Fig. 1 except for the variations in NADPH, ADP, and $FeCl_3$ concentrations. The values are the mean ± SE of four separate microsomal preparations. Peroxidation is expressed as malondialdehyde formed (MDA) in nmol/mg protein during 10 min of incubation.

Since the assay required a period of color development, it was not feasible to carry out a time-dependent measurement in order to obtain the actual linear rate for each sample. Considering the differences in the reaction progress curves for M_p and M_s, the best single time point to estimate the initial rate was judged to be 10 min, and this time was selected for all subsequent measurements. While the 10-min values provide a reasonable comparison between M_p and M_s, they are not suitable for calculation of kinetic parameters because they are only estimates of the initial rate.

As shown on the right side of Fig. 1, higher rates of malondialdehyde formation occurred with M_p at all of the microsomal protein concentrations tested when the 10-min incubation time was employed. Similar results were obtained in additional experiments in which the time of incubation was extended to 15 or 30 min; at each protein concentration tested, the peroxidation obtained with M_p was considerably greater than that obtained with M_s.

In order to determine whether the observed differences in peroxidation might be due to some limitation of the assay system, the concentrations of NADPH, ADP, and $FeCl_3$ were varied as is shown in **Fig. 2.** Under all of the conditions tested, considerably higher malondialdehyde values were obtained with M_p. For all subsequent studies, the concentrations employed were 50 μM NADPH, 1 mM ADP and 10 μM $FeCl_2$. As seen in Fig. 2, somewhat higher peroxidation values could be obtained in certain instances if larger concentrations of these cofactors were added. In no case, however, did these moderate increases lead to any alteration in the conclusion that peroxidation was greater with M_p than with M_s. In another experiment, the cofactor mixture was added five separate times to the microsome preparations, each incubation lasting for 10 min. As a result of the first exposure to cofactors, the M_p preparations produced 50 ± 2 nmol/mg protein or malondialdehyde, whereas the M_s preparations produced 11 ± 4 nmol/mg protein. When the values from the five sequential incubations were added together, the total increased only to 55 ± 2 nmol/mg protein for the M_p preparations and to 13 ± 0.8 nmol/mg protein for the M_s preparations. These results demonstrate that the observed differences between M_p and M_s are valid in spite of the fact that the cofactor concentrations employed in the routine assays were slightly suboptimal. The ability of the microsomes to oxidize the multiple additions of NADPH was not measured in this experiment. Therefore, it is possible that the failure to observe continuing increments in lipid peroxidation was due to a limitation in cofactor oxidation and not to the peroxidation process itself.

In order to determine whether the differences in peroxidation might be due to differences in the antioxidant content of the microsomes, the vitamin E content of M_p and M_s was measured. The values obtained were 0.35 ± 0.05 μg/mg protein for M_p and 0.45 ± 0.02 μg/mg protein for M_s. Although this indicated a tendency toward lower values in M_p, the difference was not statistically significant ($0.1 < P < 0.2$, n = 7).

Lipid modifications

The fatty acid compositions of M_p and M_s were considerably different as shown in **Table 1.** The phospholipids of M_p contained 22% more polyunsaturates, whereas the phospholipids of M_s contained 89% more monounsaturates. These differences were due primarily to changes in their 18:1[1], 18:2, and 20:4 contents. M_p was enriched in 18:2 and 20:4, whereas M_s was enriched in 18:1. No differences in the saturated fatty acid content of the M_p and M_s phospholipids were observed, with saturates accounting for about 45% of the fatty acyl groups in both cases. The differences in the fatty acid compositions of the M_p and M_s phospholipids were similar to those observed for the total lipid extracts of these microsomes (see Table 4). During peroxidation, 55% of the 20:4, 50% of the 22:5, and 58% of the 22:6 in M_p phospholipids were depleted. These values are very similar to those reported by Tam and McCay (9) for hepatic microsomes from rats maintained on

[1] The fatty acids are abbreviated as number of carbon atoms: number of double bonds. Therefore, 18:1 represents an 18 carbon atom acid containing one double bond.

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ordinary rodent chow. Lesser amounts of the initial 20:4, 22:5, and 22:6 content were depleted in M_s during peroxidation, the values being 18, 4, and 30%, respectively.

The phospholipid and cholesterol contents of M_p and M_s were similar, the values being 585 ± 20 and 46 ± 2 μg/mg microsomal protein, respectively. These values were not significantly decreased following the peroxidation reaction. Choline phosphoglycerides, which comprised about 70% of the microsomal phospholipids in both M_p and M_s, were not reduced following peroxidation. Ethanolamine phosphoglycerides accounted for about 17% of the microsomal phospholipids in both cases. They also were not reduced following peroxidation in M_s. In M_p, however, the ethanolamine phosphoglyceride content decreased by 41% following peroxidation.

Fractionation of the phospholipids indicated that the phosphatidylcholine fraction exhibited the most marked changes. Phosphatidylcholine in M_p had about a twofold enrichment of 18:2 and 20:4, whereas phosphatidylcholine in M_s had twofold increase in 18:1. Phosphatidylethanolamine was modified to a lesser extent and contained about the same amount of 20:4 in both M_p and M_s. Both of these fractions contributed 20:4, 22:5, and 22:6 for peroxidation. As a percentage of their initial fatty acid composition, each fraction contributed fatty acids to roughly the same extent. Since the choline phosphoglycerides accounted for about 70% of the phospholipid in the microsomal preparations, whereas the ethanolamine phosphoglycerides accounted for only about 17%, the choline fraction contributed much more total fatty acid for peroxidation. Some 18:2 was depleted from the ethanolamine fraction during peroxidation, especially in M_p, but this did not occur to any appreciable extent in the choline fraction. Fatty acid analysis of the serine plus inositol phosphoglyceride and the phosphatidic acid fractions revealed that these phospholipids did not contribute much fatty acid for peroxidation in either M_p or M_s.

Effect of added arachidonic acid

Since 20:4 was the major inherent substrate for peroxidation and appeared to be the limiting factor in M_s, we examined the effect of adding this fatty acid to the microsomes on the peroxidative activity. Arachidonate was added in the form of a complex with albumin. The amount of albumin was increased as the arachidonate concentration was raised so that the molar ratio of arachidonate to albumin was 4.0 in all cases. As shown on the left side of **Fig. 3,** addition of up to 2 mM arachidonic acid did not stimulate peroxidation in M_s, and peroxidation in

TABLE 1. Fatty acid composition of membrane phospholipids[a]

	Fatty Acyl Composition											
	Total Phospholipids				Choline Phosphoglycerides				Ethanolamine Phosphoglycerides			
	M_p		M_s		M_p		M_s		M_p		M_s	
Fatty Acid	Before Peroxidation	After Peroxidation	Before Peroxidation	After Peroxidation	Before Peroxidation	After Peroxidation	Before Peroxidation	After Peroxidation	Before Peroxidation	After Peroxidation	Before Peroxidation	After Peroxidation
	nmol/mg protein											
16:0	270 ± 22	336 ± 36	282 ± 7.4	278 ± 7.4	192 ± 24	268 ± 6.6	134 ± 8.0	196 ± 11	40 ± 5.8	58 ± 4.8	40 ± 2.6	70 ± 4.2
18:0	386 ± 26	502 ± 26	370 ± 12	420 ± 12	266 ± 9.2	274 ± 6.8	298 ± 8.6	344 ± 13	60 ± 2.8	62 ± 0.8	52 ± 1.8	60 ± 3.4
18:1	108 ± 7.2	130 ± 5.6	200 ± 16	238 ± 24	98 ± 5.0	106 ± 2.2	220 ± 8.4	238 ± 24	32 ± 3.0	30 ± 3.0	36 ± 4.0	36 ± 3.2
18:2	174 ± 4.2	182 ± 8.4	88 ± 1.4	96 ± 4.6	112 ± 7.8	102 ± 6.4	72 ± 4.8	56 ± 5.4	22 ± 1.8	11 ± 1.6	8.6 ± 1.0	7.6 ± 1.8
20:4	332 ± 36	148 ± 50	242 ± 19	206 ± 17	230 ± 13	124 ± 11	106 ± 10.2	68 ± 11	52 ± 4.4	24 ± 4.2	54 ± 4.2	40 ± 2.4
22:5	26 ± 5.6	12.6 ± 2.8	34 ± 4.4	34 ± 9.2	20 ± 2.2	10.4 ± 1.6	24 ± 5.2	14 ± 2.6	11 ± 0.2	8.8 ± 0.4	9.4 ± 1.6	7.6 ± 1.6
22:6	17 ± 4.2	7.0 ± 1.4	40 ± 3.0	30 ± 6.2	10.6 ± 1.2	3.4 ± 0.6	15 ± 5.2	11 ± 0.8	5.6 ± 0.6	3.6 ± 0.2	13 ± 2.4	7.8 ± 1.4

[a] Phospholipids were separated from neutral lipid by thin-layer chromatography. Individual phospholipids also were separated by thin-layer chromatography. After saponification and methylation, fatty acids were analyzed by gas–liquid chromatography. The values are mean ± SE of six different microsomal preparations.

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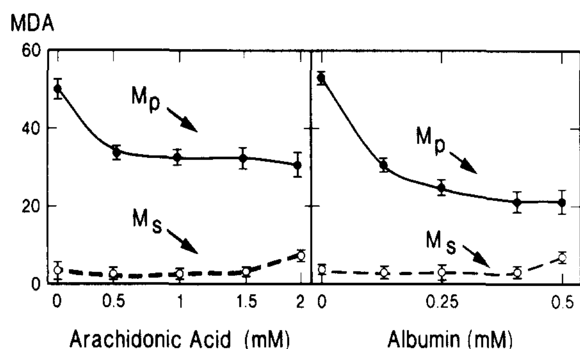

Fig. 3. Effect of arachidonic acid and albumin concentrations on microsomal lipid peroxidation. As the arachidonic acid concentration was raised, the albumin concentration was raised from 0.125 to 0.5 mM so that the molar ratio of arachidonate to albumin remained 4 in each case. The values are mean ± SE of four separate microsomal preparations. Peroxidation is expressed as malondialdehyde formed (MDA) in nmol/mg protein during 10 min of incubation.

M_p was inhibited. The degree of inhibition in M_p was about the same at all of the arachidonate concentrations tested. In order to determine whether the added albumin might be partly responsible for the inhibition noted with M_p, the effect of albumin itself was tested. As seen on the right side of Fig. 3, albumin when added in the same concentrations as in the arachidonic acid experiment inhibited the peroxidation activity in M_p, suggesting that albumin rather than arachidonate was the main inhibitory factor. Albumin itself had little effect on peroxidation in M_s.

In further experiments, ATP, CoA, and $MgCl_2$ were added together with arachidonic acid in order to determine whether stimulation of peroxidation might require a prior conversion of the added fatty acid to fatty acyl CoA, perhaps in order to facilitate

TABLE 2. Effect of added arachidonic acid on lipid peroxidation[a]

Additions	Malondialdehyde Formed	
	M_p	M_s
	nmol/mg protein	
None	56.5 ± 2.3	5.7 ± 0.3
Arachidonic acid (0.25 mM)	39.0 ± 3.4	3.4 ± 0.8
Arachidonic acid (0.25 mM) +ATP (3 mM) +CoA (0.1 mM) +$MgCl_2$ (3 mM)	36.4 ± 1.4	10.7 ± 1.3

[a] Arachidonic acid dissolved in 0.15 M KCl by sonication was incubated with microsomes in the presence or absence of ATP, CoA, and $MgCl_2$ for 10 min at 37°C. Lipid peroxidation was then initiated by adding ADP, $FeCl_3$, and NADPH. Appropriate fatty acid blanks were included, and the blank values were subtracted from experimental values. Results are the mean ± SE of three microsomal preparations.

TABLE 3. Effect of phosphatidylcholines on lipid peroxidation[a]

Addition	Malondialdehyde Formed	
	M_p	M_s
	nmol/mg protein	
None	55.6 ± 1.9	3.4 ± 0.5
Diarachidonyl phosphatidylcholine	103.6 ± 2.0	105.9 ± 5.2
Dioleoyl phosphatidylcholine	53.8 ± 1.8	13.0 ± 1.1
Dilinoleoyl phosphatidylcholine	58.6 ± 2.4	43.7 ± 1.0
Dipalmitoyl phosphatidylcholine	54.4 ± 3.8	3.8 ± 0.4

[a] Phosphatidylcholine liposomes (210 μg) were added to the assay system and malondialdehyde formation was determined after 10 min of incubation. Appropriate liposome blanks were included, and the values obtained were subtracted from the experimental values. The results are mean ± SE of four microsomal preparations.

incorporation of the added arachidonate into microsomal lipids. Preliminary experiments indicated that [^{3}H]arachidonate was esterified into microsomal lipids only when these cofactors were added. As shown in **Table 2,** a threefold stimulation in peroxidation was observed in M_s when the cofactors were present. These results suggest that added arachidonate can stimulate peroxidation in microsomes containing a relatively low inherent arachidonate content, but only if it can be activated. Even though the degree of stimulation in M_s was relatively large, the absolute peroxidation value was far below that which inherently occurred in M_p.

Effect of added phosphatidylcholine

As shown in **Table 3,** peroxidation was stimulated considerably in both M_p and M_s when the assay system contained diarachidonyl phosphatidylcholine. The concentration of diarachidonyl phosphatidylcholine used in these experiments produced maximal peroxidation activity with both M_p and M_s. Under these conditions the activity was stimulated 31-fold in M_s, but only about 2-fold in M_p. Yet, the resulting absolute activities in both microsomal preparations were similar and almost twice as high as the maximum values obtained with M_p when only inherent substrate was available. None of the phosphatidylcholines containing other fatty acyl groups stimulated peroxidation in M_p. In M_s, however, dioleoyl phosphatidylcholine produced a 3.4-fold stimulation in peroxidation, and dilinoleoyl phosphatidylcholine produced a 13-fold stimulation. In both of these cases, however, the peroxidation level to which M_s was stimulated was less than the value obtained with M_p when no substrate was added.

In an attempt to explore the mechanism whereby diarachidonyl phosphatidylcholine stimulated peroxidation, we added this phospholipid to microsomes

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TABLE 4. Microsomal fatty acid modifications produced by incubation with diarachidonyl phosphatidylcholine[a]

Fatty Acid	Fatty Acid Composition					
	M_p			M_s		
	Control	DAPC	DAPC + PLEP	Control	DAPC	DAPC + PLEP
	nmol/mg protein					
16:0	214 ± 18	200 ± 13	186 ± 15	248 ± 5.8	186 ± 38	202 ± 7.4
16:1	16 ± 4.2	16 ± 2.8	14 ± 2.8	58 ± 5.8	44 ± 4.4	46 ± 5.8
18:0	366 ± 11	346 ± 12	310 ± 12	344 ± 12	352 ± 10	298 ± 5.8
18:1	106 ± 8.6	100 ± 5.5	98 ± 5.6	298 ± 12	254 ± 3.0	224 ± 4.4
18:2	184 ± 2.8	174 ± 4.2	158 ± 7.2	92 ± 5.4	94 ± 1.4	90 ± 3.0
20:3	14 ± 1.2	13 ± 2.2	10 ± 1.6	108 ± 5.8	108 ± 4.4	90 ± 3.0
20:4	432 ± 17	494 ± 5.5	602 ± 22	184 ± 10	320 ± 5.8	444 ± 10
22:5	38 ± 5.6	32 ± 4.2	22 ± 7.2	32 ± 7.0	32 ± 0.4	24 ± 0.8
22:6	19 ± 1.2	15 ± 0.4	11 ± 0.8	30 ± 1.4	32 ± 2.2	22 ± 1.4

[a] Microsomes were incubated with diarachidonyl phosphatidylcholine liposomes (DAPC) for 60 min in the presence or absence of phospholipid exchange protein (PLEP). After incubation, the microsomes were isolated by sedimentation and their total fatty acid compositions were analyzed. The results are mean ± SE of four microsomal preparations.

in the presence of beef liver phospholipid exchange protein. Lipid analyses and assays were carried out after the microsomes were isolated from this incubation medium by sedimentation. As shown in **Table 4,** the 20:4 content of M_s was increased 2.4-fold when liposomes and phospholipid exchange protein were present. Even without phospholipid exchange protein, prior incubation with diarachidonyl phosphatidylcholine increased the 20:4 content of M_s by 1.8-fold. Lesser increases in 20:4 content were produced in M_p, although even in this case a 39% increase was observed when phospholipid exchange protein was present. There were no statistically significant increases in the total phospholipid content of any of the microsome preparations following incubation with the liposomes. Therefore, the differences in fatty acid composition result primarily from exchange of liposomal and microsomal phospholipids.

Peroxidation values for the microsomes pretreated with diarachidonyl phosphatidylcholine are presented in **Table 5.** No stimulation was produced in M_p. By contrast, there was a 5-fold stimulation in M_s when the microsomes were incubated initially with the liposomes alone and a 15-fold stimulation when phospholipid exchange protein was contained in the incubation mixture.

Length of feeding period

In order to determine the rapidity with which changes in microsomal fatty acid composition and lipid peroxidation can occur, we fed weanling rats the fat-supplemented diet for different lengths of time. As shown in **Fig. 4,** maximum changes in microsomal unsaturated fatty acid composition occurred within 3 to 7 days. Within 3 days, the 20:4 content of M_p was twice as high as in M_s. Similarly 18:2 was increased twofold and 18:1 was decreased twofold within 3 days. The changes in 22:6 content occurred more slowly, and the percentage of this acid decreased in both M_p and M_s. No significant differences in the percentage of saturated fatty acids were observed between M_p and M_s during the 40-day feeding period.

As shown in **Table 6,** maximum differences in the peroxidation activity between M_p and M_s were observed within 24 hr after the high-fat diets were begun. The values obtained with M_p were 10 to 16 times higher than those obtained with M_s. By contrast, there was no consistent difference in the NADPH-cytochrome c reductase activity of M_p and M_s. Glucose 6-phosphatase activity was higher in M_s

TABLE 5. Lipid peroxidation in hepatic microsomes modified by incubation with diarachidonyl phosphatidylcholine liposomes[a]

Treatment	Malondialdehyde Formed	
	M_p	M_s
	nmol/mg protein	
None	61.1 ± 1.8	3.0 ± 0.1
DAPC[b]	59.5 ± 5.8	14.8 ± 3.0
DAPC + PLEP[c]	65.0 ± 3.9	45.6 ± 5.8

[a] Microsomes were treated with diarachidonyl phosphatidylcholine liposomes in the presence or absence of phospholipid exchange protein. Lipid peroxidation in these modified microsomes was determined by measuring the amount of malondialdehyde formed during a 10-min incubation period. The results are mean ± SE of four microsomal preparations.
[b] Diarachidonyl phosphatidylcholine.
[c] Phospholipid exchange protein.

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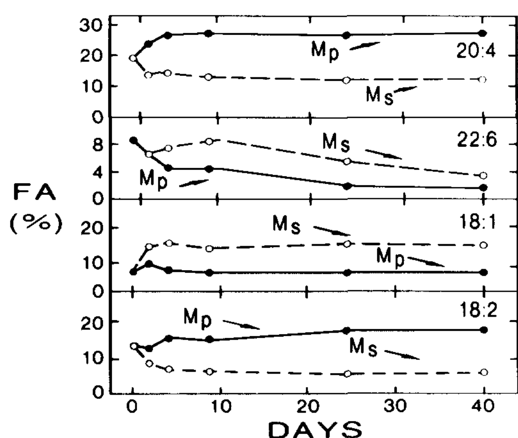

Fig. 4. Changing microsomal polyunsaturated fatty acid composition during the feeding period. Weanling rats were maintained on the high fat diet for the time intervals indicated. The values represent the amount of a given fatty acid as a percent of the total fatty acid contained in the microsomes. Each point is the average value of duplicate determinations from two microsomal preparations.

than in M_p between the 8th and 40th day, but the difference was only about 20–25%. Thus, individual enzymes respond differently and to different extents to a given microsomal fatty acid modification. Although the rapid reduction in peroxidation probably is due to the decrease in polyunsaturated fatty acid content of M_s, it is possible that it could have resulted from the presence of an antioxidant in the diet supplemented with coconut oil.

In order to determine the reversibility of the microsomal fatty acid modifications and the changes in peroxidation activity, a group of animals was transferred from one diet to the other. After rats were fed the coconut oil diet for 60 days, they were

TABLE 6. Comparison of enzyme activities in liver microsomes[a]

Length of Feeding Period	Microsomal Preparation	NADPH-dependent Lipid Peroxidation	NADPH Cytochrome c Reductase	Glucose 6-phosphatase
days		*nmol/mg protein/10 min*	*mμeq/mg protein/min*	*μmol Pi/mg protein/10 min*
1	M_p	55.8	101.6	9.9
	M_s	3.4	91.0	8.1
8	M_p	55.9	113.6	11.9
	M_s	4.8	121.2	14.5
24	M_p	50.3	96.5	9.9
	M_s	6.8	104.4	12.2
40	M_p	55.6	115.3	9.6
	M_s	5.7	101.9	12.1

[a] Microsomes were prepared from the livers of rats maintained on special diets for the time intervals indicated. Results are the average values of duplicate determinations for two microsomal preparations.

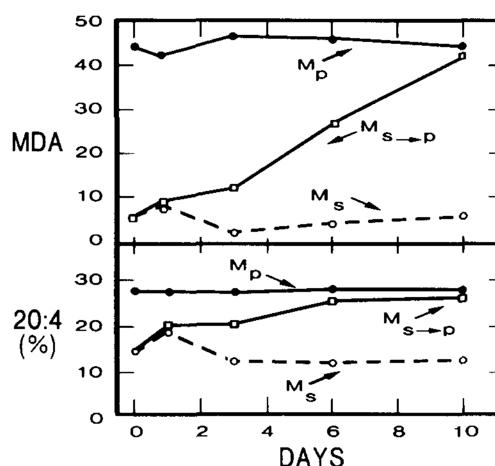

Fig. 5. Reversibility of dietary effects on lipid peroxidation and arachidonic acid content. Three groups of rats were maintained on the special diets for 60 days. One was fed the diet rich in polyunsaturated fat (P), and two were fed the diet rich in saturated fat (S). The values obtained at the end of this 60-day period for hepatic microsomal lipid peroxidation and arachidonic acid are shown as Day 0. Peroxidation is expressed as malondialdehyde formation (MDA) in nmol/mg protein during 10 min of incubation. Arachidonic acid content (20:4) is expressed as a percentage of the total microsomal fatty acids. One group of rats was maintained on the saturated and another on the polyunsaturated fat diets for an additional 10 days. The values for these two groups of rats are designated on the figure as M_s and M_p, respectively. The third group of rats was switched for the final 10-day period from the saturated to the polyunsaturated fat-rich diet. The peroxidation and arachidonate values for this group are designated on the figure as $M_{s\rightarrow p}$. Each point represents the average of triplicate values obtained from a single liver microsomal preparation.

transferred to the sunflower oil diet. As seen in the lower portion of **Fig. 5,** the 20:4 content of the microsomes in this group increased to the level present in M_p within 10 days after transfer to the sunflower oil diet. Similarly, as shown in the upper portion of Fig. 5, the peroxidation activity of the microsomes increased to the level present in M_p within 10 days. These findings indicate that both the fatty acid compositional modifications and the decrease in peroxidase activity are reversible fairly quickly even after relatively long-term feeding of a highly saturated diet. Moreover, the parallelism between repletion of the arachidonic acid content and recovery of the peroxidation activity strongly suggests that the two parameters are correlated.

DISCUSSION

These results indicate that changes in dietary fat saturation can influence the activity of NADPH-dependent lipid peroxidation in liver microsomes. A similar effect has been observed for the ascorbate-dependent peroxidation system (1). The decrease in

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peroxidation produced by feeding a diet rich in saturated fat was associated with an 18% reduction in total polyunsaturation and a 30% reduction of 20:4 in microsomal phospholipids. When the effect was reversed by transferring the rats to a diet rich in polyunsaturated fat, a doubling of the 20:4 content in microsomal phospholipids accompanied the restoration of peroxidation activity. Taken together, these findings suggest that changes in dietary fat saturation alter microsomal lipid peroxidation by modifying the polyunsaturated fatty acid composition, particularly the 20:4 content, of the microsomal phospholipids that serve as substrates for this reaction.

Several other possibilities for the lesser peroxidation activity in M_s appear to be unlikely. One of these is that M_s contains less of the enzymes involved in this reaction. When M_s was incubated with diarachidonyl phosphatidylcholine in the presence of phospholipid exchange protein, there was a 15-fold increase in peroxidation, and the maximum activity achieved was about 70% of that obtained with M_p. Furthermore, when diarachidonyl phosphatidylcholine was present during the assay, very high peroxidation rates were observed, and the activities in M_p and M_s were equalized. Therefore, M_s is capable of carrying out peroxidation at high rates provided that substrate is made available, indicating that it contains adequate amounts of functional enzymes. Another factor that argues against any difference in enzyme content is the observation that the NADPH-cytochrome c reductase activities were approximately equal in M_p and M_s. This enzyme is involved in the initiation and propagation of the peroxidation process (15, 23, 24). It is clear that M_s contains the usual amount of this activity. Another possibility that can be excluded is that M_s contains less total phospholipids, the deficiency of polyunsaturates being secondary to a decrease in these membrane lipids. Direct chemical measurements revealed that M_s contained about the same amount of phospholipid relative to either protein or cholesterol as M_p. Likewise, chemical measurements indicated that the vitamin E contents of the two microsomal preparations were not significantly different. Since these alternative possibilities can be excluded, it appears that the lesser peroxidative activity in M_s can be explained best by a reduction in the polyenoic fatty acids that normally serve as substrates for this reaction.

Arachidonate added in the form of phosphatidylcholine stimulated peroxidation, but unesterified arachidonate did not. The unesterified arachidonate was added in two forms, as an albumin complex (Fig. 3) or as a sonified dispersion (Table 2). While it is conceivable that the added arachidonate was not accessible to the microsomes, previous studies have shown that other long-chain fatty acids can be incorporated into cholesteryl esters by isolated microsomes when they were added in either of these forms (25, 26). Furthermore, the sonified solution of arachidonic acid stimulated peroxidation in M_s to some extent when ATP and CoA were added, indicating that this form of arachidonate was accessible to the microsomes under certain conditions. The failure of unesterified arachidonate by itself to stimulate suggests that the fatty acid may have to be contained in phospholipids in order to undergo peroxidation by the NADPH-dependent system. In this regard, May and McCay (8) have previously shown that peroxidation occurs while the polyunsaturated acid remains attached to phospholipids within the microsomes. It is possible that only in this form can arachidonate gain access to the peroxidation enzymes. The apparent requirement for a phospholipid substrate is unexpected because arachidonate esters undergo hydrolysis before entering into other oxidative reactions, such as those mediated by cyclooxygenase or lipoxygenase (27–29). On the other hand, Pugh and Kates (30, 31) found that polyenoic fatty acids can be further desaturated by rat liver microsomes without prior hydrolysis from phosphatidylcholine, indicating that at least one other type of microsomal lipid oxidation can utilize an esterified fatty acid substrate. As opposed to our findings, Wills (32) has observed that unesterified linoleic and linolenic acids can be utilized by liver microsomes as a substrate for peroxidation. While these acids may behave differently from arachidonate, it is more likely that they acted through a nonspecific detergent effect since the concentration employed was 16.7 mM (33, 34).

The stimulation of peroxidation produced when unesterified arachidonate was added to M_s together with ATP and CoA suggests that arachidonyl CoA may serve as a substrate for this process. Since peroxidation was not stimulated in M_p under these conditions, a more likely explanation is that acyl CoA formation enabled the arachidonate to be incorporated into phospholipids and that this stimulated peroxidation in M_s because its phospholipids are deficient in polyenoic fatty acyl groups. The addition of dioleoyl phosphatidylcholine to the assay system also stimulated peroxidation somewhat in M_s. This is difficult to explain because malondialdehyde cannot be formed from oleic acid. Therefore, the presence of these liposomes probably somehow activated the peroxidation of polyunsaturated substrates contained in M_s. Yet, this is unlikely to be a nonspecific activation because dipalmitoyl phosphatidylcholine did not produce any stimulation when added to M_s.

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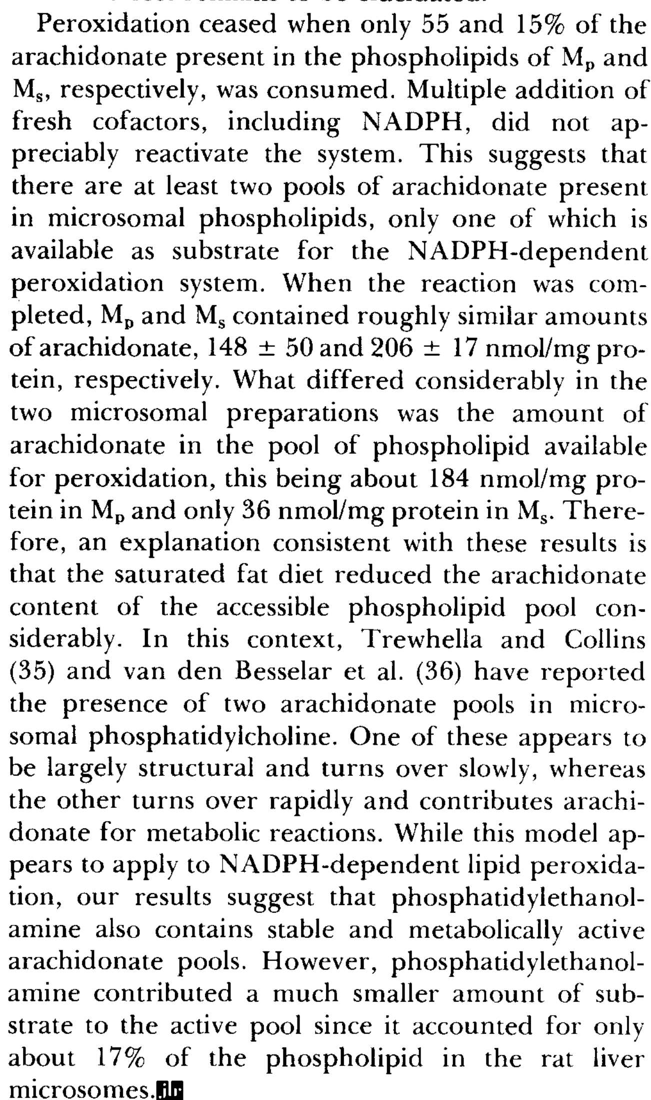

The exact mechanism of the dioleoyl phosphatidylcholine effect remains to be elucidated.

Peroxidation ceased when only 55 and 15% of the arachidonate present in the phospholipids of M_p and M_s, respectively, was consumed. Multiple addition of fresh cofactors, including NADPH, did not appreciably reactivate the system. This suggests that there are at least two pools of arachidonate present in microsomal phospholipids, only one of which is available as substrate for the NADPH-dependent peroxidation system. When the reaction was completed, M_p and M_s contained roughly similar amounts of arachidonate, 148 ± 50 and 206 ± 17 nmol/mg protein, respectively. What differed considerably in the two microsomal preparations was the amount of arachidonate in the pool of phospholipid available for peroxidation, this being about 184 nmol/mg protein in M_p and only 36 nmol/mg protein in M_s. Therefore, an explanation consistent with these results is that the saturated fat diet reduced the arachidonate content of the accessible phospholipid pool considerably. In this context, Trewhella and Collins (35) and van den Besselar et al. (36) have reported the presence of two arachidonate pools in microsomal phosphatidylcholine. One of these appears to be largely structural and turns over slowly, whereas the other turns over rapidly and contributes arachidonate for metabolic reactions. While this model appears to apply to NADPH-dependent lipid peroxidation, our results suggest that phosphatidylethanolamine also contains stable and metabolically active arachidonate pools. However, phosphatidylethanolamine contributed a much smaller amount of substrate to the active pool since it accounted for only about 17% of the phospholipid in the rat liver microsomes. JLR

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