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Studies on Three Microsomal Electron Transfer Enzyme Systems

Effects of Alteration of Component Enzyme Levels *in Vivo* and *in Vitro*

INGELA JANSSON AND JOHN B. SCHENKMAN[1]

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

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SUMMARY

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Liver microsomes contain three multicomponent enzyme systems that appear to share the same pyridine nucleotide-linked electron input enzymes. They are the mixed-function oxidase, the fatty acyl-CoA desaturate, and the lipid peroxidase. The three electron transfer pathways can be considered alternate pathways of lipid metabolism, but they also metabolize xenobiotics. Deprivation of food, a high-carbohydrate, low-fat diet, and phenobarbital administration have differential effects on the three pathways. The high-carbohydrate diet causes large increases in desaturase content, cessation of peroxidase activity, and a considerable decrease in mixed-function oxidase activity. Induction of mixed-function oxidase activity by phenobarbital markedly lowers desaturase activity but does not affect the peroxidase. Fasting causes an increase in the lipid peroxidase and reduces the desaturase activity but does not affect the level of mixed-function oxidase. These changes are accompanied by modifications of electron transfer component (FP_D, FP_T, and cytochrome b_5) levels, but no systematic relationship emerges. Similarly, alterations of the activities of these electron transfer pathways *in vitro* by fortification of microsomes with FP_D or cytochrome b_5 reveals no consistent relationship: e.g., fatty acyl-CoA desaturase is increased, mixed-function oxidase activity is inhibited, and lipid peroxidase activity is unaffected by fortification of microsomes with cytochrome b_5. These results suggest that rate limitation and electron flow direction are determined at the level of the individual terminal enzymes.

INTRODUCTION

Liver microsomes contain at least three distinct pathways of electron transfer. One of these pathways, a mixed-function oxidase system (1), is concerned with the oxidative metabolism of native lipophilic compounds like testosterone and fatty acids (2, 3), as well as compounds foreign to the body (4, 5). This pathway contains at least two components, the flavoprotein NADPH-cytochrome *c* (P-450) reductase (6) and the hemoprotein cytochrome P-450 (7). The second pathway also is involved in lipid metabolism, namely, the desaturation of fatty acids (8-10). This pathway contains the flavoprotein NADH-cytochrome b_5 reductase (11) and cytochrome b_5 (12, 13) as well as a cyanide-sensitive

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factor which is believed to be a colorless oxidase (14, 15). The fatty acid desaturase system is the mammalian system responsible for formation of Δ^9 unsaturated fatty acids from the corresponding saturated fatty acyl-CoA derivatives (e.g., stearyl-CoA→oleyl-CoA) by liver microsomes (16). A third electron transfer pathway, the lipid peroxidase system (17), is also concerned with lipid metabolism. This system is responsible for the peroxidation of constitutive fatty acids in hepatic microsomal membranes (18), mainly arachidonic acid (19), and its functioning has been variously reported to cause damage to the microsomal membranes (20) and degradation of microsomal heme (21, 22). Components of the lipid peroxidase pathway have not been well elucidated, but the flavoprotein NADPH-cytochrome *c* (P-450) reductase and perhaps an as yet undetermined component have been implicated (17, 23). Demonstration of its activity *in vitro* requires the presence of iron chelates (17), such as iron pyrophosphate or ADP-iron (24). Products of the reaction include conjugated dienes and malondialydehyde (25).

It is well established that NADPH is the electron donor of choice for the mixed-function oxidase (4, 5). Although NADH can support activity of this pathway, it does so poorly, at about 10–15% of the rate of the NADPH-driven reaction (6); addition of NADH to the NADPH-supported oxidation of certain substrates provides a superadditive rate of oxidation (26, 27). Although Holloway *et al.* (10) found NADPH and NADH to be equally effective in supporting fatty acid desaturase activity, most investigators (11, 14) have reported that NADH drives the reaction better. High levels of ascorbate (10 mM) will also support the desaturase reaction, but at about 50% of the rate of the reduced pyridine nucleotide-driven reaction (14). As with the desaturase system, ascorbate can function as an electron donor for lipid peroxidation (17, 24); however, while NADPH-supported lipid peroxidation is heat-labile, ascorbate drives the reaction equally well with boiled microsomes (17, 28). NADH was considered inactive as an electron donor for the peroxidase at 0.3 mM concentration (24, 28), but recently Pederson *et al.* (23) found that under some conditions NADH will support lipid peroxidation in microsomes.

Based upon the above studies, it is probable that these three pyridine nucleotide-dependent electron transfer pathways utilize the same two electron input enzymes, NADH-cytochrome b_5 reductase and NADPH-cytochrome *c* (P-450) reductase (FP_T). If this assumption is correct, factors affecting one pathway should have an effect on the operation of the other pathways. The purpose of this paper is to show that changing the content of component enzymes in the microsomes indeed alters the activity of all three pathways of microsomal electron transfer, but that control of the relative activities of the pathways is not at the level of the electron transfer components.

METHODS

Enzyme preparations. Microsomes were obtained from NaCl-perfused livers of 250-g male Sprague-Dawley rats (Charles River Breeders) by a rapid centrifugation technique (29, 30); microsomes obtained by this method were compared with microsomes obtained by a longer differential centrifugation technique (31) and were found not to differ in mixed-function oxidase, fatty acid desaturase, or lipid peroxidase activities. Microsomal protein was measured by the biuret procedure (32). Cytochrome b_5 was isolated from liver microsomes by modifications of the procedures of Spatz and Strittmatter (33) and Sato *et al.* (34). The detergent Emulgen 911 (Kao-Atlas, Tokyo) was used to solubilize the microsomes; they were homogenized in 1% Emulgen 911–25% glycerol–0.1 M Tris-acetate, pH 7.5, at 5 mg of protein per milliliter, and were incubated under nitrogen at 0° for 30 min. Centrifugation at 150,000 × *g* for 45 min in a Spinco refrigerated ultracentrifuge yielded only a small glycogen pellet. The supernatant was passed through a DEAE-cellulose column (Whatman DE-52, preswollen) that had been equilibrated with 0.1 M Tris-acetate (pH 7.5)–0.5% Emulgen 911–25% glycerol. When livers of 15 male rats were used, 600

ml of supernatant were obtained; this quantity was passed through a 2.6 × 30 cm column. Cytochrome b_5 and FP_T[2] were absorbed at the top of the column, and FP_D and cytochrome P-450 (60–80% of the total) were eluted without retention. No cytochrome b_5 was found in the eluate. The column was washed with 1 liter of the equilibration medium and then eluted with 400 ml of 0–0.5 M KCl gradient in the same medium. Cytochrome b_5 was eluted first, followed by FP_T with slight overlap. Cytochrome b_5 was then further purified by the methods of Spatz and Strittmatter (33). FP_D purification followed completely the method of Spatz and Strittmatter (35). The purified cytochrome b_5 was free of FP_T and FP_D activities, and the FP_D was free of cytochrome b_5 and FP_T. The resultant preparations were determined to be detergent-free by obtaining an A_{280}:A_{413} ratio of 0.39 for cytochrome b_5[3] and an A_{280}:A_{462} ratio of 9.0 for FP_D.[4] The protein content of purified enzymes was measured by the method of Lowry *et al.* (36).

Microsomes were fortified with cytochrome b_5 (purified to a specific content of 42 nmoles/mg of protein) or NADH–cytochrome b_5 reductase (3 units/mg) by incubation under nitrogen at 37° for 20 min, cooling to about 4°, centrifugation at 150,000 × *g* for 15 min, and, finally, a single wash in 0.15 M KCl. This method was based upon the technique of Enamoto and Sato (37). The amounts of cytochrome b_5 and FP_D bound to the microsomes were determined spectrophotometrically, and respectively by NADH–cytochrome *c* and NADH-ferricyanide reductase measurements (see below).

Assays. Mixed-function oxidase activity was measured in a medium containing 1.5 mg of microsomes per milliliter, 0.05 M Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM isocitrate, 18 μg of isocitrate dehydrogenase per milliliter (Sigma type IV), 0.35 mM NADP, and aniline (5 mM), ethylmorphine (8 mM), or aminopyrine (8 mM) as substrate. Assays were performed as described previously (31). Incubation times were for 7 min for aminopyrine and ethylmorphine, and up to 15 min for aniline at 37°.

Lipid peroxidation was measured by the malondialydehyde produced in that reaction according to the method of Ottolenghi (25) in a medium containing 0.15 M KCl–0.025 M Tris-HCl (pH 7.5), 50 μM ferric pyrophosphate, 0.5–1.0 mM NADPH or NADH (unless otherwise indicated), and 1 mg of microsomes per milliliter. Samples were taken at 1-min intervals up to 8 min, and the initial rates were determined from linear plots.

Stearyl-CoA desaturase activity was determined by measuring the tritiated water produced in the Δ^9 desaturation of [9,10-^{3}H]stearyl-CoA (DHOM Laboratories) (38). For standardization, oleyl-CoA production was determined by thin-layer chromatography of the methyl esters of fatty acids in the medium by the method of Oshino *et al.* (14). Since the stearyl-CoA has 1 tritium atom on C-9 and 1 on C-10 (*cis*), an isotope effect of 7.0 was obtained and corrections were made. The assay medium contained either 1 mM NADH or 1 mM NADPH in 0.1 M Tris-HCl, pH 7.25, and 70 μM stearyl-CoA (362 nCi/ml). The reaction was started by addition of 2 mg of microsomes per milliliter, and samples were removed at 0.5-min intervals up to 2.5 min.

Spectra were recorded with an Aminco-Chance dual-wavelength recording spectrophotometer as previously described (31). Kinetic measurements of spectral changes were recorded with the instrument in the dual-wavelength mode unless otherwise noted. Cytochrome *c* reductase assays were performed at 22° in 0.1 M Tris, pH 7.5, containing 60 μM horse heart cytochrome *c* (Sigma type VI) and 80 μg of microsomes per milliliter. The reaction was started by adding 0.2 mM NADH or NADPH (final concentration) and was monitored at 550 nm minus 541 nm (39). An extinction coefficient of 19.6 mM^{-1} cm^{-1} was used to calculate the activity (40). Ferricyanide reductase assays were performed in 0.1 M Tris, pH 7.5, containing 200 μM $K_3Fe(CN)_6$ and 80 μg of microsomes per milliliter. The

[2] The abbreviations used are: FP_T, NADPH–cytochrome *c* (P-450) reductase; FP_D, NADH–cytochrome b_5 reductase; CSF, cyanide-sensitive factor.

[3] J. Ozols, personal communication.

[4] M. Rogers, personal communication.

reactions were initiated in one cuvette with 0.2 mM NADH or NADPH (final concentration) and were followed at 420 nm using the split-beam mode and two cuvettes. An extinction coefficient of 1.02 mM^{-1} cm^{-1} was used to calculate activity (41). One unit of activity was defined as reduction of 1 μmole of ferricyanide per second at 22°.

Induced animals. Mixed-function oxidase induction was performed by injection of rats with 80 mg of phenobarbital per kilogram of body weight daily for 4 days prior to killing (42).

Fatty acid desaturase activity was induced by a modification[5] of the regimen of Oshino and Sato (43). Animals were fed a high-carbohydrate, fat-free diet (NBC) for 4 hr/day for 4 days. Laboratory chow (Purina) was available ad libitum at all other times. Subsequently the animals were given only the high-carbohydrate diet ad libitum for 4 hr/day for 2 days. All food was removed for the following 2 days, after which they were given the high-carbohydrate diet ad libitum for 17 hr prior to killing.

RESULTS

Incorporation of microsomal enzymes. Cytochrome b_5 and FP_D isolated from rat liver microsomes by detergents, in agreement with earlier reports (35, 37, 44, 45), could be reincorporated into fresh rat liver microsomes (Fig. 1A and B), with the amount bound dependent upon the concentration present in the medium. The content of cytochrome b_5 in rat liver microsomes could be elevated well in excess of the 6-fold increase shown in Fig. 1A. Of interest was the observation that prior 4-fold elevation of the microsomal cytochrome b_5 (Fig. 1B) lowered the amount of FP_D bound at all concentrations of FP_D in the medium, per milligram of microsomal protein. Similar results were observed by Rogers and Strittmatter (46), who reported that initial binding of either FP_D or cytochrome b_5 interfered with the binding of the second enzyme, indicating that the microsomal binding of cytochrome b_5 and FP_D is a saturable process (47) and probably occurs to the same site, microsomal phospholipid.

As shown earlier (44), detergent-isolated rat cytochrome b_5 incorporated into rat liver microsomes is fully reducible by NADH. As might be expected, full reduction by NADH takes a longer time when more hemoprotein is present, as shown by Rogers and Strittmatter (48). When NADPH is the source of reducing equivalents, full reduction can take in excess of 3 min aerobically with a 4-fold excess of cytochrome b_5 in the microsomes. The rate of electron flow from NADH is also increased, when cytochrome *c* is used as an electron acceptor, in microsomes fortified with cytochrome b_5 (Fig. 1C); the rate of NADH–cytochrome *c* reductase activity increases in proportion to the amount of cytochrome b_5 bound to the microsomes. Since cytochrome b_5 mediates electron transfer between FP_D and cytochrome *c*, it is clear that FP_D is not saturated with cytochrome b_5 at endogenous levels of this hemoprotein. Increased microsomal levels of cytochrome b_5 did not affect NADPH–cytochrome *c* reductase activity (Fig. 1C), because FP_T donates electrons to cytochrome *c* directly, without mediation of cytochrome b_5.

Desaturase. Both NADPH and NADH are capable of driving the stearyl-CoA desaturase reaction (Fig. 2A and B). Although NADH and NADPH are in general equally effective in microsomes of untreated rats (Fig. 2A and Table 1), the effectiveness can vary after different treatments *in vivo*. For example, after fasting, NADPH was the more effective donor, and after the high-carbohydrate diet NADH was more effective (Table 1). In one of our earlier experiments (Fig. 2B) NADPH permitted greater activity in microsomes of desaturase-induced rats than NADH. In no experiment did the addition of both reduced pyridine nucleotides support activity greater than the more active of the two.

Desaturase activity has been shown in reconstituted systems to require FP_D, a cyanide-sensitive factor, and cytochrome b_5 (15); when excess FP_D and CSF were used, desaturase activity increased in proportion to the amount of added detergent-

[5] R. Sato, personal communication.

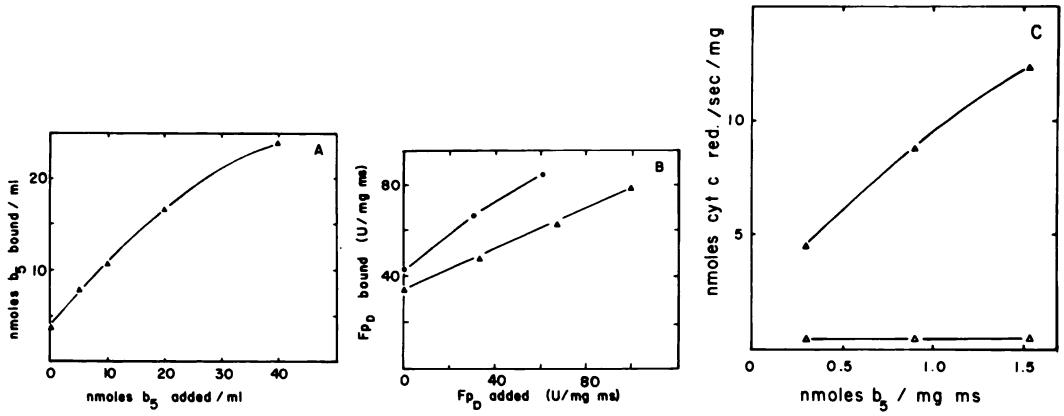

FIG. 1. *Fortification of microsomes with electron transfer components.*

A. Binding of Emulgen-solubilized cytochrome b_5 to control rat liver microsomes. For experimental details, see METHODS. Microsomal protein concentration during binding was 12 mg/ml.

B. Binding of NADH-cytochrome b_5 reductase to microsomes (ms) from control rats. ●, untreated microsomes; ▲, microsomes fortified 4-fold with detergent-solubilized cytochrome b_5. 1 U = 1 μmole of ferricyanide reduced per second by NADH at 22°.

C. Effect of fortification of microsomes (ms) with cytochrome b_5 on pyridine nucleotide-supported cytochrome *c* reduction. △, 0.5 mM NADPH; ▲, 0.5 mM NADH.

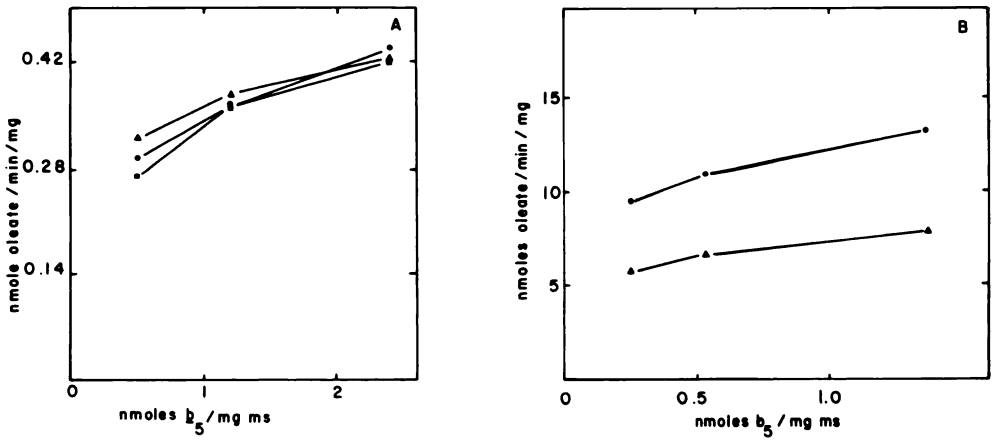

FIG. 2. *Enhancement of stearyl-CoA desaturase activity by fortification of rat liver microsomes with purified cytochrome b_5*

A. Microsomes (ms) from control rats. ○, 1 mM NADPH; □, 1 mM NADH; △, both pyridine nucleotides. B. Microsomes (ms) from rats induced by the high-carbohydrate diet. ●, 1 mM NADPH; ▲, 1 mM NADH.

isolated cytochrome b_5. However, in liver microsomes, evidence suggested that the CSF, rather than cytochrome b_5, was limiting in this pathway (49), based upon cytochrome b_5 reoxidation in the presence of phenolic compounds. Fortification of rat liver microsomes with detergent-isolated cytochrome b_5 increased both NADH- and NADPH-supported desaturase activity in control (Fig. 2A) and desaturase-induced (Fig. 2B) microsomes. In the latter preparations, desaturase activity was increased up to 20-fold by the dietary regimen. Interestingly, in these desaturase-induced rat liver microsomes, the cytochrome b_5 content per milligram of protein was 40–50% lower (Table 1). However, fortification with cytochrome b_5 still caused only a small increase in the desaturase activity (Fig. 2B), indicating that CSF is probably the main limiting component.

In the reconstituted system of Shima-

kata *et al.* (15), FP_D was the limiting component in stearyl-CoA desaturation when excess CSF and cytochrome b_5 were employed; in this case activity increased in proportion to added FP_D. However, fortification of liver microsomes with FP_D, increasing its content from 40 to 80 units/mg, was essentially without effect on stearyl-CoA desaturase activity.

Stearyl-CoA desaturase is known to be under dietary control. Oshino and Sato (43) found overnight fasting to diminish both NADH- and NADPH-supported desaturase activity by 50%. Fasting of rats for 48 hr (Table 1) caused a 60–75% drop in both NADH- and NADPH-supported desaturase activity without appreciable alteration of the cytochrome b_5 level in the microsomes. Measurements of NADH-ferricyanide reductase activity revealed only a minor change in the microsomal content of FP_D, while NADH-cytochrome *c* reductase activity was 65% lower. Fasting for 48 hr did not lower the FP_T level, as it did the NADPH-supported desaturase activity (Table 1).

Although phenobarbital treatment of animals is a potent method for elevation of the microsomal mixed-function oxidase (50, 51), at levels which increase aminopyrine demethylase, it caused a 50% decrease in both NADH- and NADPH-supported stearyl-CoA desaturase activity (Table 1).

Mixed-function oxidase. NADH poorly supports microsomal drug metabolism. However, its addition to NADPH-containing reaction media provides a stimulation of metabolism much in excess of the sum of activities obtained with either pyridine nucleotide alone. This superadditive effect is seen with aminopyrine and ethylmorphine as substrates, but usually not with aniline (Fig. 3A). Ascorbate did not support the reaction to a greater extent than NADH, and rather than providing synergism, it inhibited the NADPH-supported reaction (our observations and ref. 6).

The observed NADH stimulation of

TABLE 1

Actions of effectors of microsomal electron transfer pathways in vivo

Values are means of three separate experiments ± standard errors. All parameters were measured in each microsomal fraction.

Effector	Cytochrome P-450		Cytochrome b_5		Aminopyrine demethylation	
	nmoles/mg	%	*mole/mg*	%	*nmoles/mg*	%
Control	1.10 ± 0.04	100	0.60 ± 0.02	100	7.1 ± 0.17	100
Fasting	1.10 ± 0.03	100	0.51 ± 0.01	85	5.7 ± 0.53	80
Phenobarbital	1.75 ± 0.04	159	0.61 ± 0.02	102	12.0 ± 2.6	169
High-carbohydrate	0.59 ± 0.01	54	0.33 ± 0.003	55	4.3 ± 0.02	61

Effector	Ferricyanide reduction				Cytochrome *c* reduction			
	NADPH		NADH		NADPH		NADH	
	nmoles/sec/mg	%	*nmoles/sec/mg*	%	*nmoles/sec/mg*	%	*nmoles/sec/mg*	%
Control	1.65 ± 0.08	100	44.0 ± 2.0	100	0.93 ± 0.03	100	7.77 ± 0.54	100
Fasting	1.76 ± 0.19	107	38.1 ± 3.3	87	0.82 ± 0.08	88	2.88 ± 0.16	37
Phenobarbital	2.25 ± 0.18	136	45.3 ± 2.4	103	1.28 ± 0.06	138	4.25 ± 0.03	55
High-carbohydrate	1.46 ± 0.15	88	30.3 ± 4.4	69	0.72 ± 0.10	77	4.55 ± 0.30	58

Effector	Lipid peroxidation				Stearyl-CoA desaturation			
	NADPH		NADH		NADPH		NADH	
	nmoles/min/mg	%	*nmoles/min/mg*	%	*nmoles/min/mg*	%	*nmoles/min/mg*	%
Control	9.50 ± 0.80	100	2.16 ± 0.18	100	0.51 ± 0.06	100	0.54 ± 0.06	100
Fasting	13.60 ± 0.25	143	2.82 ± 0.09	131	0.23 ± 0.01	45	0.12 ± 0.02	22
Phenobarbital	8.83 ± 0.63	93	2.30 ± 0.28	106	0.29 ± 0.02	57	0.28 ± 0.02	52
High-carbohydrate	0	0	0.28 ± 0.06	13	5.86 ± 0.76	1147	8.40 ± 0.51	1558

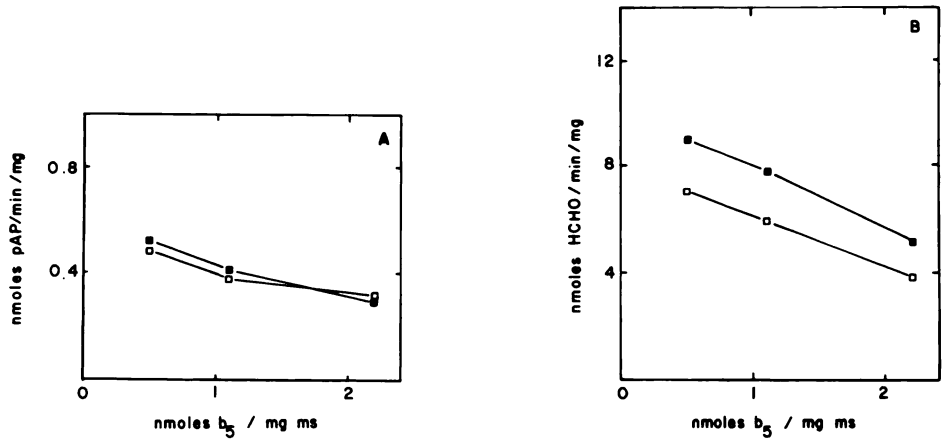

FIG. 3. *Effect of fortification of rat liver microsomes (ms) with cytochrome b_5 on mixed-function oxidations*
A. Aniline hydroxylation. B. Ethylmorphine demethylation. □, 0.35 mM (generated) NADPH-supported reaction; ■, 0.35 mM (generated) NADPH- plus 1 mM NADH-supported reaction. PAP = p-aminophenol.

NADPH-supported drug metabolism (26, 27) has been ascribed to input of a rate-limiting second electron to the reaction by a cooperative mechanism (52) via cytochrome b_5 (53, 54). However, fortification of the microsomes with functional (see Fig. 2A) cytochrome b_5 caused inhibition of the mixed-function oxidase activity (Fig. 3). The extent and pattern of inhibition of the mixed-function oxidase varied with the substrate employed. The presence of NADH did not alter the pattern of inhibition of the NADPH-supported reaction by cytochrome b_5 (Fig. 3), although it caused an increase in the dealkylation reactions (Fig. 3B).

Fortification of liver microsomes with detergent-isolated FP_D enhanced the electron flow rate from NADH (Fig. 1B), but was without effect on NADPH-cytochrome *c* reductase activity. An increase in the FP_D level in the microsomes repeatedly caused a minor increase in NADPH-supported demethylation of ethylmorphine, but no effect was obtained when both NADPH and NADH were present. Increasing the microsomal cytochrome b_5 level 4-fold considerably lowered the rate of ethylmorphine demethylase activity, and this inhibition was not overcome by doubling the microsomal content of FP_D.

Treatment of animals with the high-carbohydrate, desaturase-inducing regimen causes a diminution in the mixed-function oxidase activity per milligram of protein in proportion to a decrease in the cytochrome P-450 level (45% lower, Table 1).

As observed by Kato (55), fasting causes small but variable effects on microsomal drug metabolism. Aminopyrine demethylation, NADPH–cytochrome *c* reductase activity, and cytochrome P-450 levels were virtually unchanged (Table 1).

Treatment of rats with phenobarbital caused elevation of mixed-function oxidase activity and the cytochrome P-450 level to the usual values (56). However, control values were much above those usually observed (see ref. 44). In agreement with other investigators, phenobarbital also caused an increase in the microsomal content of FP_T. Of interest was the almost 50% drop in NADH-cytochrome *c* reductase activity (but not NADH-ferricyanide reductase activity) after phenobarbital treatment (Table 1). Increasing the cytochrome b_5 level in microsomes of phenobarbital-treated rats caused inhibition of mixed-function oxidase activity similar to that shown in Fig. 3, as was reported for aminopyrine in our preliminary communication (44); although the ratio of cytochrome b_5 to cytochrome P-450 was lower in the phenobarbital-treated rats, increasing the ratio to that of control rats, and higher, caused only inhibition of mixed-function oxida-

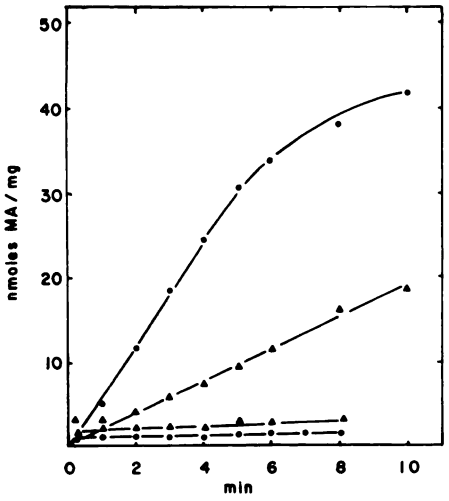

FIG. 4. *Factors affecting time course of lipid peroxidation in rat liver microsomes*

O, 1 mM NADPH; Δ, 1 mM NADH; ▲, 1 mM NADPH in the presence of 8 mM aminopyrine; ●, 1 mM NADPH with desaturase-induced microsomes. Lipid peroxidase medium was used (see METHODS). MA = malondialdehyde.

tions, indicating that cytochrome b_5 is not a required component of NADPH-supported mixed-function oxidations.[6]

Lipid peroxidase. Microsomal lipid peroxidation was shown to be an NADPH-requiring reaction (17, 28) and was found not to be supported by NADH (28). However, recent studies have demonstrated that NADH is capable of supporting lipid peroxidation (22, 23, 58). Similar observations have been made in our laboratory (Fig. 4); activities up to 25% of those obtained with NADPH were observed. In the absence of iron chelate (50 μM iron pyrophosphate) no activity (NADPH- or NADH-supported) was observed when Tris buffer was used (Fig. 4). In phosphate buffer added iron chelate is unnecessary (28); the iron present as a contaminant in the phosphate salts can apparently support the reaction (22).

Recently a close relationship between lipid peroxidation and degradation of microsomal cytochrome P-450 has been reported (21, 59, 60); lipid peroxidation was suggested as the cause of the often observed decrease with time in the drug metabolism rate *in vitro* for some substrates. However, since our assay medium for drug metabolism contains aminopyrine, and no malondialdehyde is formed in the presence of this drug (Fig. 4) while the drug metabolism rate still tails off within 10 min, lipid peroxidation cannot be the cause. Furthermore, lipid peroxidase activity is absent[7] from microsomes of rats exposed to the high-carbohydrate regimen (Fig. 4 and Table 1), but the drug metabolism rate still declines with assay time in these preparations.

Unlike its effect on the mixed-function oxidase, fortification of liver microsomes with cytochrome b_5 was without effect on either NADH- or NADPH-dependent lipid peroxidation, although the hemoprotein was suggested (58) to be a component of the lipid peroxidase system.

As indicated above, treating rats with the desaturase-inducing regimen caused complete cessation of microsomal lipid peroxidase activity. Neither NADH- nor NADPH-supported malondialydehyde production could be demonstrated during a 10-min incubation period.[7] However, NADPH-cytochrome *c* reductase activity and NADH-ferricyanide reductase activity were only 25% and 40% lower, respectively, in microsomes of these animals (Table 1).

Fasting for 48 hr caused a 45% increase in NADPH-supported lipid peroxidase and

[6] *Note in support of our observations.* Lu *et al.* (57) have found that in a reconstituted hepatic mixed-function oxidase system, consisting of cytochrome P-450, FP_T, and phospholipid, the addition of cytochrome b_5 to levels (relative to cytochrome P-450) seen in microsomes inhibited benzphetamine *N*-dealkylation. This inhibition was attributed to competition between cytochrome b_5 and cytochrome P-450 for FP_T; inhibition could be relieved by the addition of FP_D plus NADH.

[7] Subsequent findings, which will be part of another report, indicate that lipid peroxidase may be present in an inactive form; prior incubation of microsomes aerobically for about 1 hr causes the appearance of about 40% of the total activity of control animal microsomes. Incubation of desaturase-induced microsomes with NADPH for longer than 10 min results in the appearance of an increased rate of lipid peroxidation, but again only to about 40% of the total extent of lipid peroxidation.

a 30% increase in NADH-supported lipid peroxidase activities, despite the fact that NADH-cytochrome *c* reductase activity was strongly decreased. However, it should be noted (Table 1) that NADH-ferricyanide reductase activity, which measures the flavoprotein directly, was, if anything, only slightly decreased, as was cytochrome b_5 content.

Phenobarbital induction was without effect on NADPH- and NADH-dependent lipid peroxidase activity, although this treatment elevated the NADPH-cytochrome *c* reductase activity 40% and decreased the NADH-cytochrome *c* reductase activity by a similar amount.

DISCUSSION

Cytochrome b_5 has been well established as a component of the desaturase system by use both of partially reconstituted systems (12, 13) and of antibodies to the hemoprotein with liver microsomes (61). The latter study showed clearly that both NADH- and NADPH-supported desaturase activities utilized cytochrome b_5 and has been confirmed in this laboratory. This would indicate some degree of rate limitation by cytochrome b_5. Oshino and Sato (49) suggested that the cyanide-sensitive factor is the rate-limiting step in the desaturase. Indeed, their laboratory reported (12) that proteolytic removal of up to 90% of the microsomal cytochrome b_5 was necessary before inhibition of the desaturase could be demonstrated. Our observation that increasing the microsomal cytochrome b_5 causes an increase in desaturase activity (Fig. 2A) is not in agreement with their conclusions. Since 4-fold fortification of the microsomes with cytochrome b_5 caused only a 50% increase in desaturase activity, it is probable that the cytochrome b_5 concentration was close to saturation but not saturating in the control microsomes. Similarly, the above cytochrome b_5 antibody studies do not agree with the concept of Oshino and Sato (12, 49) that CSF is rate-limiting. A possible explanation for their results with proteolytic digestion may lie in the later observation in Sato's laboratory (15) that this treatment alters FP_D so that it becomes capable of transferring electrons directly to the CSF. This would then obviate the requirement for cytochrome b_5. An alternative suggestion has been made by Oshino (62) that not all cytochromes b_5 interact with the desaturase, but only about 10%, and these somehow are not as readily removed by proteolytic digestion.

Suggestions have been made that cytochrome b_5 mediates electron flow from NADH to cytochrome P-450 (53, 63–65); studies (66) have suggested that part of the cytochrome b_5 may reside near FP_T, and that after elevation of the latter enzyme in microsomes by phenobarbital treatment the cytochrome may not be able to interact with FP_D. This would explain why after phenobarbital treatment NADH-cytochrome *c* reductase activity, which requires mediation of cytochrome b_5, was decreased while NADH-ferricyanide reductase activity, which does not utilize the hemoprotein, was unaltered. It would also provide an explanation for the observed inhibition of mixed-function oxidase activity in microsomes fortified with cytochrome b_5; interaction of cytochrome b_5 with FP_T would decrease the ability of the latter to interact with cytochrome P-450.[6]

The three hepatic microsomal electron transfer pathways under consideration have been shown here to change in response to stimuli *in vivo* as well as to perturbations *in vitro*. When expressed per milligram of microsomal protein, different conditions *in vivo* affected these microsomal systems differently. Fasting followed by high-carbohydrate, low-fat intake in rats produced a 10–20-fold elevation of the fatty acid desaturase system while causing complete disappearance of the microsomal lipid peroxidase activity and an approximately 40% diminution in the microsomal mixed-function oxidase levels. Forty-eight hours of fasting without refeeding caused severe diminution of the desaturase system (60–70%) with a slight decrease, if anything, in the mixed-function oxidase content of the microsomes, but increased the lipid peroxidase level by 45% over controls. Phenobarbital treatment, which elevates the mixed-function oxidase activity (50, 51) (Table 1), reduced the microsomal desatu-

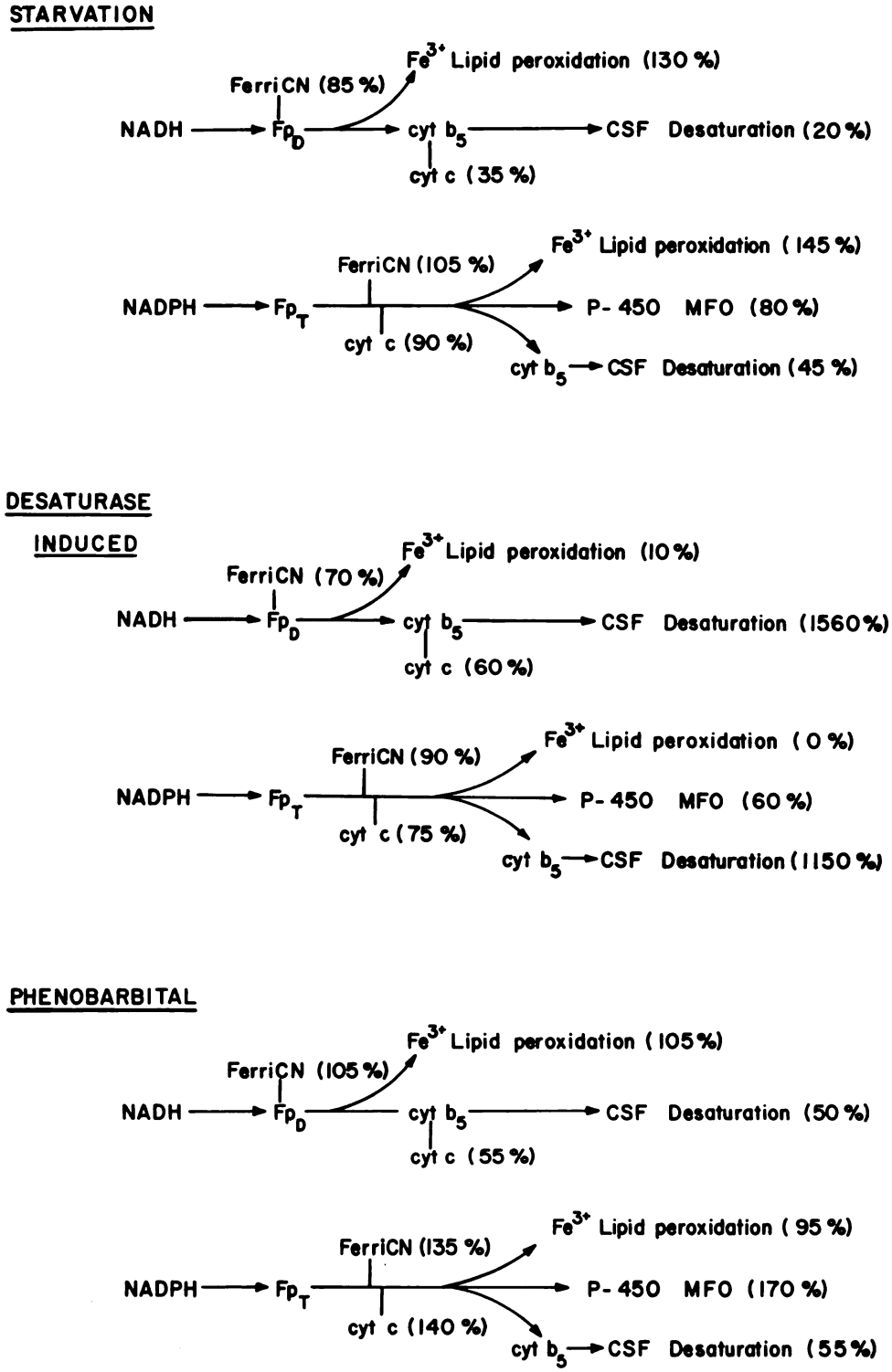

FIG. 5. *Diagram of actions of effectors of microsomal electron transfer processes in vivo*
MFO, mixed-function oxidation.

rase level 50% and did not affect the microsomal lipid peroxidase activity. These results are summarized in Fig. 5. One can see that when activity of one pathway undergoes a large increase, the activities of one or both of the other pathways generally decrease.

These effects are not due to regulation at the electron transfer level, i.e., at FP_D, FP_T, and/or cytochrome b_5, as shown by the data in Table 1 and Fig. 5. When the desaturase, which has been shown to utilize cytochrome b_5, is at half its normal activity, e.g., after fasting or phenobarbital administration, the cytochrome b_5 level is only slightly diminished or unchanged. Conversely, when the desaturase activity is elevated 15-fold, the cytochrome b_5 level is half of normal. Nor do the reductases show any discernible trend suggestive of control of the three enzyme pathways. FP_T has been shown to function in NADPH-supported mixed-function oxidations (67), and was suggested to be active in lipid peroxidase (17) and fatty acid desaturase (14) reactions. However, changes in FP_T levels (measured by cytochrome *c* reduction) are not reflected in similar changes in activities of the NADPH-supported pathways; while such changes are in the same direction as the mixed-function oxidase, they generally do not occur to the same extent and are not always in the direction of the desaturase or peroxidase. FP_D activity (measured by ferricyanide reduction) also shows no pattern of similarity to NADH-supported lipid peroxidation or desaturase activity. (Since NADH-supported mixed-function oxidase activity is so low, it was not measured in most of these studies.) Most startling of the findings concerning the reductase is that, like the cytochrome b_5 level, the FP_D content of the microsomes is decreased after the high-carbohydrate regimen, when desaturase activity is elevated 15-fold.

All these findings point to a lack of complete control over the different pathways by the electron input enzymes and cytochrome b_5. Since the electron transfer capacity of the two reductases is some 6–60 times higher than the respective rates of the three enzyme pathways under consideration, components further along the different pathways probably control the rate or ability of the pathways to function; for example, it was shown that for the mixed-function oxidation of aminopyrine, acceptance of electrons by cytochrome P-450 is the rate-controlling step (39). Limiting components would, then, by virtue of preferential electron flow direction, be expected to affect the activities of the other pathways.

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