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# DIETARY FATTY ACID-INDUCED ALTERATIONS OF HEPATIC MICROSOMAL DRUG METABOLISM*

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**Abstract**—Male and female weanling, Sprague–Dawley rats were fed synthetic fat-free (FF) or corn oil-containing diets for 3 weeks. The apparent $K_m$ and $V_{max}$ for the metabolism of ethylmorphine and hexobarbital were lower in washed hepatic microsomes from male rats fed a FF diet than in rats fed diets containing 3 or 10 per cent corn oil. The apparent $V_{max}$ for aniline hydroxylase was also depressed by feeding a FF diet to male rats, although aniline $K_m$ was not altered. Feeding a FF diet to female rats decreased $V_{max}$ for both hexobarbital oxidase and aniline hydroxylase, but the apparent $K_m$ for the substrates was not changed. Gas–liquid chromatographic analysis of fatty acids derived from microsomal membranes revealed marked alterations in the relative content of fatty acids in response to FF feeding. Content of cytochrome P-450 was lower in both male and female rats fed a FF diet. Associated with the decreased cytochrome P-450 content were decreases in the ability of microsomes to bind aniline and hexobarbital. A qualitative change produced in cytochrome P-450 was indicated by a decrease in the ratio of ethyl isocyanide peak heights (455:430 nm) in microsomes from FF-fed rats.

ENZYMES associated with the endoplasmic reticulum of mammalian liver cells are responsible for the metabolism of a variety of drugs and hormones. Alteration of the ability of the liver to metabolize these substrates will affect the degree and duration of their action. The consequences of unrecognized alterations in hepatic function range from the acquisition of unreliable data during drug testing to dangerous overdosage toxicity in man resulting from impaired metabolism of therapeutic agents.[1]

Little is known regarding precise, biochemical mechanisms of hepatic drug-metabolizing enzyme systems. The enzymes involved are intimately associated with the endoplasmic reticulum, making the isolation and identification of individual components difficult. Numerous studies, using fragments of the endoplasmic reticulum (microsomes) mechanically separated from other constituents of hepatic cells, have demonstrated nonspecific enzyme systems having diverse chemical and physical properties. An agent or environmental condition which affects any cofactors or enzymes of these systems could then stimulate or inhibit the metabolic degradation of many substrates.

Lipids are essential for the proper function of some microsomal enzymes. Treatment of microsomes with agents such as detergents or phospholipases, which attack lipid structure, destroys microsomal enzyme activity.[2] A solubilized system from

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microsomes requires phosphatidylcholine for drug-metabolizing activity, and the activity is enhanced by addition of palmitoleic and arachidonic acids.[3,4] Lipids stimulate formation of heme, a constituent of microsomal cytochrome necessary for drug hydroxylation.[5] Feeding diets containing corn oil or various quantities of saturated fatty acids increase metabolism of certain substrates by the 9000 *g* supernatant fraction of liver homogenates,[6–8] while fatty acid deficiency has been shown to depress aniline hydroxylation and cytochrome P-450 content of rat liver microsomes.[9]

The objectives of this research are to define the effects of dietary essential fatty acids on selected components of hepatic microsomal drug-metabolizing enzyme systems and to postulate possible mechanisms by which dietary lipids alter the actions of these enzymes. In order to accomplish these objectives, it was necessary to establish a degree of essential fatty-acid deficiency state; thus, measurements of the amounts and/or activities of the components of the mixed function oxidase system were made in animals fed a fat-free diet and compared to control animals receiving optimal levels of essential fatty acids (3 per cent corn oil). Higher lipid-containing diets (10 per cent corn oil) were included in order to establish the level of lipid intake in the rat required for greatest activity of these enzymes.

## METHODS

*Diets of experimental animals.* Male and female Sprague–Dawley rats (The Holtzman Company, Madison, Wis.) weighing 50–55 g were fed Purina lab chow for 3–4 days prior to receiving a synthetic diet consisting of 16·0% vitamin-free casein, 4·0% Jones–Foster salt mixture, 4·0% non-nutritive bulk (Alphacel, Nutritional Biochemicals Corp., Cleveland, Ohio), 72·0% sucrose and 4·0% of a sucrose-based mixture containing necessary vitamins.[10] Diets were identical, except that where corn oil (3 or 10 per cent by weight) was incorporated, it was substituted for an equal caloric portion of the sucrose. To decrease the possibility of lipid peroxidation occurring, 3·5 mg of alpha tocopherol was added to each kilogram of diet, the diet was stored at −15°, and was freshly prepared every two to three weeks. Fresh diet was made available to the experimental animals daily.

*Isolation of microsomes.* After 21 days feeding with synthetic diets, the rats were decapitated without anesthesia. Livers were removed, chilled in ice-cold 1·15% potassium chloride (KCl), blotted, weighed and homogenized in 2 vol. of fresh KCl solution using a motor-driven Teflon pestle-glass homogenizer. Each homogenate was centrifuged at 9000 *g* for 20 min at 0–4° in a Sorvall model RC-2B centrifuge. The floating fat layer was carefully aspirated, and the underlying supernatant fraction decanted and centrifuged at 105,000 *g* for 1 hr at 0–4° in a Beckman model L2-65 ultracentrifuge. The resulting microsomal pellet was resuspended in fresh 1·15% KCl solution, and recentrifuged at 105,000 *g* for 1 hr. The washed microsomes were resuspended in KCl using the above homogenizer, and the protein content was determined by the method of Gornall *et al.*[11] Resuspended microsomes were stored at −15° for no longer than 2 days prior to use.

*Enzyme assays.* Microsomes were thawed and diluted to a protein concentration of 5·0 mg/ml immediately prior to assay. Each incubation mixture contained the following: microsomes, 1 ml; nicotinamide adenine dinucleotide phosphate (NADP), 5 μmoles; glucose 6-phosphate (G6P), 25 μmoles; magnesium sulfate, 25 μmoles; drug

substrate, 0·5 ml; glucose 6-phosphate dehydrogenase, 2 enzyme units; enough 0·1 M phosphate buffer, pH 7·4, to make a final volume of 5·0 ml (2·0–2·5 ml). Four concentrations of each substrate were used as follows (mM): ethylmorphine—10·0, 1·0, 0·2 and 0·1; hexobarbital—2·0, 1·0, 0·2 and 0·1; aniline—0·5, 0·2, 0·1 and 0·05. Appropriate tissue and reagent blanks and standards were prepared and carried through incubation and extraction procedures. Mixtures were incubated in 25-ml Erlenmyer flasks for 15 or 20 min at 37° under air in a Dubnoff metabolic shaker at 120 oscillations/min.

When aniline or hexobarbital were used as substrates, reactions were stopped by chilling the incubation flasks in an ice bath. A 4·0-ml aliquot of the incubation mixture was then transferred to a tube containing extraction medium. The aromatic hydroxylation of aniline was assayed by measuring *p*-aminophenol formed[12] and the side chain oxidation of hexobarbital was determined by measuring disappearance of substrate.[13] When ethylmorphine was used as substrate, the reaction was stopped by adding 2·0 ml of 5·0% zinc sulfate; shaking was continued for 3 min, and then 2·0 ml of saturated barium hydroxide solution was added to precipitate protein. Ethylmorphine-*N*-demethylase activity was measured by estimation of formaldehyde formed.[14]

*Lipid analyses.* Microsomal lipids were extracted after resuspending microsomes in 17 vol. of chloroform–methanol (2:1)[15] with a motor-driven Teflon pestle–glass homogenizer. After filtration through a sintered-glass Buchner funnel, 0·2 vol. of 0·75% KCl was added, and the extracts were shaken for 5 min in Teflon-capped glass tubes. Phases were separated by centrifuging, and the lower (chloroform) layer was washed once with chloroform–methanol–water (1:16:16) containing 0·74% KCl. The top layer was aspirated, and the bottom layer evaporated to dryness under a stream of nitrogen. Ten ml of acetone saturated with magnesium chloride was added to the evaporated residue, and the tubes were placed in a freezer at −15° for 16 hr to precipitate phospholipids.

Phospholipids were settled by centrifugation at 10,000 *g* for 15 min at 0–4°. The supernatant was aspirated and the phospholipids were dissolved in 5·0 ml of chloroform–methanol (1:1) containing 0·01% α-tocopherol. Two ml of the phospholipid solution was evaporated under nitrogen, and 4·0 ml of 0·5 N methanolic sodium hydroxide added. After heating for 5 min on a boiling water bath, the solution was acidified with 2·0 ml of 1 N HCl in methanol; 2·0 ml of 14% boron trifluoride in methanol was added, and heating was continued for 2 min. After the tubes had cooled, 5·0 ml of saturated sodium chloride solution was added, then 6·0 ml of *n*-hexane, and the tubes were shaken and centrifuged. The top layer was transferred to a clean flask, dried with sodium sulfate, decanted to a tube and evaporated under nitrogen to 0·5–1·0 ml. Recoveries using this procedure for palmitic acid were 99–101 per cent.

One-μl samples of the fatty acid methyl esters were injected into a Perkin–Elmer model 881 gas chromatograph equipped with a hydrogen flame detector. Separation of the esters was accomplished on a ⅛ in. × 6 ft stainless steel column containing diethylene glycol adipate (DEGA) on 80/100 mesh Chromosorb W. For separation of short chain esters (16–20 carbon atoms) 12% DEGA on Chromosorb W was used, while 3% DEGA on Chromosorb W was used for separation of docosapentaenoic (22:5) and docosahexanenoic (22:6) methyl esters. Column conditions were as follows: injector temperature, 250°; column temperature, 190°; detector temperature, 205°; carrier gas (nitrogen) flow rate, 50–60 ml/min. Standard mixtures of fatty acid methyl

esters (Applied Science Laboratories, State College, Pa.) were used for identification of peaks. Peak areas were estimated by the method of Carroll.[16]

*Cytochrome P-450 content.* Microsomal preparations were diluted to a protein concentration of 2·0 mg/ml with 1·15% KCl containing 0·5 M Tris buffer, pH 7·4, and the concentration of cytochrome P-450 was determined by the method of Omura and Sato[17] using the split-beam mode of the Aminco–Chance dual wavelength/split beam recording spectrophotometer.

*Substrate binding studies.* The ability of microsomal preparations to bind aniline and hexobarbital was determined by the method of Remmer *et al.*[18] using microsomes diluted to 2·0 mg protein/ml with 0·3 M phosphate buffer, pH 7·4. Seven serial 1-$\mu$l additions of substrate to a cuvette containing 3·0 ml of microsomes were made and after each addition the difference in absorbance between two predetermined wavelengths was measured using the Aminco–Chance spectrophotometer in the dual wavelength mode. For aniline binding, the difference in absorbance between 430 and 417 nm was determined at final concentrations of 0·103, 0·206, 0·309, 0·412, 0·515, 0·618 and 0·721 mM. For hexobarbital binding the difference in absorbance between 430 and 400 nm was determined at final concentrations of 0·120, 0·240, 0·360, 0·480, 0·600, 0·720 and 0·840 mM.

*Ethyl isocyanide difference spectra.* Ethyl isocyanide was synthesized according to the procedure of Jackson and McKusick.[19] Ethyl isocyanide difference spectra were determined according to the procedure of Omura and Sato,[17] except that microsomes were diluted to 2·0 mg/ml with 1·5 M phosphate buffer, pH 7·5, to prevent changes in pH which affect relative sizes of absorption maxima. To 3·0 ml of diluted microsomes, 15 $\mu$l of 5% ethyl isocyanide was added, and the difference in absorbance between 430 and 490 nm and between 455 and 490 nm was determined using the Aminco–Chance spectrophotometer in either the split beam or the dual wavelength mode. Ratio of the 455 nm absorption peak to the 430 nm peak was then calculated.

*Cytochrome P-450 extinction coefficient.* Extinction coefficient for cytochrome P-450 was determined as described by Greene *et al.*[20] by assaying for total heme, cytochrome $b_5$ and absorption by cytochrome P-450.

*NADPH-cytochrome c reductase activity.* The ability of microsomes to reduce cytochrome *c* was determined by the method of Phillips and Langdon,[21] except that a NADPH-generating system was used instead of NADPH. One ml of microsomes diluted to 0·5 mg protein/ml with 0·1 M Tris buffer, pH 7·4, was mixed in a cuvette with 2·5 mg of cytochrome *c* and 3 $\mu$moles of potassium cyanide in 0·05 M Tris buffer, pH 7·4, to give a final volume of 3·0 ml. With the sample and reference monochromators of the Aminco–Chance spectrophotometer set at 550 and 539 nm, respectively, the recorder was started, and 0·05 ml of a NADPH-generating system* added. Using the extinction coefficient of 18·7 $cm^{-1}mM^{-1}$ for reduced minus oxidized cytochrome *c*, the reductase activity was determined.

*Statistical analyses.* $K_m$ and $V_{max}$ and $K_s$ and $A_{max}$ values were calculated by the method of Wilkinson[22] using an Olivetti–Underwood Programma 101 computer. The Student's *t*-test was used to analyze results, with P values of $<0{\cdot}05$ considered to represent significant differences.

* 250 $\mu$moles magnesium chloride, 219 mg G6P, 23 mg NADP, 13·5 enzyme units of G6P dehydrogenase and sufficient 0·05 M Tris buffer, pH 7·4, to make a final volume of 5·0 ml.

## RESULTS

*Effect of corn oil consumption on liver weight and microsomal protein and cytochrome content.* Increasing dietary corn oil intake from 0 to 10 per cent decreased liver weight (as percentage of body weight) in male rats and increased cytochrome P-450 per milligram of microsomal protein in both male and female rats. Microsomal protein content was not altered by changes in lipid consumption (Table 1).

TABLE 1. EFFECT OF DIETARY CORN OIL CONSUMPTION ON LIVER WEIGHT AND MICROSOMAL PROTEIN AND CYTOCHROME CONTENT*

| Parameter | Diet | | |
|---|---|---|---|
| | Fat-free | 3% Corn oil | 10% Corn oil |
| Male | | | |
| Liver weight (g/100 g body wt.) | 4·95 ± 0·10† (35) | 4·88 ± 0·08†‡ (35) | 4·61 ± 0·13‡ (35) |
| Microsomal protein (mg/g liver) | 24·6 ± 1·3† (18) | 25·8 ± 1·4† (17) | 20·7 ± 2·1† (4) |
| Cytochrome P-450§ | 0·619 ± 0·053† (10) | 0·862 ± 0·040‡ (10) | 0·880 ± 0·070‡ (4) |
| Female | | | |
| Liver weight (g/100 g body wt.) | 4·11 ± 0·39† (4) | 4·00 ± 0·30† (4) | 3·72 ± 0·38† (4) |
| Microsomal protein (mg/g liver) | 25·1 ± 4·4† (4) | 25·8 ± 1·5† (4) | 24·1 ± 3·9† (4) |
| Cytochrome P-450§ | 0·569 ± 0·060† (4) | 0·761 ± 0·067†‡ (4) | 0·993 ± 0·110‡ (4) |

* Values represent means ±S. E. Values in parentheses represent number of animals.
†‡ Values for each parameter without common superscripts differ significantly ($P < 0·05$).
§ Nanomoles of cytochrome P-450 per milligram of microsomal protein.

*Kinetics of hepatic microsomal enzyme reactions.* The addition of 3 and 10 per cent corn oil to the diets increased the apparent $K_m$ and $V_{max}$ for ethylmorphine demethylase and hexobarbital oxidase in male rats. The apparent $V_{max}$ for aniline hydroxylase was increased without altering $K_m$ in microsomes from both male and female rats receiving diets containing the corn oil. Although $K_m$ for hexobarbital oxidase tended to be higher in female rats on diets containing corn oil, only the $V_{max}$ increased significantly (Table 2).

Sex differences were obvious in these experiments. Both $K_m$ and $V_{max}$ for hexobarbital oxidase were significantly lower in female rats. Although $V_{max}$ for aniline hydroxylase was lower in the female rats, the apparent $K_m$ was higher than for male rats.

*Fatty acid analyses.* Palmitic (16:0), stearic (18:0) and docosahexaenoic (22:6) acids were not altered quantitatively by the 3 levels of dietary corn oil (Fig. 1). With increasing amounts of dietary corn oil, palmitoleic (16:1) and oleic (18:1) acid content decreased. Measurable quantities of eicosatrienoic (20:3) were found in microsomes from rats fed the FF diet, but not in microsomes recovered from those fed diets containing corn oil. The content of linoleic (18:2) and arachidonic (20:4) acids increased with increasing dietary corn oil, while docosapentaenoic acid (22:5) was highest in microsomes from 3 per cent corn oil-fed rats.

TABLE 2. EFFECT OF DIETARY CORN OIL CONSUMPTION ON THE KINETICS OF HEPATIC MICROSOMAL ENZYME REACTIONS*

| Parameter | Diet | | |
|---|---|---|---|
| | Fat-free | 3% Corn oil | 10% Corn oil |
| Male | | | |
| Ethylmorphine $K_m$ (mM) | 0·162 ± 0·011† | 0·234 ± 0·016‡ | 0·229 ± 0·020‡ |
| Ethylmorphine $V_{max}$§ | 519·7 ± 8·4† | 816·1 ± 14·1‡ | 753·7 ± 22·6‡ |
| Hexobarbital $K_m$ (mM) | 0·181 ± 0·056† | 0·326 ± 0·061† | 0·546 ± 0·065‡ |
| Hexobarbital $V_{max}$‖ | 369·3 ± 53·5† | 671·9 ± 56·0‡ | 806·0 ± 46·6‡ |
| Aniline $K_m$ (mM) | 0·078 ± 0·017† | 0·082 ± 0·014† | 0·057 ± 0·014† |
| Aniline $V_{max}$¶ | 81·7 ± 3·8† | 99·3 ± 3·3‡ | 88·5 ± 1·9† |
| Female | | | |
| Hexobarbital $K_m$ (mM) | 0·064 ± 0·033† | 0·130 ± 0·040† | 0·093 ± 0·035† |
| Hexobarbital $V_{max}$‖ | 50·1 ± 13·7† | 100·2 ± 15·3‡ | 93·9 ± 14·8†‡ |
| Aniline $K_m$ (mM) | 0·117 ± 0·014† | 0·110 ± 0·160† | 0·228 ± 0·058† |
| Aniline $V_{max}$¶ | 29·6 ± 1·3† | 32·2 ± 1·7† | 49·8 ± 6·3‡ |

* Values are means ±S.E. determined from four points on Lineweaver–Burke plots using pooled livers of four animals per group.

†‡ Values for each parameter without common superscripts differ significantly ($P < 0·05$).

§ Nanomoles of formaldehyde formed per milligram of microsomal protein per hr.

‖ Nanomoles of hexobarbital metabolized per milligram of microsomal protein per hr.

¶ Nanomoles of *p*-aminophenol formed per milligram of microsomal protein per hr.

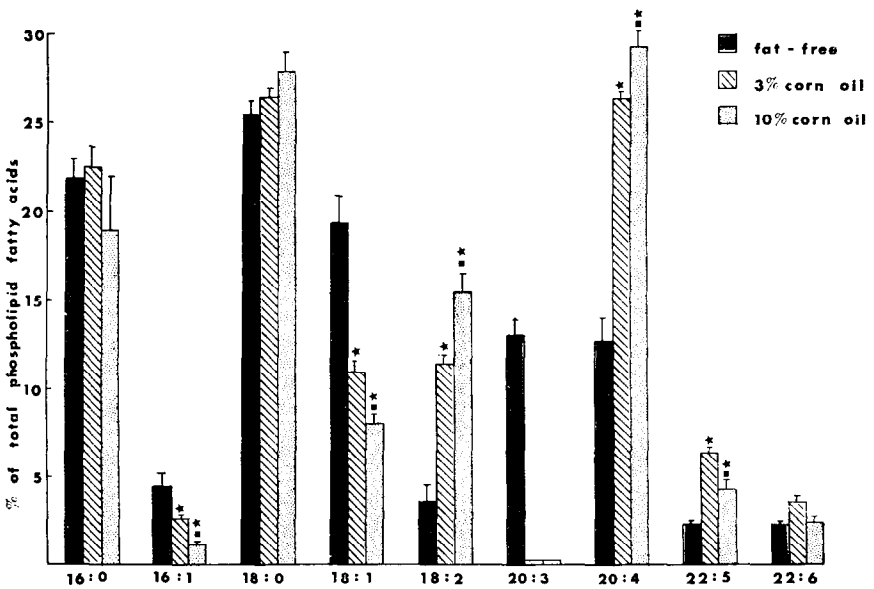


FIG. 1. Effect of dietary corn oil consumption on relative content of microsomal phospholipid fatty acids of male rats. ✱ = sig. diff. ($P < 0·05$) from FF-fed rats; ■ = sig. diff. ($P < 0·05$) from 3 per cent corn oil-fed rats.

TABLE 3. EFFECT OF DIETARY CORN OIL CONSUMPTION ON SPECTRAL BINDING PARAMETERS ASSOCIATED WITH HEPATIC MICROSOMES*

| Parameter | Diet | | |
|---|---|---|---|
| | Fat-free | 3% Corn oil | 10% Corn oil |
| Male | | | |
| Hexobarbital $K_s$ (mM) | 0·229 ± 0·014‡ | 0·196 ± 0·013‡ | 0·198 ± 0·018‡ |
| Hexobarbital $A_{max}$† | 23·2 ± 0·5‡ | 33·5 ± 0·7§ | 28·2 ± 0·7‖ |
| | (27·8 ± 0·6)‡ | (32·8 ± 0·7)§ | (28·0 ± 0·7)‡ |
| Aniline $K_s$ (mM) | 0·338 ± 0·023‡ | 0·306 ± 0·035‡ | 0·282 ± 0·029‡ |
| Aniline $A_{max}$† | 14·0 ± 0·4‡ | 24·0 ± 1·1§ | 22·1 ± 0·9§ |
| | (16·7 ± 0·5)‡ | (23·5 ± 1·1)§ | (21·9 ± 0·9)§ |
| Female | | | |
| Hexobarbital $K_s$ (mM) | 0·265 ± 0·022‡§ | 0·315 ± 0·038‡ | 0·214 ± 0·013§ |
| Hexobarbital $A_{max}$† | 17·7 ± 0·5‡ | 23·8 ± 1·1§ | 20·5 ± 0·4‖ |
| | (56·3 ± 0·8)‡ | (62·5 ± 2·0)§ | (41·7 ± 0·04)‖ |
| Aniline $K_s$ (mM) | 0·349 ± 0·058‡ | 0·326 ± 0·043‡ | 0·302 ± 0·033‡ |
| Aniline $A_{max}$† | 19·6 ± 1·4‡ | 24·3 ± 1·4‡§ | 27·0 ± 1·2§ |
| | (68·8 ± 3·2)‡ | (63·8 ± 2·3)‡ | (69·4 ± 2·1)‡ |

* Values are means ±S. E. determined from seven points on Lineweaver–Burk plots using pooled livers from four animals per group.

† Δ A per milligram of microsomal protein × $10^3$. Values in parentheses are Δ A per nanomole of cytochrome P-450 × $10^3$.

†§‖ Values for each parameter without common superscripts differ significantly ($P < 0·05$).

*Spectral binding parameters.* The spectral dissociation constant ($K_s$) for hexobarbital and aniline binding to microsomes from male and female rats was unaltered by the addition of corn oil to the diet (Table 3). Maximal spectral shift ($A_{max}$) for hexobarbital and aniline was increased in rat liver microsomes from rats receiving diets containing both 3 and 10 per cent corn oil. When calculated on the basis of maximal spectral shift per unit of cytochrome P-450, $A_{max}$ for hexobarbital was elevated in microsomes from

TABLE 4. EFFECT OF DIETARY CORN OIL CONSUMPTION ON ETHYL ISOCYANIDE DIFFERENCE SPECTRA

| Sex and diet | n* | $\Delta A_{430-490}$/mg protein | $\Delta A_{455-490}$/mg protein | Ratio† |
|---|---|---|---|---|
| Male | | | | |
| Fat-free | 14 | 0·061 ± 0·004‡ | 0·034 ± 0·002‡ | 0·561 ± 0·021‡ |
| 3% Corn oil | 13 | 0·074 ± 0·005‡ | 0·048 ± 0·004§ | 0·670 ± 0·051§ |
| 10% Corn oil‖ | | 0·080 | 0·055 | 0·688 |
| Female | | | | |
| Fat-free | 4 | 0·033 ± 0·004‡ | 0·039 ± 0·004‡ | 1·209 ± 0·038‡ |
| 3% Corn oil | 4 | 0·044 ± 0·004‡ | 0·052 ± 0·005‡§ | 1·183 ± 0·025‡ |
| 10% Corn oil | 4 | 0·052 ± 0·008‡ | 0·065 ± 0·009§ | 1·246 ± 0·057‡ |

* Represents number of animals.

† Ratio of peak at 455 nm to peak at 430 nm.

‡§ Values for each dietary group without common superscripts differ significantly ($P < 0·05$).

‖ Values obtained from duplicate determinations using pooled livers of four animals.

male and female rats receiving the 3 per cent corn oil diet, was unaltered in male rats receiving 10 per cent corn oil and was depressed in female rats ingesting 10 per cent corn oil. Aniline $A_{max}$ per unit of P-450 was elevated in male rats and unchanged in females receiving diets supplemented with corn oil.

*Ethyl isocyanide difference spectra.* Ingestion of the FF diet resulted in a decrease in the ratio of ethyl isocyanide-induced absorption maxima in male rats (Table 4). The ratio of the peak heights was not significantly decreased by the FF diet in female rats.

TABLE 5. EFFECT OF DIETARY CORN OIL CONSUMPTION ON CYTOCHROME P-450 EXTINCTION COEFFICIENT OF MALE RATS

| Diet | n* | Total heme (nmoles/mg protein ±S.E.) | Cytochrome $b_5$ (nmoles/mg protein ±S.E.) | $\Delta A_{450-490}$/mg protein | $E_{450}$† |
|---|---|---|---|---|---|
| Fat-free | 3 | 0·869 ± 0·097‡ | 0·423 ± 0·037‡ | 0·042 ± 0·004‡ | 96·2 ± 8·2‡ |
| 3% Corn oil | 4 | 1·184 ± 0·083§ | 0·537 ± 0·027§ | 0·067 ± 0·006§ | 104·0 ± 2·7‡ |
| 10% Corn oil | 4 | 1·395 ± 0·054§ | 0·610 ± 0·021§ | 0·080 ± 0·007§ | 101·1 ± 3·4‡ |

* Represents number of animals.

† $E_{450} = \frac{\Delta A_{450-490}}{\text{total heme} - b_5}\ mM^{-1}\ cm^{-1}$.

‡§ Values for each dietary group without common superscripts differ significantly ($P < 0.05$).

*Cytochrome P-450 extinction coefficient.* Total heme, cytochrome $b_5$ and the carbon monoxide-induced 450 nm absorbance peak were decreased in male rats fed the FF diet below values of 3 and 10 per cent corn oil-fed rats (Table 5). The extinction coefficient was not significantly altered by dietary corn oil.

*NADPH-cytochrome c reductase activity.* Microsomes from male rats fed the 3 per cent corn oil diet reduced 88·1 ± 9·5 nmoles cytochrome *c*/mg protein/min. Feeding a fat-free diet did not significantly alter this (79·2 ± 13·2 nmoles/mg protein/min).

## DISCUSSION

The overall appearance of rats fed the fat-free diet for 3 weeks was not different from rats fed diets containing 3 or 10 per cent corn oil. Differences observed in terminal body weights were small, but significant, in male rats and nonsignificant in female rats. Signs of essential fatty acid deficiency, such as scaliness of skin, pronounced weight loss, and hematuria were not apparent.

A decrease in $V_{max}$ of aniline, hexobarbital and ethylmorphine metabolism was observed using washed microsomes of FF-fed rats, compared to 3 per cent corn oil-fed rats, indicating that the lipid-dependent drug-metabolizing enzymes associated with the particulate microsomal fraction are altered by dietary manipulation. Since microsomal enzymes are intimately associated with the microsomal membrane, it is likely that changes in the composition of these membranes would alter activities of enzymes. Membrane alterations might alter the ability of enzymes to bind substrate molecules, and thus change the apparent $K_m$ or $A_{max}$ of microsomal reactions. Although

decreases in apparent $K_m$ for hexobarbital and ethylmorphine were produced by feeding the fat-free diet, the apparent $K_m$ for aniline was unchanged. The results are in agreement with the hypothesis that lipids are intimately involved in the binding of specific substrates, and support the findings of Chaplin and Mannering[23] that microsomes incubated with phospholipase *c* no longer bind type I substrates, such as ethylmorphine and hexobarbital, while binding of the type II substrate, aniline, is unaltered.

Minor sex differences in kinetic parameters of microsomal reactions produced by FF feeding were noted. Although the $K_m$ for hexobarbital was decreased in male rats, neither aniline nor hexobarbital $K_m$ was altered in female rats by the FF diet. This, coupled with the fact that $K_m$ for hexobarbital and aniline is significantly different in male and female rats, indicates a possible sex dependency in the ability of microsomal enzymes to interact with drug substrates.

Gram *et al.*[24] found that starvation of male rats for 72 hr decreased kinetic parameters for hexobarbital oxidation, and increased those for ethylmorphine-*N*-demethylation and aniline hydroxylation. In the present work, feeding a FF diet decreased kinetic parameters for each of these three reactions. Thus, the effects produced by FF feeding do not appear to be due to starvation. Furthermore, there were no differences in body weight gain associated with diet during the 3 weeks of these experiments.

Microsomal constituents were analyzed in an attempt to relate changes in drug metabolism with changes in membrane composition. Importance of membrane phospholipids for drug-metabolizing activity has been noted by several different investigators.[2,3,23] Dietary manipulation of the fatty acid content of phospholipids[25] might be expected to alter properties of the phospholipids, and thereby the properties of enzymes dependent on phospholipid for activity. Analysis of fatty acid content of microsomal phospholipids revealed marked changes in relative quantities of polyenoic acids after only 3 weeks feeding of the FF diet. In general, differences in fatty acid content observed between animals fed the fat-free diet and those fed 3 per cent corn oil were magnified by feeding 10 per cent corn oil. It appears that there is a direct relationship between linoleic and arachidonic acid content and rate of hexobarbital oxidation, and an inverse relationship with contents of 16:1, 18:1 and 20:3. Strobel *et al.*[3] have recently demonstrated a requirement for phosphatidylcholine by a solubilized cytochrome P-450 system capable of drug, fatty acid and steroid hydroxylation. The extent to which the phospholipid fatty acid changes observed in the present work are related to alteration of cytochrome P-450 activity remain to be clarified by determining the effect of different phosphatidylcholine species on solubilized cytochrome P-450–mediated hydroxylation. Such information should help elucidate the role of dietary lipid in drug biotransformation by indicating the relative importance of various fatty acids.

No change in microsomal protein content occurred in response to the test diets used, unlike the marked decrease in protein in response to 72-hr starvation[24] or the increase observed after induction of enzyme activity.[1] However, content of the terminal oxidase of microsomal electron transport, cytochrome P-450, was lower in both male and female rats receiving the fat-free diet. The association of cytochrome P-450 with membrane suggests the possibility that phospholipid fatty acid alterations occurring with FF feeding result in defects in microsomal membranes and loss of cytochrome P-450. Depression of cytochrome P-450 content by FF feeding was accompanied by a decrease in the maximal spectral shift resulting from the addition of aniline or hexo-

barbital to microsomes. Similar correlations between cytochrome P-450 content and binding of substrates to microsomes have been noted by other investigators.[26,27]

To investigate further the possibility of qualitative alterations in cytochrome P-450 caused by FF feeding, ethyl isocyanide difference spectra of sodium dithionite-reduced microsomes were examined. Sladek and Mannering[28] noted that the ratio of ethyl isocyanide-cytochrome P-450 peaks was altered by 3-methylcholanthrene (3-MC) pretreatment of rats. This alteration was believed to be due to formation of a new type cytochrome, termed cytochrome $P_1$-450, which resulted from administration of 3-MC. FF feeding caused a change in ratio in the opposite direction of the change produced by 3-MC. Jefcoate *et al.*[29] have indicated that both cytochrome species exist in microsomes of control animals, as indicated by the presence of high- and low-spin forms of P-450. Whether a source of dietary lipid is required to maintain a basal level of cytochrome $P_1$-450 is yet to be determined. It is possible that membrane changes alter conformation of cytochrome P-450, resulting in an alteration of the ethyl isocyanide difference spectrum occurring in the absence of a change in cytochrome $P_1$-450 content. Ability to distinguish between soluble cytochrome P-420 and $P_1$-420, and between P-450 solubilized in the presence of glycerol and similarly solubilized $P_1$-450,[30] indicates that $P_1$-450 represents a different cytochrome from P-450, not just a different association of the same cytochrome with the microsomal membrane. Solubilization of cytochromes, followed by spectral analysis, should help elucidate the effect, if any, of FF feeding on relative content of cytochrome P-450 species.

Alteration of conformation of cytochrome P-450 by FF feeding, or change in relative concentration of different forms of cytochrome P-450, could result in a change in extinction coefficient of the carbon monoxide–cytochrome complex. Extinction coefficient was increased in rats pretreated with 3-MC[20] and was lower in male rats than in females.[31] In the present studies, FF feeding did not significantly change extinction coefficient of cytochrome P-450 and the difference observed in content of P-450 between FF-fed and corn oil-fed animals appears valid.

The changes produced by FF feeding do not appear to involve all components of the microsomal electron transport system. Activity of NADPH-cytochrome *c* reductase was not affected, indicating that the rate of cytochrome P-450 reduction is unaltered. In this respect the effects of FF feeding differ from the effects of other dietary treatments. Starvation of male rats resulted in a 10 per cent reduction in NADPH-cytochrome *c* reductase activity.[12] Feeding a magnesium deficient diet to rats for 22 days reduced activity of the enzyme 21 per cent.[32] Thiamine deficiency of male rats increased the rate of cytochrome *c* reduction 75 per cent;[33] however, rats fed fat-free diets had similar cytochrome P-450 reductase and cytochrome *c* reductase activities as those whose diet was supplemented with varying levels of arachidonic acid or menhaden oil.[34] In general, changes in activity of NADPH-cytochrome *c* reductase parallel changes in drug metabolism. Absence of this relationship in FF-fed rats is evidence of a defect at some point beyond the initial oxidation of NADPH.

The above findings indicate that a source of polenoic fatty acids is necessary for optimal activity of the microsomal drug-metabolizing system. Deprivation of dietary lipid results in changes in relative content of microsomal phospholipid fatty acids, and associated with these changes are decreased metabolism of hexobarbital, aniline and ethylmorphine, decreased content of cytochrome P-450, and decreased binding of aniline and hexobarbital to microsomes. Furthermore, the changes in membrane fatty

acid distribution are associated with an apparent change in qualitative aspects of several microsomal constituents, as evidenced by a shift in the ethyl isocyanide difference spectra and by alteration of apparent $K_m$ and $K_s$ for some substrates. These findings suggest that feeding a fat-free diet results in a membrane which is deficient in one or more components of the microsomal system. The dependency of functional cytochrome P-450 on lipid, as noted by Strobel *et al.*,[3,4] implicates this hemoprotein as being one such constituent. Synthesis of new microsomal membranes in the absence of a source of polyenoic acids may result in a membrane which lacks the proper conformation for maintaining a full complement of cytochrome P-450 within the membrane. Furthermore the cytochrome P-450 which is in the membrane may itself be conformationally altered, so that it exhibits different properties from those of P-450 from microsomes of animals receiving polyenoic acids.

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