Studies on the Rate of Reduction of Hepatic Microsomal Cytochrome P-450 by Reduced Nicotinamide Adenine Dinucleotide Phosphate: Effect of Drug Substrates

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SUMMARY

Optimum conditions are described for the assay of cytochrome P-450 reduction by NADPH in rat liver microsomes. The addition *in vitro* of chemical compounds which produce type I spectral changes markedly accelerated the initial rate of NADPH-linked cytochrome P-450 reduction, while the addition of compounds which elicit type II spectral changes significantly decelerated the rate. These changes occurred in the absence of appreciable changes in the absorbance of the cytochrome P-450–CO complex, suggesting that they are not due simply to differences in the extinction coefficients of the complexes. The acceleration produced by ethylmorphine, a type I compound, was much greater in hepatic microsomes from male rats than in microsomes from female rats; this differential enhancement may play a role in determining sex differences in drug metabolism. None of the type I compounds significantly influenced microsomal cytochrome *c* reductase activity at concentrations that produced maximal enhancement of cytochrome P-450 reduction. NADPH oxidation, measured in the absence of exogenous electron acceptors, was significantly increased by type I substances. Addition of type II compounds to microsomal suspensions either did not affect or decreased cytochrome *c* reductase and NADPH oxidase activities.

The data suggest that the complexes formed between cytochrome P-450 and type I compounds are more readily reduced by NADPH than is the endogenous hemoprotein. Possible mechanisms by which type II compounds inhibit the reduction of cytochrome P-450 are discussed.

INTRODUCTION

There is considerable evidence implicating cytochrome P-450 in the hydroxylation of drugs and steroids by hepatic microsomes and in the hydroxylation of steroids by adrenal cortical mitochondria (1–6). For example, carbon monoxide (CO), which combines with the reduced form of cytochrome P-450, inhibits the hydroxylation of a variety of drugs and steroids by hepatic or adrenal enzymes, and these inhibitory effects can be maximally reversed by light at a wavelength of 450 nm (7, 8).

Changes in hydroxylase activity in hepatic microsomes have frequently been correlated with changes in cytochrome P-450 content (9–12), but there are many instances in which the activity is not directly proportional to cytochrome P-450 content (13, 14). For example, Holtzman *et al.* (15) have shown a 5-fold difference in the rate of ethylmorphine demethylation by smooth- and rough-surfaced microsomes from rabbit liver, but only a 2-fold difference in the cytochrome P-450 content.

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Since there was a 5-fold difference in the rate of cytochrome P-450 reduction, however, these authors pointed out that the rate of ethylmorphine demethylation closely paralleled the rate of cytochrome P-450 reduction. In addition, Davies *et al.* (16) found that species variations in ethylmorphine demethylation were closely paralleled by differences in the rate of cytochrome P-450 reduction and not by differences in cytochrome P-450 content, NADPH-cytochrome *c* reductase activity, or the magnitude of the type I spectral change. These findings thus raised the possibility that the rate-limiting step of ethylmorphine demethylation might be the rate of reduction of cytochrome P-450.

A number of investigators (17, 18) have shown that various compounds cause two types of changes in the visible absorption spectrum of hepatic microsomal suspensions even in the absence of NADPH. The "type I" difference spectrum is characterized by a trough at about 420 nm and a peak at about 385 nm, whereas the "type II" difference spectrum is characterized by a peak at about 430 nm and a trough at about 394 nm. Since these spectral changes presumably represent complexes of the compounds with cytochrome P-450, the rate-limiting step of reaction might be the reduction of the substrate–cytochrome P-450 complex and not necessarily cytochrome P-450 per se. It seemed possible, however, that the complexes might be reduced at different rates.

The present paper describes the kinetics of cytochrome P-450 reduction in the presence and absence of various substrates that cause type I and type II spectral changes. Preliminary reports of this work have appeared (19, 20).

METHODS

Animals. Rats of the Sprague-Dawley strain (NIH colony) were used; males weighed 180–200 g, and females, 160–170 g. The animals were allowed free access to Purina laboratory chow and tap water at all times.

Preparation of microsomes. The animals were killed by decapitation. Livers were removed, chilled in ice, and homogenized with 4 volumes of ice-cold 1.15% KCl containing 0.02 M Tris-HCl (pH 7.4) in a Potter-Elvehjem homogenizer having a motor-driven plastic pestle. The homogenate was centrifuged at $9000 \times g$ for 20 min in a Sorvall refrigerated centrifuge. The supernatant fraction was decanted and centrifuged at $105{,}000 \times g$ for 60 min in a Spinco model L ultracentrifuge. The microsomal pellet was resuspended in the ice-cold 1.15% KCl–0.02 M Tris-HCl medium by manual homogenization. All tissue manipulations were carried out at 0–4°.

Enzyme assays. The reduction of cytochrome P-450 by NADPH was assayed by a method developed in this laboratory by Davies, Gigon, and Gillette, as described by Gigon *et al.* (19). Three milliliters of a microsomal suspension (5 mg of protein per milliliter) were pipetted into an Aminco anaerobic spectrophotometric cell (A1-65085) and bubbled for 5 min with CO that had been passed through a deoxygenating solution (0.5% sodium dithionite plus 0.05% sodium anthraquinone-2-sulfonate in 0.1 N NaOH). The stopcock plunger assembly, containing 50 μl of an NADPH-generating system,[3] was then attached to the cuvette, and CO was flushed through the cuvette (i.e., the dead space above the surface of the microsomal suspension) for 3 min. The cuvette was then sealed and placed in a Gilford 2000 recording spectrophotometer at 37° for 5 min. The absorbance at 450 nm was offset so that the chart read zero. The recorder was started (chart speed of 12 inches/min), the plunger was quickly depressed, and the change in absorbance was mea-

[3] The generating system consisted of 3 mmoles of Tris-HCl buffer (pH 7.4), 10 μmoles of $MgCl_2$, 24 μmoles of isocitrate trisodium salt (Calbiochem, A grade), 1.2 μmoles of NADP, and 0.54 unit (1 unit converts 1 μmole of isocitrate to α-ketoglutarate per minute at 30°) of isocitric dehydrogenase (from porcine heart; Calbiochem, A grade) in a total volume of 50 μl. Unless otherwise specified, all routine assays of cytochrome P-450 reduction employed this NADPH-generating system.

sured until it became asymptotic at 1–2 min. The rate of cytochrome P-450 reduction by NADPH was calculated from the initial rate of increase in absorbance, and the amount of cytochrome P-450 reducible by NADPH was calculated from the final absorbance. The plunger was then removed, 2–3 mg of solid sodium dithionite were added, and the absorbance was measured. This value was used to estimate the amount of the dithionite-reducible cytochrome P-450.

In this method, it has been assumed that the rate of cytochrome P-450 reduction is considerably slower than the rate at which CO combines with reduced cytochrome P-450. The validity of this assumption is based on studies by Omura *et al.* (21) in collaboration with Gibson, who showed that after the CO–cytochrome P-450 complex is dissociated by light, the second-order rate constant for the recombination of CO and reduced cytochrome P-450 is about 3.4×10^5 M^{-1} sec^{-1}. By substituting this value for the second-order constant and the concentrations of CO (about 0.84 mM) used in our experiments into the second-order rate equation, we have calculated that the pseudo first-order rate constant for CO–cytochrome P-450 complex formation should be about 1.7×10^4 min^{-1}, which is about three orders of magnitude greater than the rate constants (approximately 10–20 min^{-1}) obtained for the reduction of cytochrome P-450 by NADPH.

The NADPH-cytochrome *c* reductase activity was determined essentially by the method of Williams and Kamin (22), as follows. To a 1-cm^2 spectrophotometric cuvette were added 1 ml of microsomal suspension (0.5 mg of protein per milliliter), 1 ml of 3 mM KCN, and 1 ml of 0.15 mM oxidized horse heart cytochrome *c* (Sigma, type III). The cuvette was warmed to 37° for 5 min in a Gilford 2000 recording spectrophotometer. The absorbance at 550 nm was then offset to zero on the chart, and 50 μl of the NADPH-generating system[3] were added. The rate of reduction was determined from the initial phase of the reaction; an extinction coefficient of 18.5 mM^{-1} cm^{-1} was used to estimate reduced cytochrome *c* (23).

NADPH oxidase activity was determined by a modification of the method described by Gillette, Brodie, and La Du (24). The reaction was carried out at 25° in 0.1 M Tris-HCl buffer, pH 7.4; the microsomal protein concentration was 2 mg/ml, and nicotinamide was omitted. Following the addition of 0.17 mM NADPH, the change in absorbance at 340 nm was monitored at 30-sec intervals for 3 min, during which time the reaction was linear.

Microsomal *N*-demethylation of ethylmorphine was assayed under conditions described by Davies, Gigon, and Gillette (25), in which liberated formaldehyde was assayed by a modification of the method of Nash (26). The kinetic constants determined (K_m, V_{max}) were obtained by a least squares method as described by Davies *et al.* (25). Microsomal protein was estimated by the method of Lowry *et al.* (27), with crystalline bovine serum albumin as a standard.

Data were analyzed for significance by Student's *t*-test (28); *p* values less than 0.05 were considered to represent significant differences between mean values.

RESULTS

Kinetics of cytochrome P-450 reduction by NADPH. In the range of 2.5–7.5 mg of microsomal protein per milliliter, the initial rate of cytochrome P-450 reduction was proportional to protein concentration; at the 7.5 mg/ml concentration it was found necessary to increase the amount of NADPH-generating system to maintain linearity. Accordingly, all subsequent assays were carried out at a microsomal protein concentration of 5 mg/ml. The adequacy of the NADPH-generating system[3] was then investigated; 50 μl of the system, which gave a final NADP concentration of 0.4 mM, were found to give maximum activity. Doubling the amount of NADPH-generating system had no influence either on the initial rate of reduction or on total cytochrome P-450

reducible by NADPH. Experiments with NADPH itself, at concentrations 10 (4 mM) and 25 (10 mM) times that of the NADP used in the generating system (0.4 mM), gave essentially the same results. Thus, the source of NADPH did not significantly affect the reduction of cytochrome P-450; moreover, stimulation of reduction by type I substrates (see below) was found to be of the same magnitude irrespective of the source of NADPH. Similarly, addition of Mg^{++} (1–7 mM) did not affect the reduction of cytochrome P-450 by NADPH (4 mM).

With the NADPH-generating system,[3] the assay of cytochrome P-450 reduction was found to be dependent on the presence of NADPH, CO, and intact microsomes. Substitution of NADH (0.4 mM) for NADPH reduced the reduction rate by about 90%. No peak formation at 450 nm was observed when microsomal suspensions were bubbled with N_2 instead of CO or when boiled microsomes (100°, 5 min) were used as the enzyme source.

Logarithmic plots of the remaining unreduced cytochrome P-450 against time showed that the rate of reduction of the cytochrome P-450 was biphasic (Fig. 1). It thus seemed possible that these findings might reflect differences in the rates of reduction of cytochrome P-446 and cytochrome P-450. Indeed, calculations based on the extinction coefficients of these cytochromes obtained by Hildebrandt *et al.* (29) with rabbit liver microsomes revealed that about 58% of the total absorbance at 450 nm may be due to cytochrome P-446.

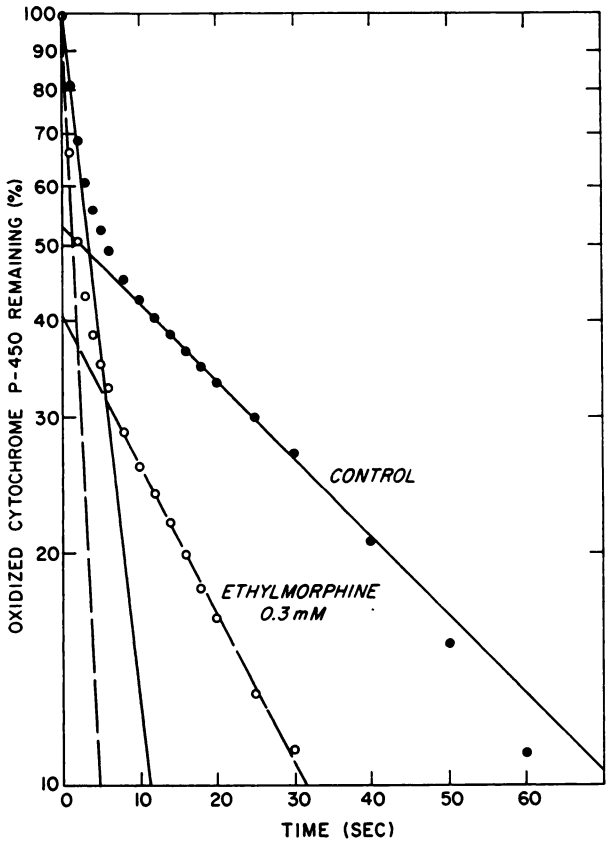

FIG. 1. *Semilogarithmic plot of the rate of reduction of hepatic microsomal cytochrome P-450 by NADPH*
Conditions were as described in METHODS. The biphasic nature of the reduction, in the absence and in the presence of ethylmorphine, is evident.

Accordingly, extrapolation of the slope of the second phase crosses the ordinate at about the 50% point. To test this possibility, rats were first treated with 3-methylcholanthrene (50 mg/kg once daily for 2 days) because Hildebrandt *et al.* (29) had found that this compound induces the formation of cytochrome P-446, but not that of cytochrome P-450. Using the extinction coefficients of cytochrome P-446 and cytochrome P-450 determined by Hildebrandt *et al.* (29), we calculated that in microsomal preparations from rats receiving prior treatment with 3-methylcholanthrene, cytochrome P-446 accounted for about 74% of the total changes in absorbance at 450 nm. If the biphasic reaction were due to the relative rates of reduction of cytochrome P-450 and cytochrome P-446, extrapolation of the slope of the second phase should intersect the ordinate axis at 26% or 74% of the total 450 nm-absorbing material, depending on which cytochrome was more rapidly reduced. As shown in Fig. 2, however, prior treatment with 3-methylcholanthrene did not significantly alter the initial rate of reduction and only slightly increased the intercept of the slope of the second phase with that of the ordinate. Since the biphasic reduction rate was apparently not due to differences in rates of reduction of cytochrome P-450 and cytochrome P-446, we have used in our calculations the apparent extinction coefficient of 91 mM^{-1} cm^{-1}, which Omura and Sato (30) deter-

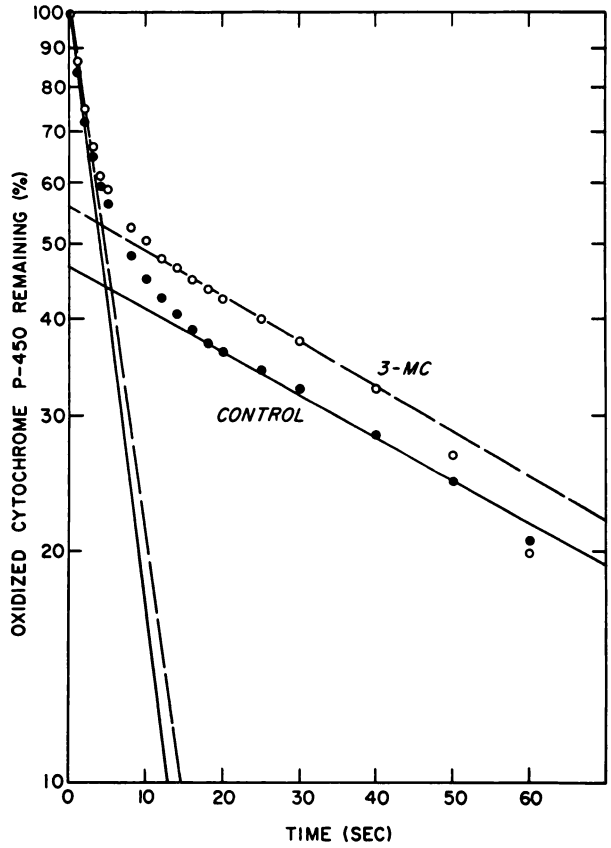

FIG. 2. *Semilogarithmic plot of the rate of reduction of cytochrome P-450 by NADPH in hepatic microsomes prepared from control and 3-methylcholanthrene-treated rats*

Conditions were as described in METHODS and RESULTS. Prior treatment with 3-methylcholanthrene (3-MC) did not influence the initial rate of reduction.

mined for the total cytochrome P-450-like material in rabbit liver microsomes.

It then seemed possible that the biphasic reaction might be due to the reoxidation of cytochrome P-450 by residual oxygen, which might have remained in the system even though the carbon monoxide had been passed through a deoxygenizer and then bubbled through the microsomal suspension for 5 min. To test this possibility, electron transport particles prepared from beef heart mitochondria[4] were added to the microsomal suspension in the anaerobic cuvette, and nitrogen which had passed through the deoxygenizer was bubbled through the mixture for 5 min. Succinate was added to the mixture, the stopcock plunger assembly containing the NADPH-generating system was attached to the cuvette, and the air space was flushed with nitrogen. The cuvette was sealed, placed in the Gilford 2000 spectrophotometer, and allowed to remain until the cytochrome oxidase in the electron transport particles was fully reduced, as determined by the increase in absorbance at 445 nm. The cuvette was removed, unsealed by turning the stopcock, and carefully gassed with carbon monoxide for 3 min through the stopcock port; diffusion of CO into the suspension was facilitated by the use of a small magnetic stirring bar. The sys-

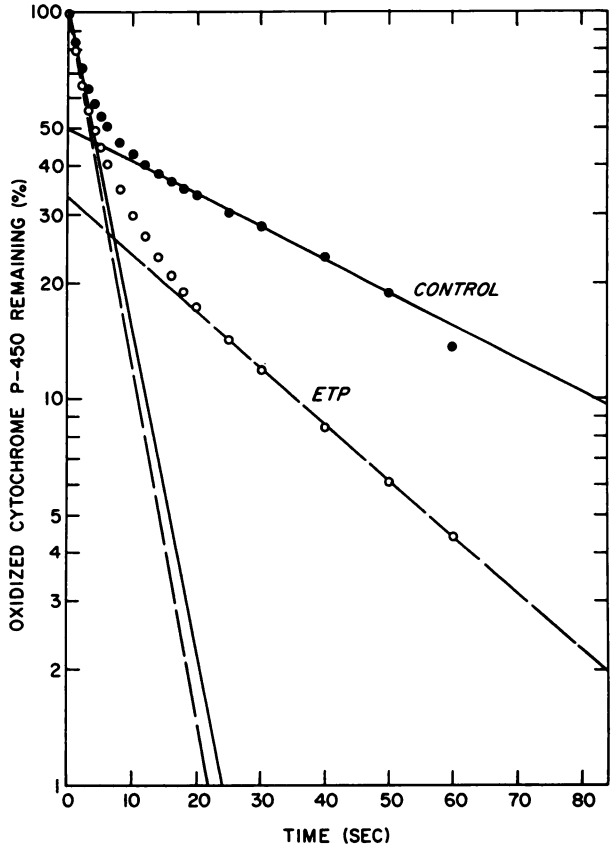

FIG. 3. *Semilogarithmic plot of the rate of reduction of hepatic microsomal cytochrome P-450 by NADPH in the absence and presence of electron transport particles (ETP) prepared from beef heart mitochondria*

Conditions were as described in METHODS and RESULTS. The presence of electron transport particle markedly decreased the second phase but had no effect on the initial rate of reduction.

[4] For this preparation we express our gratitude to Dr. Ronald W. Estabrook, Department of Biochemistry, Southwestern Medical School, Dallas, Texas.

tem was then resealed, and the rate of cytochrome P-450 reduction was determined as described in METHODS. As shown in Fig. 3, this procedure markedly decreased both the half-life of the second phase and the intercept on the ordinate. Even though these findings indicated that the treatment with succinate and electron transport particles markedly decreased the second phase of reduction, the treatment did not significantly alter the initial rate of reduction. For this reason, we concluded that the initial rate of CO–cytochrome P-450 complex formation in the absence of succinate and the electron transport particles provides a valid assay for cytochrome P-450 reduction.

Effect of substrates on cytochrome P-450 reduction. As shown in Table 1, ethylmorphine, hexobarbital, and SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl), all of which produce type I spectral changes (17, 18), significantly increased the initial rate of reduction of cytochrome P-450 by NADPH in hepatic microsomes from male rats. Moreover, in single experiments with aminopyrine (0.5 mM) and imipramine (0.5 mM), which also produce type I spectral changes (18), the rate of cytochrome P-450 reduction by NADPH was enhanced 60–80%. Thus, all the type I compounds used in this study enhanced the rate of reduction of cytochrome P-450.

The degree of stimulation varied with the substrate; SKF 525-A produced the greatest enhancement, and hexobarbital the least. The values presented for the three substrates (Table 1) represent maximum stimulation. Nevertheless, it was found that after complete reduction of cytochrome P-450 by NADPH, the addition of sodium dithionite increased the height of the peak at 450 nm about 10%. The microsomal content of NADPH-reducible cytochrome P-450 tended to be increased by all three substrates (ethylmorphine, 8.5%; hexobarbital, 7.0%; SKF 525-A, 10.8%). These increases were statistically significant only for hexobarbital and SKF 525-A. Similarly, the amounts of sodium dithionite-reducible cytochrome P-450 tended to be increased by substrates, but only the increase produced by hexobarbital was statistically significant (Table 1).

Aniline, nicotinamide, and 2,4-dichloro-6-phenylphenoxyethylamine HBr have been reported (18) to produce type II spectral shifts when added to microsomal suspensions in the absence of NADPH. As shown in Table 2, all three compounds significantly decreased the rate of cytochrome P-450 reduction by NADPH. The amount of NADPH-reducible cytochrome P-450

TABLE 1

Stimulatory effect of type I compounds on cytochrome P-450 reduction in hepatic microsomes from male rats

Values represent means ± standard error of four determinations, in which each determination was replicated two to four times. Each determination was made with a microsomal preparation obtained from the pooled livers of two to four animals.

Compound tested	Initial rate of cytochrome P-450 reduction by NADPH	NADPH-reducible cytochrome P-450	Dithionite-reducible cytochrome P-450
	nmoles reduced/min/mg protein	*nmoles/mg protein*	
Control	10.47 ± 0.74	0.84 ± 0.05	0.93 ± 0.05
Ethylmorphine, 0.3 mM	19.09 ± 1.53[a]	0.91 ± 0.05	0.96 ± 0.05
Control	7.31 ± 0.31	0.73 ± 0.01	0.82 ± 0.01
Hexobarbital, 0.3 mM	12.04 ± 0.57[a]	0.79 ± 0.01[a]	0.87 ± 0.02
Control	8.08 ± 0.12	0.78 ± 0.02	0.85 ± 0.03
SKF 525-A, 0.05 mM	16.87 ± 0.52[a]	0.86 ± 0.02[a]	0.91 ± 0.02

[a] $p < 0.05$.

TABLE 2

Inhibitory effect of type II compounds on cytochrome P-450 reduction in hepatic microsomes from male rats

Values represent means ± standard error of four determinations, in which each determination was replicated two to four times. Each determination was made with a microsomal preparation obtained from the pooled livers of two to four animals.

Compound tested	Initial rate of cytochrome P-450 reduction by NADPH	NADPH-reducible cytochrome P-450	Dithionite-reducible cytochrome P-450
	nmoles reduced/min/mg protein	*nmoles/mg protein*	
Control	9.80 ± 0.74	0.85 ± 0.05	0.95 ± 0.06
Aniline, 0.3 mM	8.74 ± 0.75	0.88 ± 0.06	0.97 ± 0.06
Aniline, 1.0 mM	5.98 ± 0.43[a]	0.85 ± 0.06	0.94 ± 0.05
Aniline, 2.0 mM	3.99 ± 0.20[a]	0.85 ± 0.07	0.95 ± 0.07
Control	7.31 ± 0.31	0.73 ± 0.01	0.82 ± 0.01
Nicotinamide, 2 mM	6.00 ± 0.40[a]	0.73 ± 0.01	0.82 ± 0.01
Nicotinamide, 5 mM	5.35 ± 0.40[a]	0.72 ± 0.01	0.82 ± 0.01
Nicotinamide, 10 mM	4.25 ± 0.29[a]	0.69 ± 0.01[a]	0.79 ± 0.01
Control	8.08 ± 0.12	0.78 ± 0.02	0.85 ± 0.03
DPEA[b], 0.005 mM	5.57 ± 0.38[a]	0.74 ± 0.02	0.85 ± 0.02
DPEA, 0.02 mM	4.11 ± 0.22[a]	0.70 ± 0.02	0.83 ± 0.03
DPEA, 0.1 mM	2.95 ± 0.09[a]	0.62 ± 0.02[a]	0.81 ± 0.02

[a] $p < 0.05$.

appeared to decrease as the concentration of nicotinamide and DPEA[5] was increased. The changes, however, were significant only at the highest concentration of both these compounds. Aniline produced no significant change in NADPH-reducible cytochrome P-450 at any of the three concentrations studied. Dithionite-reducible cytochrome P-450 was not significantly influenced by any of the three substrates.

The stimulatory and inhibitory effects of the various compounds on the rate of cytochrome P-450 reduction are presumably not caused by their metabolism. When the microsomal suspension was made anaerobic by the treatment with succinate and electron transport particle, ethylmorphine still increased the initial rate of cytochrome P-450 reduction; the increase in rate elicited by ethylmorphine was of the same magnitude, expressed either as percentage of control or in absolute terms, in the presence or absence of electron transport particle. Moreover, when 3,4-benzpyrene was added to the NADPH-generating system and thus added to the liver microsomes at the same time as NADPH, no hydroxylation products were detected by the sensitive method of Kuntzman *et al.* (31).

In order to rule out gross spectral shifts as an explanation for the effects of type I and type II compounds on cytochrome P-450 reduction rates, difference spectra (350 — 500 nm) were obtained both before and after addition of CO and NADPH. The addition of CO and NADPH to microsomal suspensions produced no qualitative change in the difference spectra obtained in the presence of ethylmorphine or aniline. Similarly, the absorption maximum of the reduced CO–cytochrome P-450 complex was at 450 nm in both the absence and presence of type I and type II compounds.

Sex differences. In accord with previous work from this laboratory (25), the *N*-demethylase activity (V_{max}) was about 3 times as high in microsomes from males as

[b] The abbreviation used is: DPEA, 2,4-dichloro-6-phenylphenoxyethylamine HBr.

TABLE 3

Sex differences in ethylmorphine N-demethylation and in the effects of ethylmorphine on cytochrome P-450 reduction in rat liver microsomes

Values represent means ± standard error of four determinations, in which each determination was replicated two to four times. Each determination was made with a microsomal preparation obtained from the pooled livers of two to four animals.

Sex	Cytochrome P-450 reduction			Ethylmorphine *N*-demethylation (V_{max})[a]
	Control	Ethylmorphine (0.3 mM)	Difference	
	nmoles reduced/min/mg protein			
Male	10.74 ± 0.74	19.09 ± 1.53[b]	8.62	10.84 ± 0.82
Female	8.21 ± 0.04[c]	11.41 ± 0.04[b,c]	3.20	3.33 ± 0.41[c]
Male/female	1.27	1.67	2.70	3.25

[a] Expressed as nanomoles of formaldehyde formed per minute per milligram of protein.
[b] $p < 0.05$ (*t*-test) compared with control.
[c] $p < 0.05$ (*t*-test) compared with male value.

in those from females (Table 3). There was a slight, but significant, sex difference in the basal rate of cytochrome P-450 reduction by NADPH, the activity being about 25% higher in males than in females (Table 3). Moreover, ethylmorphine enhanced the rate of reduction of cytochrome P-450 about 82% in microsomes from male rats, but enhanced the rate only 39% in those from females. The amount of NADPH-reducible cytochrome P-450 was significantly higher in males than in females in both the absence and presence of ethylmorphine, and was only slightly increased in both sexes by ethylmorphine (less than 10%) (Table 4). Similarly, the amount of dithionite-reducible cytochrome P-450 was significantly greater in males than in females, but was not significantly increased by the addition of ethylmorphine (Table 4).

Clearly, the sex difference in the absolute rate of cytochrome P-450 reduction in either the presence or absence of ethylmorphine could not account for the 3-fold difference in ethylmorphine demethylation (Table 3). But the sex difference in the substrate-induced increase in the reduction rate of the cytochrome closely paralleled the sex difference in ethylmorphine metab-

TABLE 4

Effect of ethylmorphine on NADPH-reducible and sodium dithionite-reducible cytochrome P-450 in hepatic microsomes from male and female rats

Values represent means ± standard error of four determinations, in which each determination was replicated two to four times. Each determination was made with a microsomal preparation obtained from the pooled livers of two to four animals.

Sex and compound tested	NADPH-reducible cytochrome P-450	Dithionite-reducible cytochrome P-450
	nmole/mg protein	
Male		
Control	0.84 ± 0.05	0.93 ± 0.05
Ethylmorphine (0.3 mM)	0.91 ± 0.05	0.96 ± 0.05
Female		
Control	0.68 ± 0.01[a]	0.74 ± 0.01[a]
Ethylmorphine (0.3 mM)	0.74 ± 0.02[a,b]	0.74 ± 0.02[a]

[a] $p < 0.05$ compared with male value.
[b] $p < 0.05$ compared with control.

olism. These findings thus suggest the possibility that ethylmorphine combines with the cytochrome P-450 in liver microsomes to form a complex which is more readily reduced by NADPH-cytochrome P-450 reductase than is cytochrome P-450 in the absence of the substrate.

Effect of type I and type II compounds on NADPH-cytochrome c reductase. It has been proposed that cytochrome P-450 is reduced either directly or indirectly through NADPH-cytochrome *c* reductase (32). In an attempt to delineate the step or steps which were altered by type I and type II substrates (Tables 1 and 2), we examined the influence of these compounds on the rate of reduction of cytochrome *c* by NADPH. As shown in Table 5, none of the type I compounds affected this activity at concentrations which caused maximal enhancement of cytochrome P-450 reduction by NADPH. Similarly, the type II compounds nicotinamide and DPEA had no significant effect on NADPH-cytochrome *c* reductase activity, suggesting that tions found to cause marked inhibition of cytochrome P-450 reduction. Aniline, however, caused significant inhibition of cytochrome *c* reductase activity, suggesting that the mechanism by which it inhibits cytochrome P-450 reduction by NADPH (Table 2) may be different from that of nicotinamide and DPEA. Thus, of the six compounds which either enhanced or diminished cytochrome P-450 reduction by NADPH, five were without effect on NADPH-cytochrome *c* reduction. These five compounds must therefore exert their effects on cytochrome P-450 reduction at a step beyond cytochrome *c* reductase.

Effect of type I and type II substances on NADPH oxidation by liver microsomes. Several investigators (33, 34) have noted that the addition of various drugs to liver microsomes enhances the rate of NADPH oxidation and have suggested that this increase was due to the metabolism of the drug. As shown in Table 5, only type I substances significantly stimulated NADPH oxidation. Of the type II substances examined, aniline and nicotinamide did not alter NADPH oxidation and DPEA significantly inhibited it. Similar results were obtained at both 25° and 37°.

TABLE 5

Effect of type I and type II compounds on NADPH-cytochrome c reductase and NADPH oxidase activities in hepatic microsomes from male rats

Values represent means ± standard error obtained from four animals.

Additions	Concentration	Cytochrome *c* reductase	NADPH oxidase
	mM	*nmoles/min/mg protein*	
Control		119.4 ± 6.6	18.0 ± 0.9
Type I			
Ethylmorphine	0.3	117.0 ± 8.4	32.1 ± 1.0[a]
Hexobarbital	0.3	109.2 ± 5.4	29.0 ± 0.9[a]
SKF 525-A	0.05	117.6 ± 7.8	39.6 ± 2.1[a]
Type II			
Aniline	2.0	89.4 ± 7.8[a]	20.5 ± 0.9
Nicotinamide	10.0	114.0 ± 6.0	19.1 ± 0.9
DPEA	0.1	118.2 ± 6.0	13.9 ± 0.3[a]

[a] $p < 0.05$.

DISCUSSION

In investigations of the mechanism of drug metabolism by cytochrome P-450-dependent enzymes in liver microsomes, it has generally been assumed that electron transport proceeds through the following sequence. First, cytochrome P-450 is reduced by NADPH either directly or indirectly by NADPH-cytochrome *c* reduc-

tase. The reduced cytochrome then reacts with oxygen to form an active oxygen complex (O_2–cytochrome P-450), which in turn either oxidizes a drug attached to another enzyme or forms an O_2–cytochrome P-450–substrate complex that decomposes with the oxidation of both the drug and the cytochrome P-450.

Trivus and Spirtes (33) and Ernster and Orrenius (34) reported that substrates such as hexobarbital and aminopyrine enhance the rate of NADPH oxidation. According to the above mechanism, however, a stoichiometric relationship should occur only when the O_2–cytochrome P-450 complex is relatively stable and little if any of the endogenous NADPH oxidation is mediated by cytochrome P-450. This conclusion is readily apparent from the following considerations. Let R_c represent the steady-state rate of NADPH oxidation associated with the formation, rearrangement, and decomposition of the O_2–cytochrome P-450 complex; R'_c, the steady-state rate of NADPH oxidation associated with that of the O_2–cytochrome P-450–substrate complex; and R_o, the steady-state rate of NADPH oxidation by other pathways. Thus the endogenous rate of NADPH oxidation (R_t) in the absence of a substrate would be

$$R_t = R_o + R_c$$

and the rate of NADPH oxidation in the presence of substrate (R'_t) would be

$$R'_t = R_o + R'_c$$

The difference in the rates of NADPH oxidation is therefore

$$R'_t - R_t = R'_c - R_c$$

Since according to the mixed-function oxidase mechanism the rate of metabolism of the drug should equal R'_c, there should be a stoichiometric relationship between the increase in the rate of NADPH oxidation and rate of drug metabolism only when R_c is negligible.

A number of facts, however, make it difficult to accept the view that the rate-limiting step in the oxidation of drugs is the breakdown of O_2–cytochrome P-450–substrate complex and not the rate of reduction of the cytochrome P-450. Carbon monoxide blocks NADPH oxidation about 30–50% (32), indicating that a significant amount of the endogenous NADPH oxidase activity is mediated by cytochrome P-450. Indeed, Ullrich, Cohen, Cooper, and Estabrook (35) have shown that the first-order rate constant for the oxidation of reduced cytochrome P-450 is about 10 sec^{-1}. Even though NADH reduced cytochrome P-450, it is not very effective in replacing NADPH in the drug oxidative systems. In addition, Sasame[6] found that under an atmosphere containing both CO and O_2, the steady-state level of CO–cytochrome P-450 is lower in the presence of NADH than it is in the presence of NADPH. As pointed out earlier, Davies *et al.* (16) found that the maximal rates of ethylmorphine demethylation by liver microsomes from various species are more closely related to the rates of reduction of cytochrome P-450 than they are to the rates of cytochrome *c* reduction, the amount of cytochrome P-450, or the magnitude of the type I spectral change caused by ethylmorphine. Similarly, differences in the rate of cytochrome P-450 reduction account for the differences in the rate of ethylmorphine demethylation between smooth- and rough-surfaced microsomes from rabbit liver (15) and between microsomes from adrenalectomized and cortisol-treated, adrenalectomized rats (36).

The finding that various substances enhanced NADPH oxidation thus prompted the present investigation into the effects of the substances on various components of the NADPH-dependent electron transport system in liver microsomes. The possibility that the effects were due to alterations in the activity of NADPH-cytochrome *c* reductase was rejected because, of the six compounds shown to alter cytochrome P-450 reduction (Tables 1 and 2), five did not alter cytochrome *c* reductase (Table 5). Thus, the effects occur either at a hypothetical carrier or at cyto-

[6] H. Sasame, unpublished observations (see ref. 32).

chrome P-450 itself. The finding that compounds which cause type I spectral changes enhance cytochrome P-450 reduction, whereas those which cause type II spectral changes decelerate cytochrome P-450 reduction, lends support to the view that the alterations in the rate of change in absorbance at 450 nm after addition of NADPH reflect changes in either the structure or the kinetic properties of cytochrome P-450. The possibility that the extinction coefficients of the CO–cytochrome P-450–substrate complexes were sufficiently different from the extinction coefficient of CO–cytochrome P-450 to account for the rate of absorbance change was excluded because the absorbance at the end of the reaction was changed only about 10% in the presence of the various substrates. It is likely, therefore, that alterations in the rate of absorbance change after addition of NADPH represent alterations in the rate of cytochrome P-450 reduction.

It is interesting in this regard that type I substances stimulated the anaerobic reduction of *p*-nitrobenzoate by cytochrome P-450-dependent nitroreductase, whereas type II substances inhibited the reaction (37).

The concept that cytochrome P-450–substrate (type I) complexes are more rapidly reduced than cytochrome P-450 suggested the possibility that the degree of stimulation might be more closely related to the activity of oxidative enzymes than any other parameter thus far measured. Although Remmer *et al.* (12) found a correlation between the magnitude of the spectral changes and the metabolism of aminopyrine and hexobarbital, Davies *et al.* (16) were unable to account for the sex difference in ethylmorphine demethylation by sex differences in the cytochrome P-450 content, cytochrome *c* reductase activity, cytochrome P-450 reductase activity, or the magnitude of the type I spectral change. As shown in Table 3, ethylmorphine stimulated the rate of cytochrome P-450 reduction in liver microsomes from male rats to a greater extent than it did in liver microsomes from female rats. Clearly, there was no relationship between the sex difference in the maximum velocity of the ethylmorphine demethylation and the sex difference in either the total rate or the percentage increase in the rate of cytochrome P-450 reduction. It seemed possible, however, that not all of the cytochrome P-450 in liver microsomes was capable of catalyzing the demethylation of ethylmorphine. As shown in Table 3, the sex difference in the magnitude of the stimulatory effect was closely correlated with the sex difference in the maximal velocity of the ethylmorphine *N*-demethylation. Thus far, however, we have found that the K_m for the stimulatory effects on cytochrome P-450 reduction is about one-fifth to one-third the K_m for ethylmorphine demethylation. It is therefore possible that the kinetics of ethylmorphine demethylation is more complex than hitherto supposed. It could very well be that the apparent dissociation constant for the reduced cytochrome P-450–substrate complex differs from that of the oxidized cytochrome P-450–substrate complex, but these possible interpretations have not yet been evaluated for ethylmorphine.

The decelerating effects of type II substances on cytochrome P-450 reduction may be interpreted in at least two ways: (a) the oxidized cytochrome P-450–substrate complex is reduced more slowly than oxidized cytochrome P-450; (b) only the free cytochrome P-450 is reduced, but the reduced form, once it is formed, combines with the type II compound to form the reduced cytochrome P-450 complex by combining with the heme (18). If the latter interpretation is correct, however, there must be at least some cytochrome P-450 present which does not bind the type II substance, because we have never been able to inhibit cytochrome P-450 reduction completely even at very high concentrations of DPEA. Regardless of the mechanism involved, however, the finding that type II substances slow cytochrome P-450 reduction may account for the general impression that these substances are generally poorer substrates for the microsomal oxidative systems than are the

$$\text{NADPH} + \text{cytochrome } c \text{ reductase} + H^+ \rightleftharpoons \text{NADP}^+ + H_2\text{-cytochrome } c \text{ reductase} \quad (1)$$

$$H_2\text{-cytochrome } c \text{ reductase} + \text{cytochrome P-450 } (Fe^{3+}) \xrightarrow{\text{carrier?}} \text{cytochrome } c \text{ reductase} + \text{cytochrome P-450 } (Fe^{2+}) + 2H^+ + e^- \quad (2)$$

$$\text{Cytochrome P-450 } (Fe^{2+}) + O_2 \rightleftharpoons \text{cytochrome P-450 } (FeO_2) \quad (3)$$

$$\text{Cytochrome P-450 } (FeO_2) + \text{substrate} \rightleftharpoons \text{cytochrome P-450 } (FeO_2)\text{-substrate} \quad (4)$$

$$\text{Cytochrome P-450 } (FeO_4)\text{-substrate} + 2H^+ + e^- \rightarrow \text{cytochrome P-450 } (Fe^{3+}) + \text{oxidized substrate} + H_2O \quad (5a)$$

$$\text{Cytochrome P-450 } (FeO_2) + 2H^+ + e^- \rightarrow \text{cytochrome P-450 } (Fe^{3+}) + H_2O_2 \quad (5b)$$

FIG. 4. *Interrelationships between hepatic microsomal electron transport and drug metabolism*

type I substances. In fact, many type II substances, such as DPEA, are potent inhibitors of the reductive as well as the oxidative systems (32).

These findings thus provide evidence for a view of the mechanism of drug oxidation which differs from the one commonly considered. Oxidized cytochrome P-450 reacts with substrates to form a cytochrome P-450–substrate complex. Type I complexes are then reduced more rapidly, but type II complexes are reduced more slowly than the free cytochrome. The reduced form of the cytochrome P-450–substrate complex then rapidly reacts with oxygen to form O_2–cytochrome P-450–substrate complexes, which in turn rapidly decompose with the formation of the oxidized substrate and oxidized cytochrome P-450 (Fig. 4). Hence, type I substances may enhance NADPH oxidation by stimulating the reduction of the complex, the rate-limiting step of the reaction. A similar sequence of events has been proposed for the hydroxylation of aniline (4), but as noted above it may be possible that only the free form of cytochrome P-450 is reduced in the presence of type II substrates and hence the mechanism may not be applicable to these substrates.

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