Mechanism of *p*-Nitrobenzoate Reduction in Liver: The Possible Role of Cytochrome P-450 in Liver Microsomes

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SUMMARY

The following evidence suggests that the reduction of *p*-nitrobenzoate to *p*-aminobenzoate by liver microsomes is mediated by cytochrome P-450: (a) carbon monoxide blocks nitroreduction, and the degree of inhibition is proportional to the amount of cytochrome P-450 bound as the carbon monoxide complex; (b) the rate of nitroreduction is proportional to the amount of cytochrome P-450 in liver microsomes from animals previously treated with either phenobarbital or carbon tetrachloride.

INTRODUCTION

Nitro compounds such as chloramphenicol, *p*-nitrobenzoate, and nitrobenzene are reduced to primary amines by a liver enzyme system that can use either NADH or NADPH as its electron donor (1). Presumably the reaction proceeds through the formation of nitroso and hydroxylamine derivatives (Fig. 1). Unlike most reductases, nitroreductase is active under anaerobic conditions but is virtually inactive in air. This oxygen sensitivity may be due in part to autoxidation of the hydroxylamine derivatives presumably formed as intermediates in the reductive pathway. For example, phenylhydroxylamine is rapidly oxidized in air to nitrosobenzene, which in turn is reduced back to phenylhydroxylamine by NADPH, either enzymatically (2) or nonenzymatically (3). This cyclic process of oxidation and reduction could therefore lead to depletion of NADPH in the system when the NADPH-generating system is inadequate to maintain the cofactor in the reduced form. It also seemed possible, however, that nitro compounds are reduced by an enzyme which is readily oxidized by air. According to this view, oxygen competes with the nitro compounds for the reduced form of the enzyme.

As shown in the present study, reduction of *p*-nitrobenzoate to *p*-aminobenzoate is catalyzed by an enzyme localized in liver microsomes. It therefore seemed possible that the reduction might be mediated by one of the autoxidizable cytochromes in liver microsomes, cytochrome b_5 or cytochrome P-450. Since carbon monoxide combines with the reduced form of cytochrome P-450 but not that of cytochrome b_5, the finding that CO blocks nitroreduction suggested that the reaction was mediated by cytochrome P-450. Accordingly, various treatments that altered the amount of microsomal cytochrome P-450 also affected the activity of nitroreductase. Since most of the NADPH-dependent liver microsomal enzyme systems that catalyze the oxidation

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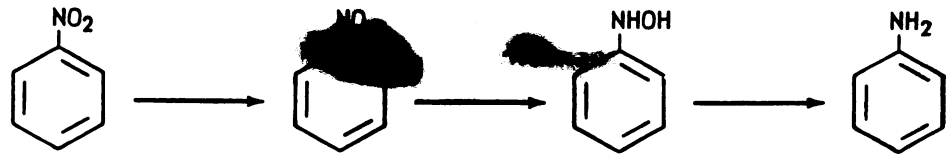

FIG. 1. *Pathway of nitrobenzene reduction*

of various steroids and drugs are also mediated by cytochrome P-450, these findings suggest that the reduction of nitro compounds and the oxidative reactions are catalyzed by similar electron transport systems.

MATERIALS AND METHODS

Animals. The animals used in these experiments were male NIH general-purpose mice (25–30 g), male Sprague-Dawley rats (160–180 g), and male New Zealand white rabbits (2 kg). In some experiments, mice received carbon tetrachloride (2.5 ml/kg orally) 3 hr before death, and rats (60–70 g) were treated with three daily doses of phenobarbital (80 mg/kg intraperitoneally) and killed 24 hr after the last dose. The animals were killed by breaking their necks, and the livers were perfused with a Krebs-Ringer-phosphate solution.

In studying the intracellular distribution of nitroreductase, the liver was homogenized with 4 volumes of 0.25 M sucrose and the homogenates were fractionated according to the method of Schneider and Hogeboom (4). In most experiments, however, the perfused livers were homogenized in 4 volumes of a 1.15% KCl solution containing Tris-HCl buffer (0.02 M, pH 7.4). The homogenates were centrifuged for 20 min at 9000 $\times$ *g* in a Sorvall centrifuge. The 9000 $\times$ *g* supernatant fraction was then centrifuged for 1 hr at 105,000 $\times$ *g* in a Spinco model L preparative ultracentrifuge (all *g* values were calculated for the center of the centrifuge tube). The microsomal pellet was suspended in the ice-cold KCl–Tris-HCl solution.

Incubation system. Typical incubation mixtures consisted of enzyme equivalent to 300 mg of liver, NADP (1.0 μmole), *p*-nitrobenzoic acid (6 μmoles), $MgCl_2$ (15 μmoles), nicotinamide (40 μmoles), glucose 6-phosphate (30 μmoles), glucose 6-phosphate dehydrogenase (1 unit, 1 μmole of NADP reduced per minute), 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.4), and water to a final volume of 3.0 ml. In assaying the reduction of nitrobenzene, nitrosobenzene, or phenylhydroxylamine, the amount of substrate was 2 μmoles.

Assay of nitroreductase. The anaerobic experiments under either N_2 or CO atmosphere were carried out in either square Thunberg tubes or rubber-stoppered Erlenmeyer flasks. The incubation mixtures were alternately evacuated *in vacuo* and flushed with nitrogen, which was passed through a deoxygenizer system containing a solution of 0.5% sodium dithionite, 0.05% 2-anthroquinone sodium sulfonate, and 0.4% sodium hydroxide. The mixtures were incubated for 20 min at 37°, the reaction was stopped by the addition of 1.0 ml of 20% trichloracetic acid, and the amount of *p*-aminobenzoate formed was assayed according to the method of Bratton and Marshall (5) as modified by Fouts and Brodie (1). Known amounts of sulfanilamide and *p*-nitrobenzoic acid added to unincubated mixtures and carried through the method served as standards and blanks, respectively. This method was also used to assay aniline formed from nitrobenzene, nitrosobenzene, and phenylhydroxylamine. In these instances aniline was used to obtain standard values.

Assay of cytochrome P-450. The method was essentially that used by Omura and Sato (6). Microsomal suspensions (approximately 2–3 mg of protein per milliliter) in potassium phosphate buffer, pH 7.4, were added to a reference cuvette and a sample

cuvette. A baseline, which reflects a differential absorption of two cuvettes, was obtained in a Shimadzu recording spectrophotometer by scanning from 400 mμ to 500 mμ. Carbon monoxide was gently bubbled through the sample cuvette for 30 sec, followed by addition of a few milligrams of sodium dithionite, and the mixture was gassed with carbon monoxide for another minute. The reference cuvette was treated with sodium dithionite only. The quantity of cytochrome P-450 was calculated from the absorbance difference ($A_{450} - A_{490}$) and the molar extinction coefficient of 91 mM^{-1} cm^{-1} (7).

Assay of cytochrome c reductase. The method used was that described by Williams and Kamin (8). The number of micromoles of cytochrome *c* reduced per minute per milliliter was calculated from the molar extinction coefficient of 19.1 mM^{-1} cm^{-1} for the difference in absorbance between reduced and oxidized cytochrome *c* at 550 mμ.

Assay of protein. Protein was determined by the method of Sutherland *et al.* (9). Crystalline bovine serum albumin was used as the protein standard.

RESULTS

Intracellular distribution of nitroreductase activity in mouse liver homogenates. Fouts and Brodie (1) reported that nitroreductase activity in liver was about equally distributed between the microsomal and soluble fractions. As subsequently reported by Fouts (10), however, only the microsomal fraction possessed significant nitroreductase activity (Table 1). The activity in the microsomal fraction, however, was considerably lower than that present in the 9000 × *g* supernatant fractions, which in turn was lower than that in the whole homogenate. Addition of the boiled preparations of nuclei and mitochondria to the 9000 × *g* supernatant fraction restored the activity to that of the control, whereas addition of the boiled soluble fraction restored the activity to that of the reconstituted 9000 × *g* supernatant. These findings thus suggest that nuclei, mitochondria, and soluble fractions of liver contain unidentified activators of nitroreductase. Other experiments showed that boiled microsomes added to the assay system also enhanced nitroreductase activity of unboiled liver microsomes (Figs. 2 and 3).

Inhibition of nitroreductase by carbon monoxide. Carbon monoxide almost completely blocked the reduction of *p*-nitrobenzoate by liver microsomes from mouse, rat, or rabbit (Table 2). Similarly, the increased activity caused by the addition of boiled preparations to liver microsomes was

TABLE 1

Intracellular distribution of nitroreductase activity in mouse liver homogenates

Boiled and unboiled fractions were equivalent to 300 mg of liver. The preparations were mixed with cofactors and incubated as described in the text. The measurements are expressed as the mean of duplicate values ± half the difference between these values.

Fraction	*p*-Aminobenzoic acid formed	Relative activity
	mμmoles/g liver/hr	%
Whole homogenate	8.66 ± 0.24	100
Nuclear fraction	0.71 ± 0.07	8
600 × *g* supernatant fraction	7.60 ± 0.02	88
Mitochondrial fraction	0.52 ± 0.05	6
9000 × *g* supernatant fraction	5.68 ± 0	66
Microsomal fraction	2.81 ± 0.03	32
105,000 × *g* supernatant fraction	0.135 ± 0.01	2
600 × *g* supernatant fraction + boiled nuclei	8.92 ± 0.08	103
9000 × *g* supernatant fraction + boiled nuclei + boiled mitochondria	8.35 ± 0.07	96
Microsomes + 105,000 × *g* supernatant	4.35 ± 0.15	52
Microsomes + boiled 105,000 × *g* supernatant	4.43 ± 0.03	51

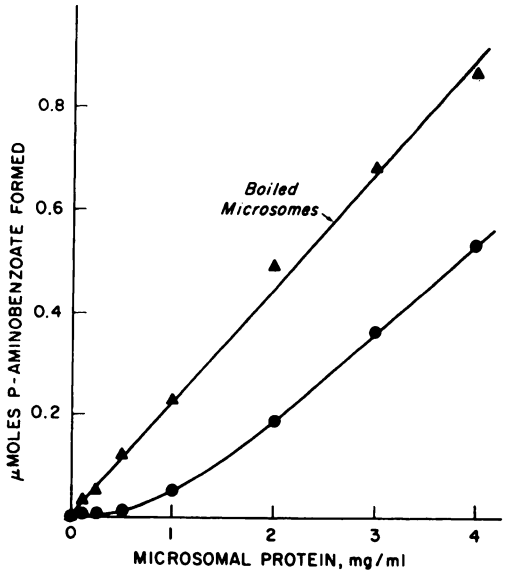

FIG. 2. *Effect of boiled microsomes (4 mg/ml) on the activity of various amounts of mouse liver microsomes*

●——●, Microsomes without boiled microsomes; ▲——▲, microsomes with boiled microsomes.

also blocked by carbon monoxide (Table 3).

Effects of carbon monoxide on various steps in the reduction of nitro compounds. Nitroreductase presumably converts nitro compounds first to nitroso derivatives and then to phenylhydroxylamine derivatives before aromatic amines are formed. As shown in Table 4, the formation of aniline from nitrobenzene was considerably slower than it was from nitrosobenzene or phenylhydroxylamine, suggesting that the rate-limiting step is the conversion of the nitro group to the nitroso group. Table 4 also shows that carbon monoxide blocked the formation of aniline from nitrosobenzene and phenylhydroxylamine about as well as it inhibited the reduction of nitrobenzene. These findings did not preclude the possibility that only the third step was blocked by CO. If this were true, however, CO should block the oxidation of NADPH associated with nitroreduction only about 33%. As shown in Table 5, oxidation of NADPH in the presence of *p*-nitrobenzoate under anaerobic conditions was blocked by CO about 85%, indicating that CO blocks both the first and third steps of the reaction.

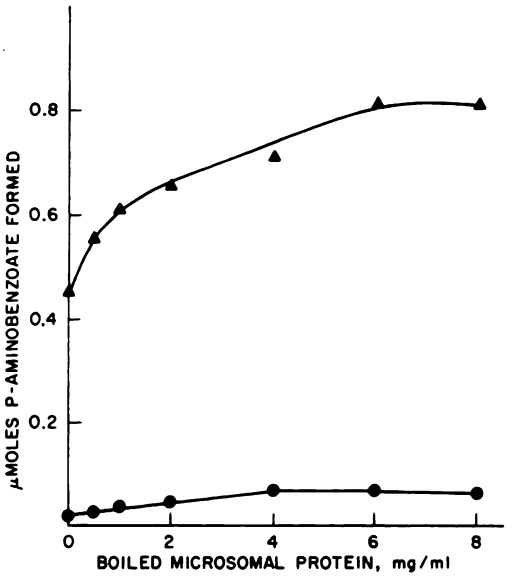

FIG. 3. *Effect of various amounts of boiled microsomes on the activity of low (0.5 mg/ml;* ●——●*) and high (4.0 mg/ml;* ▲——▲*) concentrations of mouse liver microsomes*

TABLE 2

Effect of carbon monoxide on nitroreductase activity from various species

Microsomes equivalent to 300 mg of liver were incubated with cofactors and *p*-nitrobenzoate as described in the text. The results are typical of at least two experiments.

Species	*p*-Aminobenzoic acid formed		Inhibition
	In N_2	In CO	
	mμmoles/min/mg microsomal protein		%
Mouse	2.98	0.24	92
Rat	1.53	0.28	82
Rabbit	0.92	0.13	86

Effect of prior treatment with phenobarbital on nitroreductase activity and cytochrome P-450 in liver microsomes. Rats received phenobarbital intraperitoneally in three daily doses of 80 mg/kg and were killed 24 hr after the last dose. As shown in Table 6, the enhancement of nitroreductase activity closely paralleled the increase in liver microsomal cytochrome P-450. Moreover, prior treatment of rats with pheno-

TABLE 3
Effect of CO on the increase in nitroreductase activity of mouse liver microsomes produced by boiled enzyme preparations
The conditions were those described in Table 1.

Mouse liver preparation	*p*-Aminobenzoate formed		Inhibition
	In N_2	In CO	
	mμmoles/g liver/hr		%
Microsomes	1.54 ± 0.12	0.10 ± 0.04	94
Microsomes + boiled 9000 × *g* precipitate and boiled 105,000 × *g* supernatant	4.59 ± 0.02	0.36 ± 0.00	92
Microsomes + boiled 105,000 × *g* supernatant	3.49 ± 0.00	0.12 ± 0.02	95

barbital did not markedly alter the effectiveness of carbon monoxide in blocking the reduction of *p*-nitrobenzoate.

Effect of prior treatment with CCl_4 on nitroreductase activity and the level of liver microsome cytochrome P-450. The administration of CCl_4 to rats is known to impair the metabolism of drugs by decreasing the amount of cytochrome P-450 (11, 12), as measured by the method of Omura and Sato (6) and by the decrease in heme (13). Mice received carbon tetrachloride (2.5 ml/kg orally) and were killed 3 hr afterward. The administration of carbon tetrachloride did not alter the activity of NADPH-cytochrome *c* reductase as markedly as it decreased the amount of cytochrome P-450 in liver microsomes (Table 7). The impairment of nitroreductase activity paralleled the decrease in cytochrome P-450 more closely than it did the decrease in NADPH-cytochrome *c* reductase activity.

Relationship between carbon monoxide inhibition and CO–cytochrome P-450 complex formation. After the addition of carbon monoxide to the incubation mixture, the Thunberg tubes were alternately evacuated and gassed with nitrogen until the amount of CO–cytochrome P-450 complex was significantly reduced. The addition of *p*-nitrobenzoate from a side arm of the Thunberg

TABLE 4
Effect of carbon monoxide on formation of aniline from nitrobenzene, nitrosobenzene, and phenylhydroxylamine by mouse liver microsomes
Microsomes (3 mg of protein per milliliter) from mouse liver were incubated with cofactors and the various substrates (2 mM) as described in the text.

Substrate	Aniline formed		Inhibition
	In N_2	In CO	
	mμmoles/min/mg microsomal protein		%
Nitrobenzene	6.05	0.92	85
Nitrosobenzene	24.5	3.85	84
Phenylhydroxylamine	27.9	7.48	73

TABLE 5
Effect of CO on NADPH oxidation associated with p-nitrobenzoate reduction by mouse liver microsomes
Incubation mixtures containing mouse liver microsomes (6 mg) with or without *p*-nitrobenzoate (2 μmoles) in 3 ml of 0.067 M potassium phosphate buffer, pH 7.4, were placed in an anaerobic cell (Aminco, Al-65085) and gassed for 7 min with nitrogen or carbon monoxide which had been deoxygenated by passage through three successive gas towers containing alkaline dithionite. The stopcock assembly was attached, and air space above the mixture was flushed with the gas for an additional 4 min. The reaction was started by addition of 25 μl of 8.0 mM NADPH from the reservoir of the stopcock assembly, and NADPH oxidation was followed at 340 mμ in a Gilford model 2000 spectrophotometer. The results are expressed as the mean ± standard deviation obtained with three replicates.

Atmosphere	NADPH oxidized	
	Control	*p*-Nitrobenzoate
	mμmoles/mg protein/min	
Air	19.4 ± 1.4	
N_2	0.098 ± 0.089	4.59 ± 0.22
CO	0 ± 0	0.65 ± 0.01

TABLE 6

Effect of prior treatment with phenobarbital on cytochrome P-450 content and microsomal nitroreductase activity in rat liver microsomes in nitrogen or carbon monoxide

Rats were treated with phenobarbital as described under MATERIALS AND METHODS. The results represent the mean ± standard deviation obtained from four separate groups (N) of three pooled livers per group.

Treatment and gas phase	*p*-Aminobenzoic acid formed	N_2 − CO difference	Cytochrome P-450	(N_2 − CO)/P-450
	mμmoles/mg protein/min		*mμmoles/mg*	
Phenobarbital				
N_2	3.98 ± 0.26	3.74 ± 0.20[a]	1.98 ± 0.03[b]	1.90 ± 0.23[c]
CO	0.24 ± 0.10			
Saline				
N_2	1.64 ± 0.14	1.29 ± 0.07[a]	0.74 ± 0.02[b]	1.74 ± 0.07[c]
CO	0.35 ± 0.04			

[a,b] $p < 0.001$.
[c] $p > 0.05$.

TABLE 7

Effect of prior treatment with carbon tetrachloride on nitroreductase, NADPH-cytochrome c reductase, and cytochrome P-450 in mouse liver microsomes

Mice were treated with carbon tetrachloride as described under MATERIALS AND METHODS. The results represent the mean ± standard deviation obtained from five separate groups (N) of three pooled livers per group.

Treatment	Cytochrome *c* reduced	*p*-Aminobenzoic acid formed	Cytochrome P-450	*p*-Aminobenzoic acid formed/ P-450 concentration
	μmoles/mg protein/min	*mμmoles/mg protein/min*	*mμmoles/mg protein*	
CCl_4	1.84 ± 0.15[a]	2.03 ± 0.43[b]	0.46 ± 0.13[c]	4.62 ± 1.28[d]
Control	2.13 ± 0.34[a]	6.46 ± 0.88[b]	1.53 ± 0.26[c]	4.14 ± 0.30[d]

[a] $p > 0.1$.
[b,c] $p < 0.001$.
[d] $p > 0.5$.

TABLE 8

Relationship between binding of CO to mouse liver microsomes and the inhibition of nitroreductase

	P-450–CO complex			(P-450–CO complex)/ P-450		*p*-Aminobenzoic acid	
[CO][a]	A[b]	B	C	B	C	formed	Inhibition
	mμmoles/mg protein/ml			%	%	*mμmoles/mg protein/min*	%
M							
0				0	0	1.96	0
6×10^{-8}	0.361	0.291	0.259	22	23	1.53	21.5
4.2×10^{-7}	1.15	0.980	0.784	74	68	0.56	71.5
1.0×10^{-3}	1.36	1.32	1.12	100	100	0.204	89.5

[a] Concentrations were calculated on the basis of the dissociation constant, 2×10^{-7} M, of the CO–cytochrome P-450 complex determined by Omura *et al.* (14).

[b] A = before the addition of *p*-nitrobenzoic acid (6 μmoles); B = after the addition of *p*-nitrobenzoic acid (6 μmoles); C = after a 10-min incubation at 37°.

tube caused a further decrease in the amount of CO–cytochrome P-450 complex. The mixtures were incubated for 10 min, and the amounts of the CO–cytochrome P-450 complex and *p*-aminobenzoate were determined. As shown in Table 8, the degree of inhibition of nitroreductase closely paralleled the proportion of cytochrome P-450 present as the carbon monoxide complex.

DISCUSSION

The observation by Fouts and Brodie (1) that the conversion of *p*-nitrobenzoate to *p*-aminobenzoate did not occur in air suggested that nitroreductase might be mediated by metalloenzymes other than cytochrome P-450, for carbon monoxide is known to inhibit a number of metalloenzymes such as xanthine oxidase (15), tyrosinase (16), and β-phenethylamine hydroxylase (17). As shown in the present paper, however, there is a relationship between the degree of inhibition of nitroreductase and formation of the CO-cytochrome P-450 complex in the presence of various concentrations of the inhibitor. The relationship between cytochrome P-450 and nitroreductase was further supported by the finding that changes in nitroreductase activity paralleled changes in the amount

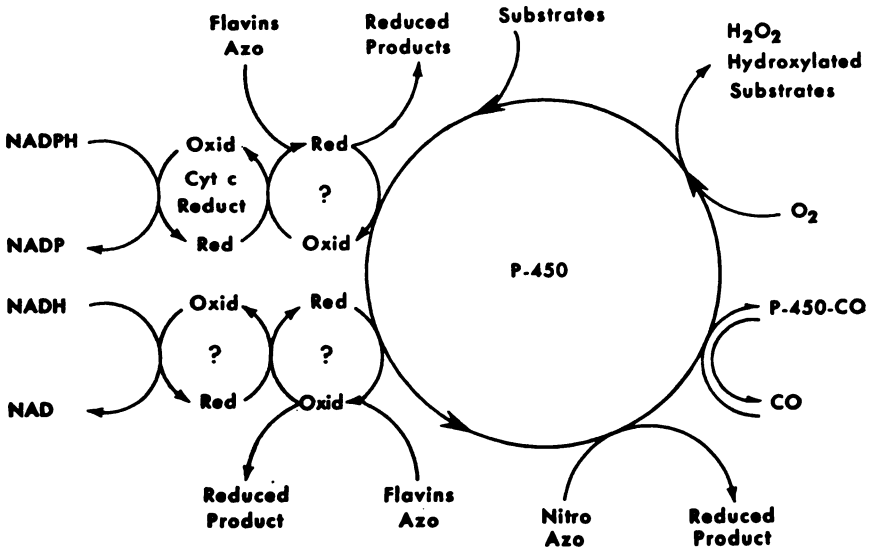

FIG. 4. *Proposed electron transport system in liver microsomes*

lated to the NADPH-dependent enzymes in liver microsomes which catalyze the oxidation of a wide variety of steroids and drugs. Support for this view was obtained by showing that nitroreductase was blocked by carbon monoxide. This inhibitor blocks a variety of NADPH-dependent oxidative reactions by combining with the reduced form of cytochrome P-450 in liver microsomes. Hence, according to our view, oxygen blocks nitroreductase by reacting with cytochrome P-450.

Evidence that carbon monoxide blocked microsomal nitroreductase did not preclude the possibility that nitroreductase might be of cytochrome P-450 in liver microsomes: (a) impairment of the enzymatic activity caused by prior administration of CCl_4 closely paralleled the decrease in microsomal cytochrome P-450, and (b) induction of nitroreductase by prior treatment of rats with phenobarbital paralleled increases in cytochrome P-450 in liver microsomes.

Our current view of the NADPH-dependent electron transport system in liver microsomes is illustrated in Fig. 4. Since carbon monoxide does not block the reduction of cytochrome *c*, flavins, and tetrazolium salts (18), these substances ap-

parently accept electrons directly from NADPH-cytochrome *c* reductase or indirectly through an intermediate electron carrier, which may or may not be involved in reduction of cytochrome P-450.

Carbon monoxide inhibits the reduction of Neoprontosil [disodium 2-(4′-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate] only about 40% (19, 20), suggesting that azo compounds are reduced not only by cytochrome P-450 but also by NADPH-cytochrome *c* reductase either directly or indirectly through an endogenous electron carrier. Although purified NADPH-cytochrome *c* reductase is known to reduce azo compounds, there is evidence suggesting that an endogenous carrier is required for azo group reduction in intact hepatic microsomes; treatment of rats with phenobarbital, which increases NADPH-cytochrome *c* reductase, does not increase carbon monoxide-insensitive azoreductase activity, whereas prior treatment with 3-methylcholanthrene, which does not affect cytochrome *c* reductase activity, enhances carbon monoxide-insensitive azoreductase (20). Since carbon monoxide almost completely blocks nitroreductase activity in liver microsomes, we conclude that cytochrome P-450 mediates the transfer of electrons to aromatic nitro compounds. However, the finding that boiled preparations of various fractions of liver homogenate cause an increase in nitroreductase activity that is blocked by carbon monoxide suggests the possibility that an intermediate is required to mediate the transfer of electrons between cytochrome P-450 and the substrate.

REFERENCES

1. J. R. Fouts and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **119**, 197 (1957).
2. M. Kiese, *Ann. N. Y. Acad. Sci.* **123**, 141 (1965).
3. J. R. Gillette, *Ann. N. Y. Acad. Sci.* **123**, 154 (1965).
4. W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **183**, 123 (1950).
5. A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.* **128**, 537 (1939).
6. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370 (1964).
7. T. Omura and R. Sato, *Biochim. Biophys. Acta* **71**, 224 (1963).
8. C. H. Williams, Jr. and H. Kamin, *J. Biol. Chem.* **237**, 587 (1962).
9. E. W. Sutherland, C. F. Cori, R. Haynes and N. S. Olsen, *J. Biol. Chem.* **180**, 825 (1949).
10. J. R. Fouts, *Biochem. Biophys. Res. Commun.* **6**, 373 (1961).
11. E. A. Smuckler, E. Arrhenius and T. Hultin, *Biochem. J.* **103**, 55 (1967).
12. J. A. Castro, H. A. Sasame, H. Sussman and J. R. Gillette, *Life Sci.* **7**, 129 (1968).
13. F. E. Greene, B. Stripp and J. R. Gillette, *Biochem. Pharmacol.* in press.
14. T. Omura, R. Sato, D. Y. Cooper, O. Rosenthal and R. W. Estabrook, *Fed. Proc.* **24**, 1181 (1965).
15. S. J. Adelstein and B. L. Vallee, *J. Biol. Chem.* **233**, 589 (1958).
16. D. Keilin and T. Mann, *Proc. Roy. Soc. Ser. B. Biol. Sci.* **125**, 187 (1938).
17. S. Friedman and S. Kaufman, *J. Biol. Chem.* **240**, 4763 (1965).
18. J. R. Gillette, in "Experimental Medicine and Surgery," p. 105. Brooklyn Medical Press, New York, 1965.
19. P. H. Hernandez, P. Mazel and J. R. Gillette, *Biochem. Pharmacol.* **16**, 1859 (1967).
20. P. H. Hernandez, J. R. Gillette and P. Mazel, *Biochem. Pharmacol.* **16**, 1877 (1967).