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FATTY ACID REQUIREMENTS AND TEMPERATURE DEPENDENCE OF MONOOXYGENASE ACTIVITY IN RAT LIVER MICROSOMES

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Summary

The effect of variation in the microsomal membrane fatty acid composition on Arrhenius plot phase transition temperatures for *p*-nitroanisole O-demethylation and benzo[*a*]pyrene hydroxylation has been investigated. In liver microsomes from normal-dieted rats, *p*-nitroanisole *O*-demethylase activity has a break temperature at 24°C, while that of benzo[*a*]pyrene hydroxylase occurs at 29°C indicating that these two enzymes may exist in different patches of membrane. The microsomal membrane fatty acid composition was altered by starving rats for 48 h and then refeeding them a fat-free diet for 4 or 5 days. In microsomes having diet-altered fatty acid compositions, benzo[*a*]pyrene hydroxylase has a break temperature at 33°C, a value higher than that observed in normal-dieted rats. This observation correlates with the increase in saturation observed in the diet-altered fatty acid composition and thus may correspond to a phase transition roughly dependent on the fatty acid melting point. Induced and basal levels of cytochrome *P*-450 and *P*-448 in animals having different microsomal fatty acid composition are reported. Phenobarbital-induced levels of *p*-nitroanisole *O*-demethylase in normal microsomes were six times higher than those in microsomes having diet-altered composition, whereas 3-methylcholanthrene-induced levels of benzo[*a*]pyrene hydroxylase were similar regardless of diet. The low level of *p*-nitroanisole *O*-demethylase activity in membranes with altered fatty acid compositions suggests that a particular type(s) of fatty acid was not present in sufficient quantity to permit the induction of maximal enzyme activity. Since the induced benzo[*a*]pyrene hydroxylase activity was the same regardless of diet, there was presumably sufficient quantities of the appropriate fatty acids present in the membrane for induction of this activity. Therefore, particular fatty acids may be necessary for the

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Abbreviations: Me_2SO, dimethylsulfoxide; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; C-GC-MS, computerized gas chromatographic-mass spectrometry.

induction of maximal activity of particular enzymes in the mixed function monooxygenase system.

Introduction

The mixed function monooxygenase system metabolizes drugs and carcinogens in mammalian tissue [1,2]. Several investigations have identified lipid, cytochrome *P*-450, and NADPH-cytochrome *P*-450 reductase as the three components required to obtain maximal monooxygenase activity in a solubilized reconstituted system [3—7]. The lipid has been further identified as phosphatidylcholine, with the type of diacyl chains influencing the amount of reconstituted enzyme activity [3]. Furthermore, dietary lipid has been shown to have an effect on rat liver microsomal mixed function monooxygenase activities [8,9]; rats fed a fat-free diet had less cytochrome *P*-450 content and lower levels of drug-metabolizing enzymes than those fed a normal diet.

Studies of the kinetics of chemical modification of the reductase in microsomes [10], as well as temperature-dependent measurements of microsomal [11—13] and liposomal [14] membrane-bound enzyme activities showing break temperatures have supported the proposal that lateral mobility of the reductase and the cytochrome proteins in the lipid is the mechanism whereby several cytochromes can be reduced by one reductase.

Using physical techniques such as spin-label and fluorescence polarization measurements, a succession of transition temperatures has been measured in the endoplasmic reticulum membranes from rat liver [15] and from cells grown in culture [16]. In addition, Arrhenius plots of rat liver microsomal membrane-bound enzyme activities have yielded various break temperatures depending on the enzyme assayed [15,11—13], and these breaks have been attributed to phase transitions of the fatty acids in the membrane lipid.

The mixed function monooxygenase system consists of various enzyme activities. Little is known about the lipid requirement for each of these various activities and the location of these enzymes in the endoplasmic reticulum membrane. In an effort to determine the spacial arrangement and lipid fatty acid requirements of these membrane-bound enzymes, we have studied the physical properties of the lipid environment and the effect of the membrane fatty acid composition on enzyme activity. The enzyme activities studied were: (1) *p*-nitroanisole *O*-demethylase, which contains cytochrome *P*-450 and can be induced by the drug phenobarbital; and (2) benzo[*a*]pyrene hydroxylase, which contains cytochrome *P*-448 and can be induced by the chemical carcinogen 3-methylcholanthrene.

In this paper we report Arrhenius plots of enzyme activity which depend on the lipid fatty acid environment of these particular enzymes. In rats fed a normal diet, the transition temperatures of these two membrane-bound enzymes were found to be significantly different, indicating that the fatty acid environments are not the same and that *P*-450- and *P*-448-dependent enzymes may exist in different patches of microsomal membrane. The Arrhenius plot break temperature of enzyme activity in microsomal membranes with diet-altered fatty acid composition was also measured. Moreover, induction studies in

microsomes having normal and diet-altered fatty acid compositions suggest that the lipid fatty acid requirement of these enzymes is different.

Materials and Methods

Animals. Male Sprague-Dawley weanling rats (Simonsen Laboratories, Gilroy, Calif.) weighing approx. 60 g were fed a balanced diet for a few days after arrival. They were then placed in groups, half the group received a normal balanced diet (Berkeley diet containing 7% fat from Foodstuffs Processing Co., Laboratory Animal Food Div., San Francisco, Calif.) throughout, while the other half were fasted for 2 days and then refed a fat-free diet (Fat-free Diet No. 11270T from U.S. Biochemical Corp., Cleveland, Ohio) for 5—6 days. Both diets contained supplemental vitamins and minerals. Food and water were accessible ad libitum throughout, except for food during the fast.

The groups of normal-dieted, and fasted-and-refed rats were each subsequently divided into groups of 4—6 animals. One group received a daily intraperitoneal injection of sodium phenobarbital (80 mg/kg body weight) in 0.9% saline for 3 days prior to killing, while another group received a single intraperitoneal injection of 3-methylcholanthrene (25 mg/kg body weight) in 0.5% methylcellulose in saline one day prior to killing. Control groups received saline, methylcellulose, or nothing. The animals were killed by decapitation and the livers were immediately removed and chilled in ice-cold saline. Microsomes were prepared in batches of 4—6 livers as previously described [17], except the final microsomal pellet was washed once.

Analytical procedures. The temperature dependence of enzyme activity was obtained by incubating the reaction mixtures simultaneously at various temperatures between approx. 5 and 50°C. This was accomplished by performing the incubation in water filled holes which were drilled into an aluminum bar approx. 1.5 m long with a cross-section of 7.5 × 10 cm. A heat source at one end and a heat sink at the other provided the necessary temperature gradient along the bar. Incubation temperatures were monitored along the bar in control reaction mixtures with a copper-constantan thermocouple and mercury thermometers. A least squares fit of the data to two straight lines was done on a Sigma 2 computer.

Benzo[a]pyrene hydroxylase assay. This assay was basically that of Nebert and co-workers [18,19] and is described in detail elsewhere [20] except that the substrate was added in 10 μl Me_2SO to give a concentration of 4 μM. Final concentration of Me_2SO in the assay was 1%. Following a 4-min pre-incubation period for temperature equilibration, the reaction was initiated by the addition of 50 μg microsomal protein in 100 μl of Tris buffer, pH 7.5. The samples were incubated for 10 min at all temperatures and under these conditions the reaction was linear with respect to time and protein. The reaction was stopped by the addition of 1.0 ml acetone. Controls had acetone added at the start of the reaction. The effect of temperature on pH was found to be negligible.

p-Nitroanisole O-demethylase assay. This assay is based on the procedure reported by Netter and Seidel [21]. The reaction mixture consisted of 3 mM glucose 6-phosphate, 10 units glucose-6-phosphate dehydrogenase, 1 mM *p*-nitroanisole, 1 mg protein, and 1 ml of 25 mM phosphate buffer, pH 7.85. A 1.0

ml volume was preincubated for 3 min at the specific temperature. The reaction was started by addition of 0.025 mol $NADP^+$ in 10 μl buffer and it was stopped by addition of 200 μl of 1 M NaOH. The controls had the NaOH added at the start of the reaction.

The NaOH also served to clarify the assays presumably by dissolving the protein and thus reduced one cause of the light scattering. The absorbance of the reaction product, *p*-nitrophenol, was measured at 400 nm using the control as a reference in a Cary 118 spectrophotometer. The absorbance of the substrate, *p*-nitroanisole, at 400 nm was negligible. An effective molar extinction coefficient of $1.5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ was determined by measuring the change in absorbance at 400 nm upon addition of known amounts of *p*-nitrophenol after the reaction was stopped. The reaction was generally run for 3—5 min and was linear at all temperatures used. The non-linear region usually seen at the start of the *O*-demethylation of *p*- or *o*-nitroanisole according to Netter [22] is not observed under the conditions described above. The reaction was linear in protein up to 1 mg at all temperatures used in this study. Addition of nicotinamide to obtain maximum rates was found to be unnecessary. Indeed, when 20 μM nicotinamide was present in the reaction mixture the measured velocities were approx. 30% lower, presumably due to inhibition by nicotinamide [23].

Fatty acid compositions. Lipids were extracted from liver microsomes equivalent to 10 mg of microsomal protein in chloroform/methanol/water (8 : 4 : 3, v/v) by vortexing for several minutes [24]. The aqueous layer was removed by suction and the chloroform layer evaporated to dryness under nitrogen and the residue dissolved in 2 ml chloroform for loading onto a silicic acid column. The column was 5 mm inside diameter and contained approx. 6 g of activated silicic acid 100 mesh (Mallinckrodt, Inc., St. Louis, Mo.). The column was washed with 30 ml chloroform before each sample was run. The sample was then eluted with 10 ml chloroform (neutral lipid, fraction 1) and 10 ml methanol (complex lipid fraction 2). The flow rate was approx. 0.5 ml/min. Both fractions were evaporated to dryness and then saponified by heating to 100°C in a capped tube for 1 h in 2 ml of 2 M KOH in ethanol/water (1 : 1, v/v). Free cholesterol was extracted three times from fraction 1 with 10 ml light petroleum. The combined ether extracts were evaporated to dryness with nitrogen. The cholesterol when silylated was heated in a capped tube to 65°C for 10 min in the presence of 100 μl pyridine, 300 μl *N,O*-bis (trimethylsilyl)-trifluoroacetamide (BSTFA), and 25 μl trimethylchlorosilane. The reaction mixture was evaporated to dryness with nitrogen and immediately dissolved in 0.5 ml chloroform for gas chromatographic analysis. Some samples were run without silylation. The aqueous phase of the neutral lipid (fraction 1) was acidified with H_2SO_4 and the fatty acids extracted with 10 ml light petroleum. The organic phase was washed three times with water and evaporated to dryness with nitrogen prior to methylation.

The non-saponifiable lipid was extracted from the complex lipid (fraction 2) with 10 ml light petroleum. The saponified fatty acids were then acidified, extracted, washed, and dried as described above for the neutral lipid (fraction 1). The fatty acids from fractions 1 and 2 were then separately methylated by heating to 100°C for 4 min in a capped tube in the presence of 2 ml benzene and 2.5 ml BF_3 (14% in methanol). The reaction was stopped by addition of

10 ml water. The methyl esters were extracted with 10 ml heptane, evaporated to dryness with nitrogen and dissolved in 200 μl ethyl acetate for gas chromatographic analysis.

Gas chromatographic (GC) analysis. The analyses of the methyl esters of fatty acids were performed on a Varian Model 2700 gas chromatograph at 180°C, with helium carrier gas flowing at 6 ml/min. A 10 ft long, 0.030 inch inside diameter glass capillary column containing HI-EFF-2BP (Applied Science Labs) on 100/120 mesh Gaschrom Q was used. Peaks were identified by comparison with mobilities of known standards and by computerized gas chromatographic-mass spectrometric (C-GC-MS) analysis. The C-GC-MS analyses were performed using a Dupont 491-2 double focusing mass spectrometer coupled directly to a Varian aerograph 204 GC equipped with the same glass capillary column described above. Cholesterol levels in the microsomal membranes were measured by extraction and GC analysis of its silyl derivative. A Hewlett-Packard GC model 5750 was used with temperature programmed from 130 to 300°C at 30°C/min. Carrier gas was helium flowing at 12 ml/min. A 12 ft long, 1/16 inch inside diameter stainless steel column containing OV-17 (Applied Science Labs) on Chromosorb W 100/120 mesh was used. Known amounts of cholesterol were used as standards.

Cytochrome *P*-450 and *P*-448 content was measured by the method of Omura and Sato [25] using a molar extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$. Protein was measured by the method of Lowry et al. [26] using bovine serum albumin as a standard.

All solvents used were chromatoquality (MC/B, Norwood, Ohio). GC standards were purchased from Sigma Chemical Co. (St. Louis, Mo.) and initially checked for purity by thin-layer chromatography [27].

Results

Lipid analysis

The relative (mol percent) composition of the major saponified fatty acids from rat liver microsomes maintained on a normal diet and those fasted for 48 h and then refed a fat-free diet is shown in Table I. The fatty acid compo-

TABLE I

FRACTION 2 SAPONIFIABLE FATTY ACIDS (relative mol percent)

N, normal diet; R, fasted and refed the fat-free diet; C, non-induced control; PB, phenobarbital-induced; MC, 3-methylcholanthrene-induced. Double bond, average number of double bonds per fatty acid molecule.

Diet and pre-treatment	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	20 : 3	20 : 4	Double bond
NC	16.2	0.8	31.6	6.3	16.8	0	28.4	1.5
NPB	19.6	1.9	33.5	7.6	16.2	0	20.8	1.3
NMC	17.9	2.0	33.9	6.8	17.7	0	21.8	1.3
RC	24.2	6.7	20.3	29.1	4.8	9.7	7.4	1.0
RPB	19.3	7.1	23.0	28.1	3.4	10.1	9.1	1.0
RMC	18.8	3.8	25.4	28.2	4.3	9.9	9.5	1.1

sition is shown for non-induced animals, as well as for those induced with phenobarbital and with 3-methylcholanthrene. The inducing compounds do not have a measurable effect on the fatty acid composition of rat liver microsomes. Microsomes from the rats maintained on the two different diets, however, have markedly different fatty acid compositions. Compared to the normally fed animals, the fasted and refed animals have elevated levels of palmitoleic acid (16 : 1), oleic acid (18 : 1), and eicosatrienoic acid (20 : 3), and decreased levels of the polyunsaturated acids 18 : 2 and 20 : 4, which is in agreement with results reported by others [28,29]. There is an overall increase in saturation of the fatty acids in the animals that were fasted and refed the fat-free diet. These diet-induced changes in fatty acid composition are similar to those observed in rats fed a diet deficient in essential fatty acids [30].

When the neutral lipid (fraction 1) free fatty acids were analyzed (data not shown), changes in relative composition were observed similar to those measured in the complex lipid (fraction 2) saponified fatty acids. Total cholesterol levels were also measured, and rats fed the normal diet had approximately twice that of the rats fasted and refed the fat-free diet. (Normal diet: 0.030 μmol cholesterol/mg protein; fasted and refed: 0.014 μmol cholesterol/mg protein.) In addition, total complex lipid (fraction 2) saponified fatty acid levels were measured, and rats fed the normal diet had approximately twice that of the rats fasted and refed the fat-free diet. (Normal diet: 0.690 μmol/mg protein; fasted and refed: 0.309 μmol/mg protein.) Thus the mol ratio of total complex lipid (fraction 2) saponified fatty acid to cholesterol is essentially the same in both the normal-dieted rats and those fasted and refed the fat-free diet.

Temperature dependence

In normal-dieted rats induced with phenobarbital the break point in the Arrhenius plot of *p*-nitroanisole *O*-demethylation (Fig. 1) is at 24.2 ± 2.1°C (n = 5, 95% confidence) which agrees with the break temperature of 23.9 ± 1.6°C recently reported by Yang and coworkers [13]. In contrast, the *P*-448-dependent benzo[*a*]pyrene hydroxylase (Fig. 2) has a significantly different break temperature at 29.3 ± 1.2°C (n = 3, 95% confidence) in normal-dieted 3-methylcholanthrene-induced and non-induced rats suggesting that these two enzymes may be located in different environments within the membranes.

The temperature dependence of benzo[*a*]pyrene hydroxylase activity was also measured in microsomes from animals which were fasted and refed the fat-free diet (Fig. 3). These microsomal membranes have an overall increase in saturation in their fatty acids, and have a significantly (99% confidence) higher break temperature at 33.4 ± 0.1°C (n = 2, 95% confidence). Owing to low levels of activity, the temperature dependence of *p*-nitroanisole *O*-demethylase could not be measured in phenobarbital-induced microsomes from rats fed the fat-free diet nor in non-induced microsomes regardless of diet.

In Figs. 1—3, the calculated activation energies above and below the break temperature are shown. In all cases regardless of enzyme type or membrane fatty acid composition, the value of the activation energy above the break temperature is less than that below it. Since the membrane is in a more fluid state above the phase transition temperature this may facilitate electron transfer between the reductase and the cytochrome, resulting in a smaller activation

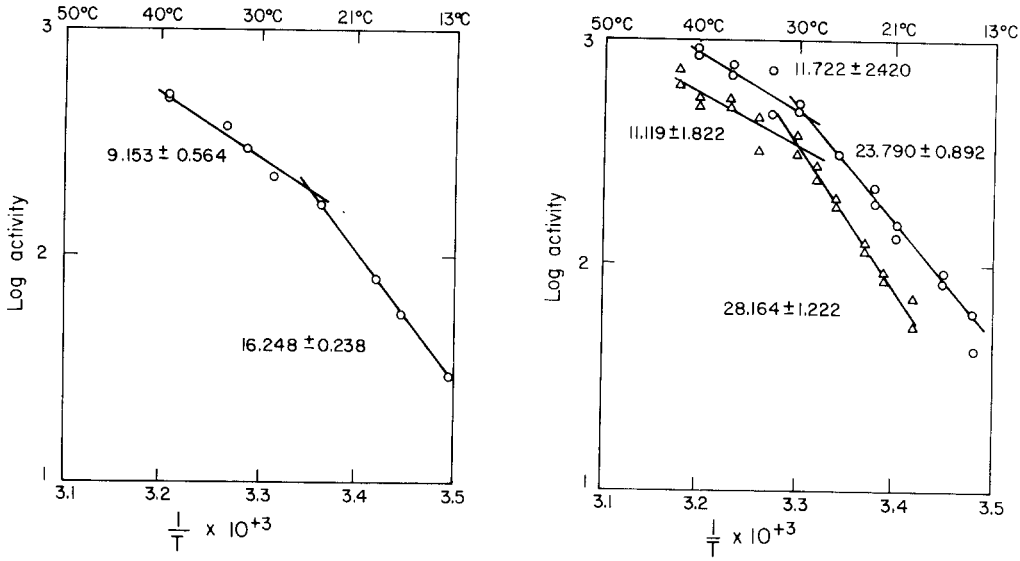

Fig. 1. Arrhenius plot of *p*-nitroanisole O-demethylation activity in liver microsomes from phenobarbital-induced rats fed a normal diet. Specific activity in units of nmol *p*-nitrophenol · min^{-1} · mg^{-1} are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperature are shown in kcal/mol.

Fig. 2. Arrhenius plot of benzo[*a*]pyrene hydroxylase activity in liver microsomes from 3-methylcholanthrene-induced (○) and non-induced (△) rats fed a normal diet. Specific activity in units of nmol 3-hydroxybenzo[*a*]pyrene · min^{-1} · mg^{-1} are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperatures are shown in kcal/mol.

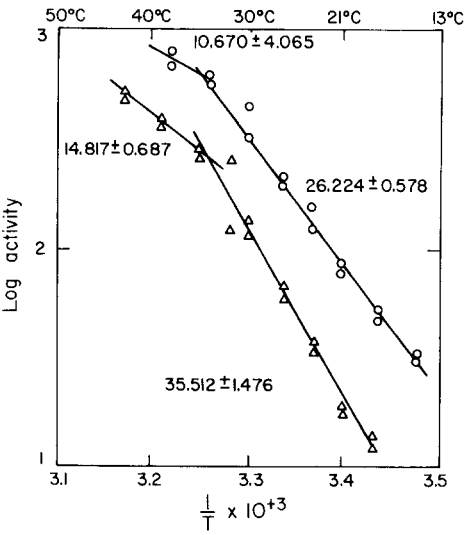

Fig. 3. Arrhenius plot of benzo[*a*]pyrene hydroxylase activity in liver microsomes from 3-methylcholanthrene-induced (○) and non-induced (△) rats fasted and refed a fat-free diet. Specific activity in units of nmol 3-hydroxybenzo[*a*]pyrene · min^{-1} · mg^{-1} are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperatures are shown in kcal/mol.

TABLE II

CYTOCHROME *P*-450 AND *P*-448 CONTENT AND SPECIFIC ENZYME ACTIVITY AT 37°C

Diet and pre-treatment *	nmol *p*-nitrophenol · mg^{-1} · min^{-1}	Cytochrome *P*-450 content (nmol/mg protein)
NC	0.18 ± 0.17	0.38 ± 0.10
NPB	2.41 ± 0.65	1.41 ± 0.01
RC	0.02 ± 0.03	0.34 ± 0.09
RPB	0.42 ± 0.22	0.36 ± 0.01
	nmol 3-hydroxybenzo[*a*]pyrene · mg^{-1} · min^{-1}	Cytochrome *P*-448 content (nmol/mg protein)
NC	0.089 ± 0.005	0.38 ± 0.10
NMC	0.225 ± 0.024	0.98 ± 0.08
RC	0.033 ± 0.004	0.34 ± 0.09
RMC	0.197 ± 0.018	0.64 ± 0.04

* Nomenclature is the same as in Table I.

energy above the transition. Electron transfer, the first or the second electron, has been considered as a rate-limiting step in the overall enzyme catalyzed reaction [10—13,31—35]. Presumably electron transfer rates would vary between different patches of lipid (or fatty acid) and thus might explain the large variation in metabolic rates from approx. 0.3 nmol · min^{-1} · mg^{-1} for benzo[*a*]-pyrene hydroxylation to approx. 30 nmol · min^{-1} · mg^{-1} for benzphetamine N-demethylation [13].

Induced enzyme activity

In rats fed a normal diet the levels of 3-methylcholanthrene-induced benzo[*a*]pyrene hydroxylase activity are approximately the same as those in membranes with altered fatty acid compositions. Altered membrane fatty acid composition does not affect the ability of the animal to synthesize the benzo[*a*]-pyrene hydroxylation enzyme in the monooxygenase system. However, phenobarbital-induced levels of *p*-nitroanisole *O*-demethylation activity in normal-dieted rats are approximately six times greater than in rats with diet-altered fatty acid compositions, thus indicating that membrane fatty acid composition may affect the ability of the rat to synthesize certain types of enzyme in the mixed function monooxygenase system.

Cytochrome *P*-450 and *P*-448 content was measured by absorption of the reduced protein · CO complex and this data is shown in Table II.

Discussion

The results reported herein indicate that the physical characteristics of the endoplasmic reticulum membrane affect the activity of those enzymes bound to it. This type of behavior has also been observed in purified and reconstituted enzyme systems. Strobel and coworkers [3] have shown that *P*-450-dependent benzphetamine hydroxylation in a reconstituted system requires phosphatidylcholine, and the type of fatty acid chains on the phospholipid affected the amount of enzyme activity. Lu and coworkers [4] came to the same conclusion

for *P*-448-dependent benzo[*a*]pyrene hydroxylation.

With normal-dieted rats, the different break temperatures (Figs. 1 and 2) for the two enzyme activities indicates that the enzymes are associated with different fatty acids and therefore may be physically located in different patches or areas of the membrane. The higher phase transition temperature observed for benzo[*a*]pyrene hydroxylase activity implies that its fatty acid environment is more saturated than that associated with the *p*-nitroanisole O-demethylation enzyme. In both control and 3-methylcholanthrene-induced fasted and refed rats the break temperature for benzo[*a*]pyrene hydroxylation (Fig. 3) is approx. 4°C higher than that observed in normal rats. This change in phase transition temperature indicates that the *P*-448-dependent enzyme is in a lipid environment that is sensitive to the overall fatty acid composition of the membrane. A change to more saturated fatty acids is accompanied by an increase in their melting point temperature. This type of compositional change was observed when rats were fasted and refed the fat-free diet, and there was a corresponding increase in the Arrhenius break temperature for benzo[*a*]pyrene hydroxylase. This correlation indicates that the fatty acid composition at or near the hydroxylase is influencing the observed changes in phase transition temperature. Others [11,13] have shown that such phase transition temperatures, observed in the monooxygenase enzyme system, are due to a phase transition of membrane phospholipids and are not due to enzyme-substrate interactions nor protein conformational changes. The role of fatty acids in these enzyme systems is unknown but they may be needed for proper orientation between reductase and cytochrome.

A comparison of the levels of induced enzyme activities (Table II) in microsomes having normal fatty acid composition with those membranes having altered fatty acid composition gives an indication of the fatty acid required for maximal enzyme activity. The fact that the *P*-450-dependent enzyme did not increase to the same extent upon phenobarbital induction in the refed animals to the level that was attained in normal-dieted animals suggests that a particular type(s) of fatty acid was not present in sufficient quantity to permit the induction of maximal enzyme activity. On the other hand, the *P*-448-dependent enzyme activity was inducible to a high level in the refed rats. Presumably sufficient quantities of the appropriate fatty acids were present in the membrane for induction of this activity. Minimum levels of particular types of fatty acids may, therefore, be necessary for induction of maximal activity of particular enzymes in the mixed function monooxygenase system. Induction of *P*-450-dependent enzyme activity may require membrane proliferation with unsaturated fatty acids, while induction of *P*-448-dependent enzyme activity may require membrane proliferation with saturated fatty acids. It should be noted that the normal diet and the fat-free diet were different and although both contained vitamin and mineral supplements, the possibility exists that the low levels of induced *P*-450-dependent enzyme activity might be due to some nonlipid dietary factor. However, since the induced *P*-448-dependent benzo[*a*]pyrene hydroxylase activity was approximately the same regardless of diet, both diets are sufficient to supply the nutrients necessary to synthesize the components of this enzyme system.

The induced cytochrome content in animals having different microsomal

fatty acid composition is shown in Table II. One interpretation is that phenobarbital causes no increase in cytochrome *P*-450 content, and thus results in low *p*-nitroanisole *O*-demethylase activity. However, the measured cytochrome content represents multiple forms [36—39] of the cytochromes, and this fact makes the data difficult to interpret. For example, in rats refed the fat-free diet, the specific activity of *p*-nitroanisole O-demethylation increased upon phenobarbital induction while the measured cytochrome content remains essentially constant. Thus, the synthesis of *p*-nitroanisole *O*-demethylase enzyme must have been induced while synthesis of other cytochromes was repessed in order for the overall cytochrome content to remain constant.

Recent work by Davison and Wills [40] indicates that in rat liver microsomes, monooxygenase induction by phenobarbital and by 3-methylcholanthrene is associated with increases in membrane linoleic and oleic acids, respectively. Our results are consistent with their observations and suggest that *P*-450- and *P*-448-dependent enzymes are associated in the membrane with different fatty acids, i.e. they are located in different patches of non-uniformly distributed membrane lipid [41], or are on different sides of a membrane with an asymmetric lipid distribution [42].

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