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RELATIONSHIP BETWEEN MALONDIALDEHYDE PRODUCTION AND ARACHIDONATE CONSUMPTION DURING NADPH-SUPPORTED MICROSOMAL LIPID PEROXIDATION*

RONALD A. JORDAN and JOHN B. SCHENKMAN†
Department of Pharmacology, University of Connecticut, Health Center, Farmington, CT 06032, U.S.A.

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Abstract—Fatty acid concentrations and malondialdehyde formation were determined before and during NADPH-supported lipid peroxidation in liver microsomes from rat, mouse, guinea pig and rabbit. In agreement with earlier reports, malondialdehyde production was greatest for rat, followed by mouse, and much less for guinea pig and rabbit. The microsomal content of total unsaturated fatty acids (18:1, 18:2, 18:3, 20:3, 20:4, 20:5, 22:5, 22:6) was approximately the same for rat and mouse and was lower in guinea pig and rabbit. Lipid peroxidation caused a time-dependent decrease in the polyunsaturated fatty acids, particularly 20:4 and 22:6, for all species. These decreases were most pronounced for rat and mouse. Alterations in the dietary regime for rat produced marked changes in microsomal fatty acid content as reported by others, and also caused changes in the rates of malondialdehyde production and polyunsaturated fatty acid consumption during lipid peroxidation. A comparison between the rates of malondialdehyde production and the rates of individual unsaturated fatty acid consumption was performed for each animal species and for rats fed different diets. A linear relationship was found between malondialdehyde production and 20:4 disappearance in individual microsomal preparations and in different species. A similar relationship was seen for the initial microsomal concentration of 20:4 and the initial rate of malondialdehyde formation. Other unsaturated fatty acids did not exhibit linear relationships. Various microsomal mixed-function oxidase variables were measured for the different species. No direct relationship between these values and malondialdehyde production was found.

Enzymic lipid peroxidation is dependent upon the presence of chelated iron [1] for the production of malondialdehyde (MDA) or MDA precursors, which react with thiobarbituric acid to yield a colored complex [2, 3]. Such products have also been observed during liver microsomal lipid peroxidation, as well as during nonenzymatic oxidation of fatty acids with three or more double bonds [4] and in the enzymatic oxidation of tri- and tetraenoic fatty acids by platelets, presumably during breakdown of prostaglandin endoperoxide intermediates [5].

Studies showing changes in fatty acid composition in liver microsomes during lipid peroxidation have not identified the source of malondialdehyde since several polyunsaturated fatty acids decrease during the process. Most of the polyunsaturated fatty acids have been suggested to be MDA precursors. May and McCay [6] examined changes in fatty acid composition and noted decreases in certain polyunsaturated fatty acids, but primarily arachidonate (20:4) and docosahexaenoate (22:6). Malondialdehyde production, however, accounted for only 12% of the fatty acids consumed [7]. Similar decreases in phospholipid unsaturated fatty acids were also noted by Högberg *et al.* [8].

Niehaus and Samuelsson [9] demonstrated that addition of tritiated arachidonyl phosphatidylcholine to liver microsomes could give rise to MDA of specific activity similar to the added arachidonate. They concluded that phospholipid bound arachidonate and probably docosahexaenoate are precursors of MDA. In support of this suggestion, Lokesh *et al.* [10] recently found that MDA production is enhanced by fortification of rat liver microsomes with diarachidonyl phosphatidylcholine.

Although many studies have related polyunsaturated fatty acid changes to malondialdehyde production by liver microsomes, the fact that the concentration of many polyunsaturated fatty acids decreases during lipid peroxidation makes delineation of specific precursors difficult. The studies reported here examine this relationship and indicate that arachidonate content and rate of disappearance can be linearly related to MDA production.

MATERIALS AND METHODS

Animals. Male animals from various sources were used: CD (caesarean derived) Sprague–Dawley rats (200–220 g) and CF1 mice (24–29 g), Charles River Breeding Laboratories, Wilmington, MA; New Zealand rabbits (2–2.3 kg), Jenks, Feedings Hill, MA; and guinea pigs (330–350 g), Buckberg Laboratories, Tonkins Cove, NY. Animals had free access to water and Purina laboratory chow (Ralston Purina Co.).

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† Author to whom correspondence should be addressed.

For studies involving dietary changes, rats were given normal laboratory chow and then either were fasted 24 hr prior to their being killed or were fed a fat-free (high carbohydrate) diet (ICN Nutritional Biochemicals, Cleveland, OH) for 5 days prior to sacrifice. These treatments markedly influence lipid peroxidation [11] and liver microsomal lipid composition [12, 13].

Preparation of liver microsomes. Animals were killed by decapitation and the livers were perfused *in situ* with ice-cold saline before removal. Microsomes were prepared by the calcium chloride aggregation procedure [14]. The microsomal pellet was washed and suspended in cold 0.15 M potassium chloride at a protein concentration of 10 mg/ml. The microsomal protein concentration was determined by the biuret method. Freshly prepared microsomes from rat, mouse, guinea pig and rabbit were used for all studies except those involving dietary changes in rats, where both fresh and frozen microsomes (2 days at −70°) were used. Freezing did not have any apparent effect on the rate of MDA formation or on the fatty acid composition.

Enzyme assays. Assay mixtures for lipid peroxidation were incubated in flasks open to air in a shaking water bath at 37°. Assays contained 0.5 mg microsomal protein per ml, 20 mM sodium phosphate buffer (pH 7.5), 0.15 mM potassium chloride, and 50 μM ferric pyrophosphate (K & K Laboratories Inc., Plainview, NY). The reactions were initiated by the addition of 0.5 mM NADPH (Type III, Sigma Chemical Co., St. Louis, MO) after a 2-min temperature equilibrium period. In some experiments a glucose-6-phosphate dehydrogenase NADPH-regenerating system was added, but it did not enhance or prolong MDA formation. Aliquots (1 ml) removed at various time intervals for MDA measurements were added to 30% trichloroacetic acid (1 ml) and allowed to react with thiobarbituric acid reagent at 100° for 15 min according to the method of Ottolenghi [15]. The color complex was determined spectrophotometrically at 535 nm. The extinction coefficient for the chromagen under our assay conditions was determined by using tetraethoxypropane (malonaldehyde tetraethylacetal) from Matheson, Coleman & Bell Manufacturing, Norwood, OH, as a reference standard [16]. The measured value was the same as previously reported, 156 mM^{-1} cm^{-1} [17].

Samples (1 ml) for fatty acid analysis were added to 2 ml of 10% potassium hydroxide in methanol containing butylated hydroxytoluene (5 mg/100 ml), sealed, and incubated at 50° for 40 min. The saponified microsomal lipids were cooled to room temperature and acidified with 6 N hydrochloric acid, and 74 nmoles heptadecanoic acid (Nu Check Prep, Inc., Elysian, MN) was added to each sample as an internal standard. Samples were mixed and extracted two to three times with ethyl ether (7–10 ml each). The extracts were combined and evaporated to dryness under a gentle stream of nitrogen. Diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine using the methodology and apparatus developed by the Aldrich Chemical Co. (Milwaukee, WI). Diazomethane dissolved in ether during its generation was added to each dried sample for methylation and allowed to react at room temperature for 20–40 min. After evaporating the samples as described above, a small volume of spectral quality *n*-hexane (Burdick & Jackson Laboratories, Inc., Muskegon, MI) was added to dissolve the fatty acid methyl esters. The microsomal fatty acid composition was analyzed by gas chromatography/mass spectroscopy (GC/MS) with a Hewlett Packard instrument, model 5992B. Separation of the fatty acids was achieved on a 6-ft column, packed with 3% Silar-10C on Gas-Chrom Q (100–120 mesh) with helium as the carrier gas at a flow rate of 15 ml/min. Maximum resolution of the fatty acids was accomplished by using a temperature program from 120 to 220° at 10°/min. The injection port was maintained at 240°. Fatty acids were identified by their fragmentation patterns, and relative retention times were compared with known standards (Nu Chek Prep, Inc.). Calculation of the microsomal fatty acid concentrations was based on the ratio of each fatty acid to the internal standard, heptacedanoate (17:0). Standard curves were determined for each fatty acid. Recoveries of the different fatty acids were generally between 60 and 70%, in part due to the conditions used during saponification to protect the polyunsaturated fatty acids from oxidation. Higher yields of saturated fatty acids could be obtained using higher temperatures and longer heating, but these conditions caused greater loss of polyunsaturated fatty acids. Corrections for these losses were made for each of the fatty acids.

Spectrophotometric assays of microsomes were performed with a temperature regulated (25°) Aminco DW-2 spectrophotometer. The cytochrome P-450 content in microsomes was measured by the method described by Omura and Sato [18] and the cytochrome b_5 level in microsomes by that of Raw and Mahler [19] using extinction coefficients of 91 mM^{-1} cm^{-1} and 111 mM^{-1} cm^{-1} respectively. NADPH-Cytochrome *c* reductase activity was measured according to the procedure of Shimakata *et al.* [20], using any extinction coefficient of 19.6 mM^{-1} cm^{-1}. Dealkylation of *p*-nitroanisole was determined at 412 nm by the formation of *p*-nitrophenol [21].

Mixed-function oxidase activity was also measured by aniline hydroxylation over a 10-min time course at 37° in a shaking water bath. The hydroxylation of aniline was measured as described earlier [22].

RESULTS

When microsomes are incubated in the presence of NADPH and chelated iron, the levels of unsaturated fatty acids decrease and malondialdehyde (MDA) is generated. To discern the fatty acid sources of MDA, microsomal lipids were examined by gas chromatography/mass spectroscopy after extraction, saponification and methylation. A typical chromatogram depicting the elution profile, relative retention times, and individual fatty acid composition of rat liver microsomes is shown in Fig. 1 (−NADPH). Incubation at 37° for 30 min in the absence of NADPH did not change the fatty acid composition. When reaction mixtures were incubated with 0.5 mM NADPH for 30 min, MDA was produced and a marked alteration in the fatty acid

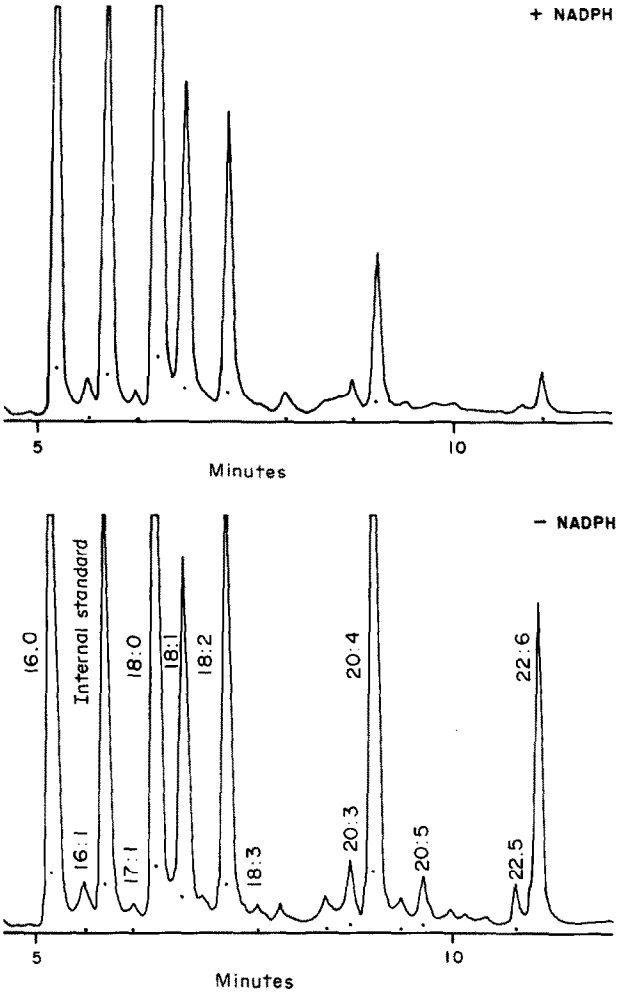

Fig. 1. Rat liver microsomal fatty acid profile after incubation in the presence and absence of NADPH (0.5 mM). Assay conditions were as described in Materials and Methods. Identifications and analyses of fatty acids were by GC/MS, as described in Materials and Methods. Dots under the peaks represent peak heights at 1/10 the tracing sensitivity.

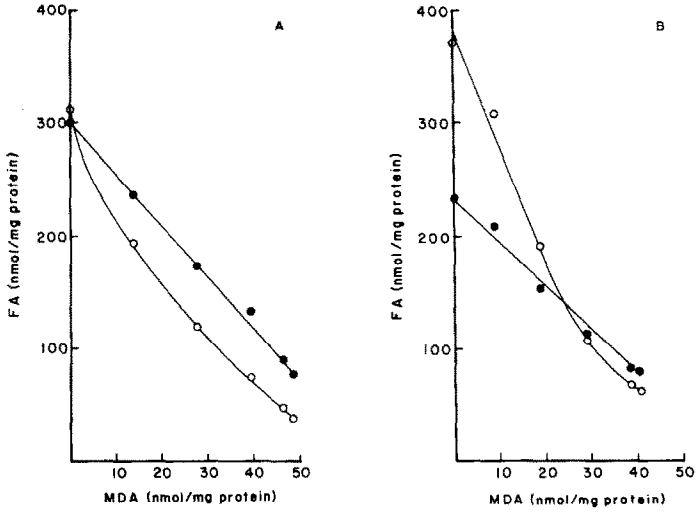

Fig. 2. Comparison of the amounts of polyunsaturated fatty acids, i.e. 20:4 (●) and 22:6 (○), consumed with MDA production for individual liver microsomal preparations from rat (A) and mouse (B). Assays and analyses of fatty acids were by GC/MS, as described in Materials and Methods.

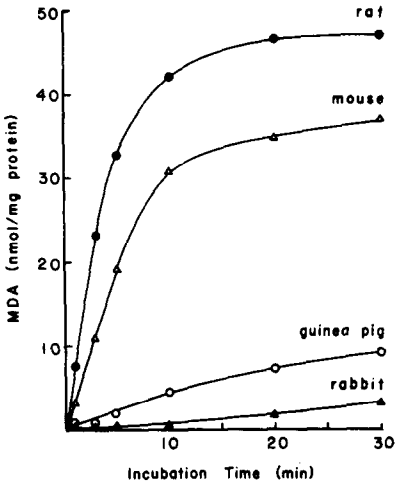

Fig. 3. Species differences in the formation of MDA during NADPH-supported lipid peroxidation. Samples were removed from assay mixtures containing microsomes (0.5 mg/ml) from rat (●), mouse (△), guinea pig (○) or rabbit (▲) at various times. Assays and analyses were as described in Materials and Methods.

composition was observed as illustrated in Fig. 1 (+NADPH). The changes were predominately in the polyunsaturated fatty acids, with the saturated and monounsaturated fatty acids showing little or no change. Since several polyunsaturated fatty acids were observed to decrease during the incubation period, the task of identifying the fatty acid source of MDA was complicated.

In an attempt to determine whether one or both of the two proposed [6–10] fatty acids, 20:4 and 22:6, are sources of MDA, the disappearance of these fatty acids was plotted against the MDA production for individual liver microsomal preparations. Typical plots are shown in Fig. 2. The decline in 20:4 was related linearly to MDA production both in rat liver microsomes ($r = 0.998$, Fig. 2A) and in mouse liver microsomes ($r = 0.991$, Fig. 2B). The decline in 22:6; however, consistently appeared curvilinear (Fig. 2, A and B), although straight lines with fairly high correlation coefficients (0.981–0.987) could be obtained. In mouse liver microsomes, the amount of 22:6 was greater than in rat microsomes, and its decline much steeper, although production of MDA with these microsomes was lower.

The rates of lipid peroxidation in liver microsomes of different species have been reported previously [23]. As shown in Fig. 3, a dramatic difference in the initial rate and extent of MDA formation was observed between species, with rat exhibiting the greatest activity followed by mouse, guinea pig and rabbit. These large differences in lipid peroxidation activity did not parallel activities of other microsomal electron transfer enzymes. Several microsomal electron transfer pathways and enzymes were examined for the different species and are compared with lipid peroxidation activity in Table 1. Neither the rates nor the extents of MDA formation could be related to NADPH–cytochrome *c* reductase activity or to cytochromes P-450 or b_5 content. In addition, monooxygenase activity toward aniline and *p*-nitroanisole showed no apparent correlation with MDA formation.

A comparison of the hepatic microsomal fatty acid composition for the different species is shown in Table 2. Considerable difference in the fatty acid profiles was observed. For example, guinea pig liver microsomes were relatively low in palmitate (16:0), oleate (18:1), arachidonate (20:4) and docosahexaenoate (22:6), but they were relatively more abundant in linoleate (18:2) than the other species tested. Rabbit liver microsomes were also relatively low in 20:4 and had unmeasurable levels of 22:6. A comparison of the fatty acid composition of rat liver microsomes used in this study with that in the literature [24–28] reveals a remarkable similarity in the proportion of most fatty acids. The two fatty acids with the greatest variability were 20:4 and 22:6. This is apparent in the literature where the ratio of 22:6 to 20:4 varied widely (1.04, [28]; 0.78, this study; 0.69, [24]; 0.48, [25]; 0.36, [26]; and 0.14, [27]). It is possible that these two fatty acids vary more than the others in response to diet composition and conditions. To establish further a relationship between MDA production and its fatty acid precur-

Table 1. Microsomal enzymic activities for rat, mouse, guinea pig and rabbit*

Variable	Percent of activity			
	Rat	Mouse	Guinea pig	Rabbit
Lipid peroxidation†	100 (7.8)‡	49	6	1
Cytochrome P-450	100 (0.72)§	96	86	179
Cytochrome b_5	100 (0.41)§	71	134	139
NADPH–Cytochrome *c* reductase	100 (161)‡	106	82	56
p-Nitroanisole *O*-demethylation	100 (0.49)‡	224	284	263
Aniline hydroxylation	100 (2.34)‡	124	97	55

* Values are from an experiment typical of microsomal preparations for each animal species.
† Lipid peroxidation was measured by the initial rate of MDA formation in the presence of NADPH as described in Materials and Methods.
‡ Values in parentheses are specific activities for rat liver microsomes, nmoles/min-mg protein.
§ Values in parentheses are the concentrations in rat liver microsomes, nmoles/mg protein.

Table 2. Fatty acid concentrations in rat, mouse, guinea pig, and rabbit microsomes

	Concentration (nmoles/mg protein)					
	Rat			Mouse	Guinea pig	Rabbit
Fatty acid	Normal diet*	Normal diet and 1-day fast†	Fat-free diet†	Normal diet‡	Normal diet§	Normal diet‖
16:0	447 ± 5	404	560	429	187	448
18:0	581 ± 16	664	572	413	534	513
18:1	198 ± 11	165	421	269	176	308
18:2	314 ± 20	300	67	362	489	422
18:3	9 ± 2	7	7	16	26¶	33¶
20:3	30 ± 4	18	96	69	18¶	9¶
20:4	303 ± 22	399	210	223	97	116
20:5	35 ± 8	35	11	16	3	0
22:5	35 ± 7	31	9	17¶	14	0
22:6	237 ± 31	303	276	354¶	12	0

* Data are from four microsomal preparations with five rats per preparation. Each value from a microsomal preparation is the mean ± S.E.M. of five determinations.

† Data are from one experiment with five rats per group. Each value is the mean of five determinations.

‡ Values are given for one of two experiments with fifty mice per group. Each value is the mean of five determinations.

§ Values are given for one of two experiments with five guinea pigs per group. Each value is the mean of five determinations.

‖ Values are given for one of two experiments with one rabbit per group. Each value is the mean of five determinations.

¶ These values differed from a duplicate preparation by about a factor of 2. All other values in the table varied by 25% or less.

sor, rats were fasted 24 hr prior to being killed or were fed a fat-free (high carbohydrate) diet for 5 days to alter the microsomal fatty acid composition. Fasting produced some changes in the unsaturated fatty acids, as compared with normal diet and non-fasting (Table 2) but 20:4 showed the most dramatic increase. The fat-free diet produced an increase in 16:0 and 18:0 contents, but a decrease in 18:2 and 20:4 contents, i.e. a decrease in the degree of unsaturation of microsomal lipid.

Incubation of microsomes at 37° for 30 min in the presence of iron pyrophosphate and NADPH caused little or no change in the levels of saturated and monoenoic fatty acids for rat, mouse, guinea pig and rabbit fed the normal laboratory diet. However, marked changes were noted in the polyenoic fatty acids for the different species (Fig. 4). Both rat and mouse microsomes exhibited a pronounced decrease in arachidonic (20:4) and docosahexaenoic (22:6), as mentioned above, but much less in linoleic (18:2), linolenic (18:3), eicosatrienoic (20:3) eicosapentaenoic (20:5), and docosapentaenoic (22:5) fatty acids. Unlike rat and mouse, guinea pig and rabbit showed little change in 20:4 and 22:6 fatty acids (0–30% vs 3 to 6-fold decrease).

Changes in other measurable microsomal polyunsaturated fatty acid concentrations for each of the species were also determined after the maximum extent of MDA formation was achieved (Table 3). Some fatty acids, including 20:3, 20:5, and 22:5, underwent relatively small decreases, in comparison to 20:4 and 22:6 in each of the animal species (Fig. 4).

Since each of the species exhibited unique microsomal fatty acid profiles (Table 2) and also demonstrated different time courses for MDA formation (Fig. 3), further analysis of the relationship of fatty acid change and composition to MDA formations was examined. By varying the dietary regime for rats it was possible to alter the microsomal fatty acid composition (Table 2), which altered the amount of lipid peroxidation as well. Fasting for 24 hr prior to killing the animals enhanced the rate of MDA production [11 nmoles·min^{-1}·(mg protein)$^{-1}$] and the extent (65 nmoles/mg protein) of MDA formation when compared to the rate (6.2 nmoles·min^{-1}·

Table 3. Decreases in amount of other polyunsaturated fatty acids after MDA production ceased*

	Final concentration† (nmoles/mg protein)			
Fatty acid	Rat	Mouse	Guinea pig	Rabbit
20:3	30 (71)‡	30 (43)	4 (22)	1 (11)
20:5	25 (81)	11 (69)	0 (−)	0 (−)
22:5	32 (74)	11 (65)	7 (50)	0 (−)
Total	87 (66)	52 (51)	11 (31)	1 (−)

* Microsomes were incubated in the presence of NADPH as described in Materials and Methods. Samples for fatty acid analysis were removed after the maximum MDA formation was observed.

†Determinations were the same as described in Table 2.

‡ Values in parentheses are percentages of the initial concentration for the individual fatty acid.

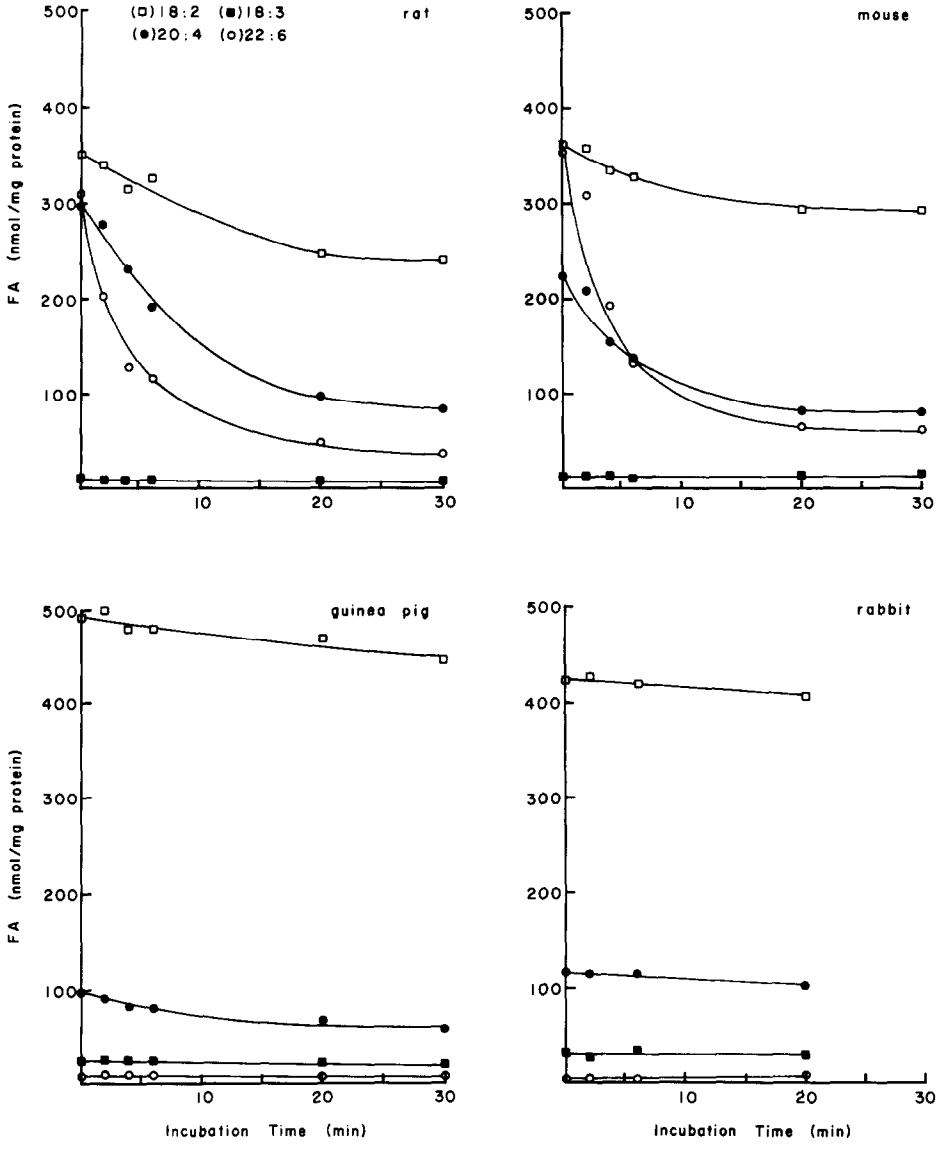

Fig. 4. Changes in the liver microsomal unsaturated content during NADPH-supported lipid peroxidation for different species fed a normal laboratory diet. Fatty acids shown are 18:2 (□), 18:3 (■), 20:4 (●) and 22:6 (○) from rat, mouse, guinea pig, and rabbit liver microsomes. Assays and analyses of fatty acids were by GC/MS, as described in Materials and Methods.

mg protein)$^{-1}$ and extent (53 nmoles/mg) in rats fed a normal diet until the time of use. Feeding rats a fat-free diet for 5 days prior to use caused an initial lag in the formation of MDA, a slower rate (2.9 nmoles·min^{-1}·mg^{-1}) and a lesser extent (44 nmoles/mg) than observed for rats on a normal diet. The differences in the formation of MDA by microsomes from rats on varied dietary treatments were accompanied by different rates of unsaturated fatty acid disappearance, shown in Fig. 5 for 18:2, 18:3, 20:4 and 22:6. The fat-free diet, as noted earlier [12, 13], caused marked changes in the contents of the different fatty acids in microsomal lipids (Table 2 and Fig. 5).

When the rates of consumption of the individual fatty acids in microsomes were plotted against the rates of appearance of MDA for the same microsomes, a linear relationship was found for arachidonate (Fig. 6A), despite the fact that values for microsomes from four animal species, i.e. rat, mouse, rabbit and guinea pig, as well as microsomes from starved and fat-free diet rats, were used in the figure. The line drawn for 20:4 had a correlation coefficient of 0.988 whereas for 22:6 the relationship had a correlation coefficient of 0.811. No relationship was apparent for 18:2 and 18:3. The slope of the line for 20:4 indicated that the rate of arachidonate consumption was 3-fold greater than MDA production. A similar linear relationship could also be demonstrated between the initial concentration of arachidonate in the different microsomes and the initial rate of MDA production (Fig. 6B). The correlation coefficient for the line drawn for 20:4 was 0.990. The correlation coefficient for the relationship

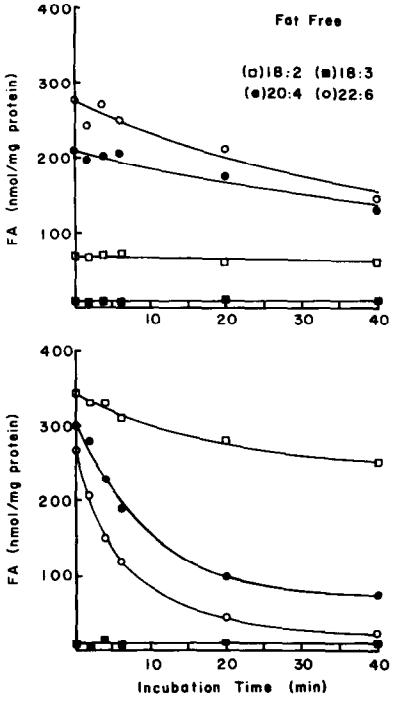

Fig. 5. Comparison of liver microsomal unsaturated fatty acid content changes in rats fed a fat-free diet (top) with normal diet (bottom) controls. Fatty acids shown are 18:2 (□), 18:3 (■), 20:4 (●) and 22:6 (○). Assays and analyses of fatty acids were by GC/MS as described in Materials and Methods.

between 22:6 and MDA production was 0.641. No relationship was apparent for 18:2 or 18:3.

DISCUSSION

In the present study, liver microsomal fatty acid concentrations were determined for rat, mouse, guinea pig and rabbit and also for rats starved or fed a fat-free diet. During NADPH-supported lipid peroxidation in microsomes, it was observed that rat and mouse exhibited the greatest decline in polyunsaturated fatty acids, particularly 20:4 and 22:6, and guinea pig and rabbit showed much smaller changes (Table 3). Similar differences were seen in MDA production between species. When the rates of individual polyunsaturated fatty acid consumption were compared with MDA formation in microsomes from the same (Fig. 2) or different species (Fig. 6A), only 20:4 demonstrated a linear relationship. A similar relationship was obtained when the initial microsomal concentrations of the fatty acids were compared with MDA production (Fig. 6B). In animals starved or maintained on a fat-free diet, the lipid composition of the microsomes changes [12, 13]. In Table 2 the changes shown mainly involve some of the polyunsaturated fatty acids. Whereas 22:6 is hardly changed, 20:4 is increased 30% by starvation and decreased 30% by the fat-free diet. The changes in MDA formation are such that when related to 20:4 disappearance the coordinates fit the linear relationship seen in Fig. 6. This would suggest that

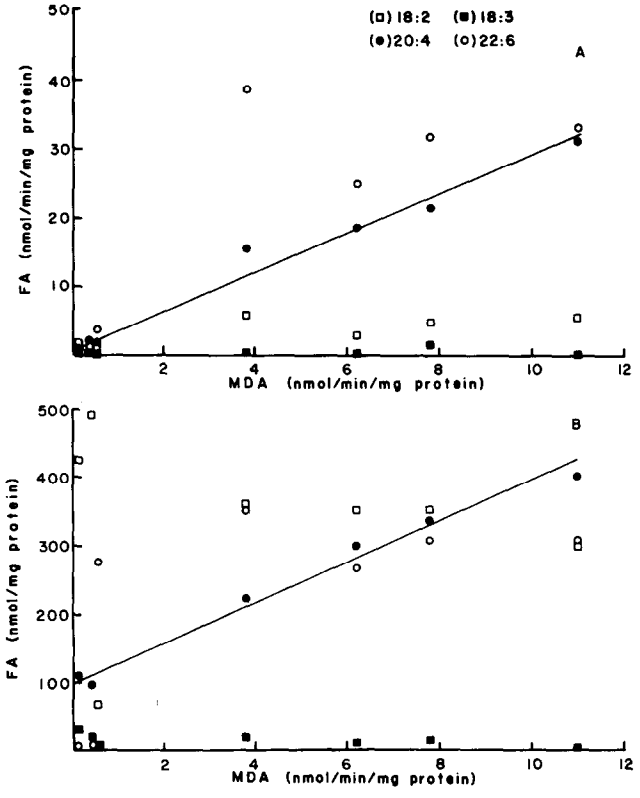

Fig. 6. Relationship between the initial rate of MDA formation and the initial rate (A) or the initial concentration (B) of polyunsaturated fatty acids in microsomes from rat, rabbit, mouse and guinea pig, and after different dietary treatments of rats. Fatty acids shown are 18:2 (□), 18:3 (■), 20:4 (●) and 22:6 (○). Assays and analyses of MDA and fatty acids were as described in Materials and Methods.

the MDA produced enzymatically is solely from 20:4. However, the ratio of 20:4 utilization to MDA formation was approximately 3 to 1, indicating that pathways of 20:4 breakdown, other than that yielding MDA, may also exist, unless subsequent MDA consumption or reactions occur. The latter possibility does not appear to occur, since continued incubation of the microsomes and MDA with or without NADPH for up to 15 min did not result in loss of the MDA (data not shown). Other polyunsaturated fatty acids also were consumed but their consumption showed no relationship to MDA formation, and probably yields non-MDA products, the natures of which are unknown at present. *In vivo*, under conditions known to cause hepatic lipid peroxidation, ethane, pentane and other volatile hydrocarbons are present in the expired air [29, 30]. It is possible that precursors to these metabolites will eventually be found *in vitro*. Whereas the MDA may be the precursor of the ethane, the nature of the pentane precursor is not as yet apparent. Although a linear relationship could be shown for MDA appearance and 20:4 disappearance, the fact remains that the portion of any 20:4 molecule going to MDA represents only a small part of the total fatty acid molecule. The rest of the molecule or its products remains to be identified.

The microsomal NADPH-dependent formation of MDA could not be related to the components of the hepatic monooxygenase system or microsomal electron transfer. This in itself would not eliminate the monooxygenase or its components from the reaction. Since antibody to NADPH–cytochrome P-450 reductase can inhibit the NADPH-supported reaction [31], it is apparent that this electron transfer enzyme is involved, but is not normally rate limiting. The fact that the initial rates of MDA formation were linear with increasing initial microsomal content of 20:4 suggests a non-saturated process. Whether the reaction is enzymatic at the limiting step remains to be determined; the linearity of MDA production with microsomal arachidonate level over several different species casts doubt upon this possibility. In studies in this laboratory we are currently examining this question.

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