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STUDIES ON THE MECHANISM OF ACTION OF MAMMALIAN HEPATIC AZOREDUCTASE—II

THE EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON CARBON MONOXIDE SENSITIVE AND INSENSITIVE AZOREDUCTASE ACTIVITIES*†

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Abstract—The effect of carbon monoxide and steapsin solubilization on rat hepatic microsomal azoreductase and NADPH–cytochrome c reductase previously induced with 3-methylcholanthrene and phenobarbital has been investigated. Microsomal azoreductase was inhibited 30–40 per cent in the presence of carbon monoxide, whereas NADPH–cytochrome c reductase and neotetrazolium diaphorase were uneffected. Upon solubilization by steapsin, azoreductase, NADPH–cytochrome c reductase or neotetrazolium diaphorase was not inhibited by carbon monoxide. The percentage of protein solubilized by steapsin in control microsomes did not differ significantly in microsomes obtained from 3-methylcholanthrene or phenobarbital-pretreated animals. Phenobarbital pretreatment increased NADPH–cytochrome c reductase and azoreductase activity and cytochrome P-450. The increased azoreductase activity was blocked by carbon monoxide in intact microsomes, whereas the solubilized preparation was unaffected. The increased azoreductase activity seen after phenobarbital could be correlated directly with the increased cytochrome P-450.

Although 3-methylcholanthrene pretreatment did not increase NADPH–cytochrome c reductase or neotetrazolium diaphorase in either the microsomal or solubilized fraction, it did increase both cytochrome P-450 and carbon monoxide sensitive and insensitive microsomal azoreductase. In contrast to phenobarbital, the inductive effect of 3-methyl cholanthrene on azoreductase was destroyed upon solubilization and could not be correlated with increased cytochrome P-450. The data indicate that azo dyes are reduced via: (1) a carbon monoxide sensitive pathway (cytochrome P-450), which is destroyed upon solubilization; (2) a carbon monoxide insensitive pathway (NADPH–cytochrome c reductase), which is not destroyed upon solubilization; and (3) a 3-methylcholanthrene inducible pathway, which is insensitive to carbon monoxide but sensitive to solubilization.

IN THE preceding paper[1] we have presented evidence which supports the view that purified microsomal azoreductase is probably identical with NADPH–cytochrome c reductase. Therefore, the same enzyme system that catalyzes the reduction of cyto-

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chrome c and other electron acceptors catalyzes the reduction of neoprontosil with NADPH. The previous experiments[1] further suggested that NADPH–cytochrome c reductase could not be responsible for all of the azoreductase found in hepatic microsomes since solubilization of the microsomal fraction by steapsin decreased azoreductase activity but did not alter NADPH–cytochrome c reductase, which is completely recovered in the solubilized protein. This could be due to destruction of cytochrome P-450, which upon solubilization is converted into an inactive form,[2] or to the removal of an inhibitor or essential cofactor.

The studies of previous investigators also suggest that microsomal azoreductase and NADPH–cytochrome c reductase may be different, since 3-methylcholanthrene (3-MC) pretreatment increased azoreductase activity,[3] but NADPH–cytochrome c reductase was unaffected.[4] On the other hand, phenobarbital (PB) pretreatment stimulates both azoreductase[5] and NADPH–cytochrome c reductase activities.[5, 6] Therefore, for azoreductase a distinction has to be made between the organized microsomal electron transport system and azoreductase activity of purified NADPH–cytochrome c reductase.

The purpose of this paper is to characterize further a microsomal azoreductase which is distinct from microsomal NADPH–cytochrome c reductase ('azoreductase'). The differential inductive pattern of 3-MC and PB coupled with CO inhibition of cytochrome P-450 and steapsin solubilization was therefore utilized to further characterize microsomal azoreductase.

MATERIALS AND METHODS

The isolation of liver microsomes, enzymatic assays employed, solubilzation procedures, and the source of reagents have been previously described.[1] 3-Methylcholanthrene (3-MC) was obtained from Mann Research Laboratories, Inc.; sodium phenobarbital from Merck & Co.; and carbon monoxide from Matheson Co.

Pretreatment of animals with 3-methylcholanthrene and phenobarbital

Female Sprague–Dawley rats weighing 50 g were used. 3-MC (7 mg/ml) was dissolved in corn oil, with gentle heating, and administered i.p. (35 mg/kg) once daily for 3 days in a volume of 0·5 ml/100 g body wt. Control animals were given 0·5 ml corn oil. Phenobarbital (80 mg/kg) was given once daily in saline. The control group was given a daily injection of saline (0·5 ml/100 g body wt.) i.p. Control and experimental groups were deprived of food on the third day of treatment in both 3-MC and PB experiments. Water was supplied *ad libitum*. The animals were sacrificed by decapitation on the morning of the fourth day. In order to obtain enough material for all the assays, the livers from 5 weanling rats were pooled.

Determination of carbon monoxide-binding pigment (P-450) *in rat liver microsomes*

The method employed was essentially that used by Omura and Sato.[7] The microsomes were obtained as previously described.[1] In order to removed as much of the contaminants as possible (i.e. hemoglobin), the isolated microsomes from 500 mg liver (wet wt.) were resuspended in cold isotonic KCl (10^{-3} M EDTA). The resuspended microsomes were centrifuged at 198,000 *g* for 30 min in a Spinco model L-2 ultracentrifuge at 2°. The sedimented pellet was then resuspended in 6 ml of 0·1 M

NaH_2PO_4–K_2HPO_4 buffer, pH 7·6 (10^{-3} M EDTA). The protein concentration of this microsomal suspension was approximately 2 mg/ml. Half of the suspension was placed in the reference cuvette and half in the sample cuvette of a Beckman DB recording spectrophotometer, and a baseline obtained by scanning from 510–410 mμ. Carbon monoxide was gently bubbled through the sample cuvette for 20 sec, followed by addition of a few milligrams of sodium dithionite and gassing with carbon monoxide for another 20 sec. The reference cuvette was treated with sodium dithionite only. Both cuvettes were sealed with Teflon stoppers, and 2–3 min later the spectrum was recorded from 510–410 mμ. All of the above procedures were done at room temperature (20–25°). The quantity of cytochrome P-450 was calculated from the optical density difference (450 minus 480 mμ) and the molar extinction coefficient of 91 mM^{-1} cm^1 (Omura and Sato[7]).

RESULTS

Effect of phenobarbital and 3-methylcholanthrene on liver weight, microsomal protein and steapsin solubilized protein

The data of Table 1 indicate that phenobarbital pretreatment markedly increased liver weight and microsomal protein, whereas 3-MC pretreatment increased liver weight without affecting microsomal protein. The percentage of microsomal protein solubilized in control animals did not differ significantly from that in rats pretreated with PB or 3-MC. An increase in liver weight, microsomal protein, and total protein after phenobarbital administration has also been found by Conney *et al.*[8] and hypertrophy of rat liver after 3-MC administration, without an increase in protein per g of liver, has also been observed.[3]

Effect of carbon monoxide on azoreductase, NADPH–*cytochrome c reductase and neotetrazolium diaphorase activities*

The effect of carbon monoxide on azoreductase, NADPH–cytochrome c reductase and neotetrazolium diaphorase at all stages of purification is shown in Table 2. Since carbon monoxide affects only azoreductase activity in intact microsomes, the reduction of azo dyes is mediated, at least in part, via microsomal cytochrome P-450. This interpretation is further supported by the finding that inactivation of cytochrome P-450 by steapsin pretreatment of the microsomal fraction causes partial loss of azoreductase[1] activity, whereas NADPH–cytochrome c reductase is completely recovered in the solubilized protein. The data further indicate that the reduction of cytochrome c and neotetrazolium is not mediated via carbon monoxide-binding pigment. Earlier reports[1, 9] and these data suggest the existence of a microsomal azoreductase that is (1) destroyed upon solubilization and (2) sensitive to carbon monoxide, namely cytochrome P-450.

Effect of phenobarbital on microsomal NADPH–*cytochrome c reductase*

The effect of phenobarbital pretreatment on microsomal NADPH–cytochrome c reductase is shown in Table 3. The stimulatory effect (approximately 90 per cent was also observed in the solubilized fraction after steapsin treatment, indicating that the increased azoreductase activity of this fraction corresponded to stimulation of NADPH–cytochrome c reductase.

TABLE 1. EFFECT OF 3-METHYLCHOLANTHRENE OR PHENOBARBITAL PRETREATMENT ON LIVER WEIGHT, MICROSOMAL PROTEIN, AND THE PERCENTAGE OF MICROSOMAL PROTEIN SOLUBILIZED*

	3-Methyl-cholanthrene	Corn oil control	% Difference from control	Phenobarbital	Saline	% Difference from control
Microsomal protein (mg/g liver)	20 ± 0·94	18 ± 0·43	+11†	28·0 ± 0·19	16·0 ± 0·55	+74 0‡
Grams wet liver wt/. 100 g body wt.	5 ± 0·06	4 ± 0·06	+25‡	5·7 ± 0·06	4·1 ± 0·10	+38·0‡
Percentage of microsomal protein solubilized	42 ± 2·1	48 ± 1·6	−13†	45·0 ± 1·0	44·0 ± 1·7	+0·8†

* Pretreatment of rats with 3-MC or PB and steapsin solubilization treatment are described under Materials and Methods. The results are expressed as the mean ± S.E. of the values obtained from 4 separate groups of 5 pooled livers per group.

† Not significant.

‡ $P < 0·001$.

TABLE 2. EFFECT OF CARBON MONOXIDE ON ENZYMATIC ACTIVITY‡

Fractions* tested	Azoreductctase	NADPH–cyt. c reductase	Neotetrazolium diaphorase
Microsomes	Inhibited 30 – 40%	0†	0
Steapsin supernatant	0	0	0
Ammonium sulfate 40–80%	0	0	0
DEAE-cellulose eluate	0	0	0

* Fractions obtained as described under Materials and Methods.

† 0 = No effect.

‡ These experiments have been repeated a number of times with the same results.

TABLE 3. EFFECT OF PHENOBARBITAL PRETREATMENT ON NADPH–CYTOCHROME C REDUCTASE*

Fractions*	Cytochrome c reduced†	Difference (%)
Microsomes		
Phenobarbital	221 ± 4·4	+ 76·0
Saline control	126 ± 5·1	($P < 0.001$)
Solubilized microsomal protein		
Phenobarbital	594 ± 25·2	+ 91·0
Saline control	310 ± 19·8	($P < 0.001$)

* Pretreatment of rats and preparation of fractions are described under Materials and Methods. The results are expressed as the mean ± S.E. of the values obtained from 4 separate groups of 5 pooled livers per group.

† Expressed as mμmole cytochrome c reduced/min/mg protein.

Effect of phenobarbital on the formation of microsomal cytochrome P-450 *and its relationship to microsomal and solubilized azoreductase activity*

The formation of microsomal cytochrome P-450 was markedly stimulated by phenobarbital pretreatment, which resulted in a marked stimulation of microsomal azoreductase (Table 4). Azoreductase activity of the steapsin-solubilized fraction did not differ whether incubation was performed under nitrogen or under carbon monoxide. However, the inductive effect of phenobarbital is no longer seen in microsomes when incubation is performed under carbon monoxide, and the amount of sulfanilamide formed under nitrogen minus that formed under carbon monoxide is proportional to the amount of cytochrome P-450. This would imply that the inductive effect of PB on azoreductase was entirely due to increased formation of cytochrome P-450. This interpretation is supported by the findings that solubilization converts cytochrome P-450 to an inactive form and that P-450 is completely inhibited by carbon monoxide.[2, 10]

TABLE 4. EFFECT OF PHENOBARBITAL PRETREATMENT ON CYTOCHROME P-450, MICROSOMAL AND SOLUBILIZED AZOREDUCTASE UNDER NITROGEN OR UNDER CARBON MONOXIDE*

Treatment	Sulfanilamide formed†	Difference (%)	Sulfanilamide formed†	Difference (%)	Difference (N_2-CO)	Cytochrome P-450‡	N_2-CO/P-450
	Nitrogen atmosphere		CO atmosphere				
Microsomes							
Phenobarbital	398 ± 5·5	+ 89·2	143 ± 0·7	+ 6·5	255	2·22 ± 0·05	115
Saline control	210 ± 5·7	(P < 0·001)§	134 ± 6·3	(P < 0·2)	76	0·65 ± 0·05	117
Solubilized microsomal protein							
Phenobarbital	128 ± 6·0	+ 88·0	123 ± 7·2	+ 98·4			
Saline control	68 ± 2·7	(P < 0·001)	62 ± 5·0	(P < 0·001)			

* Rats were pretreated with phenobarbital as described under Materials and Methods.

† Express as mμmole of sulfanilamide/30 min/mg protein.

‡ Expressed as mμmole/mg protein.

§ P < 0·001 for (N_2-CO) differences and for the difference between cytochrome P-450 from phenobarbital-treated animals and controls. The results represent the mean ± S.E. obtained from 4 separate groups of 5 pooled livers per group.

Effect of 3-MC *on* NADPH–*cytochrome c reductase and neotetrazolium diaphorase*

The data in Table 5 indicate that 3-MC pretreatment did not increase NADPH–cytochrome c reductase or neotetrazolium diaphorase (NT). The difference observed after steapsin solubilization (15 per cent) in Table 5 may be due to a change in the ratios of NADPH–cytochrome c reductase to inactive solubilized protein. This would explain the small increase in specific activity. Since NT activity did not change when either nitrogen or carbon monoxide was the gas phase (Table 5), this would imply that no secondary pathway utilizing cytochrome P-450 was involved in the reduction of neotetrazolium. This is in agreement with the hypothesis that NT is reduced by NADPH–cytochrome c reductase in the presence of NADPH.

Effect of 3-MC *on the formation of microsomal cytochrome* P-450 *and its relationship to microsomal solubilized azoreductase activity*

In contrast to NADPH–cytochrome c reductase and NT microsomal azoreductase activity was significantly increased by 3-MC (Table 6) pretreatment. Microsomal cytochrome P-450 was increased 170 per cent above controls. The microsomes from 3-MC-treated animals exhibited 35 per cent greater activity than the controls when incubated under nitrogen. The azoreductase activity of steapsin-solubilized microsomal protein from 3-MC-treated rats did not differ from the controls. Under carbon monoxide, azoreductase was decreased 37 per cent in the controls and 32 per cent in the 3-MC-treated group. This decrease reflects the azoreductase activity which is probably mediated via carbon monoxide-binding pigment (P-450). If 3-MC induction is attributed solely to its effect on cytochrome P-450, the azoreductase activity under CO should be identical for controls and 3-MC-treated animals. This did not occur; rather, the difference between controls and 3-MC-treated rats increased to 60 per cent under CO, whereas it was 35 per cent under nitrogen. The inductive effect was not evident after solubilization, indicating that the pathway induced was sensitive to steapsin treatment. Since NADPH–cytochrome c reductase was not stimulated by 3-MC (Table 5), it cannot account for the stimulatory effect of 3-MC on liver microsomal azoreductase. In addition, the ratios shown in Table 6 on the relationship between cytochrome P-450 and azoreductase induction do not agree for controls and 3-MC-treated animals, indicating that a pathway other than microsomal cytochrome P-450 was stimulated by 3-MC pretreatment. The data of Table 6, therefore, indicate that 3-MC pretreatment stimulates azoreductase via a pathway which is insensitive to CO but sensitive to solubilization.

DISCUSSION

The previous communication in this series[1] presented evidence that NADPH–cytochrome c reductase could not account for all of the azoreductase activity found in hepatic microsomes, since solubilization causes partial loss of azoreductase activity, while NADPH–cytochrome c reductase is completely recovered in the solubilized protein. In the present communication, a microsomal azoreductase distinct from the azoreductase activity of purified microsomal NADPH–cytochrome c reductase has been further characterized.

The marked increase in microsomal azoreductase activity observed after phenobarbital pretreatment (Table 4) can be attributed to significant stimulation of both NADPH–cytochrome c reductase and cytochrome P-450. Upon solubilization of the

TABLE 5. EFFECT OF 3-METHYLCHOLANTHRENE PRETREATMENT ON NADPH-CYTOCHROME C REDUCTASE AND NEOTETRAZOLIUM DIAPHORASE

Treatment*	Cytochrome c reduced†				Formazan formed‡			
	Microsomes	Diff. (%)	Solubilized microsomal protein	Diff. (%)	Microsomes			
					Nitrogen	Diff. (%)	Carbon monoxide	Diff. (%)
3-Methylcholanthrene	100 ± 3·3	−15·0 ($P > 0·02$)	339 ± 7·3	+15·5 ($P < 0·01$)	2328 ± 120	−11·0 ($P > 0·1$)	2676 ± 152	−2·6 ($P > 0·6$)
Corn oil	117 ± 5·7		294 ± 5·8		2615 ± 99		2748 ± 80	

* Rats were pretreated as described under Materials and Methods. The results are expressed as the mean ± S.E. of the values obtained from 4 separate groups of 5 pooled livers per group.

† Express as mμmole cytochrome c reduced/min/mg protein.

‡ Expressed as mμmole formazan formed/10 min/mg protein.

TABLE 6. EFFECT OF 3-METHYLCHOLANTHRENE PRETREATMENT ON CYTOCHROME P-450, MICROSOMAL AND SOLUBILIZED AZOREDUCTASE UNDER NITROGEN OR UNDER CARBON MONOXIDE*

Treatment	Sulfanilamide formed†	Difference (%)	Sulfanilamide formed†	Difference (%)	Difference (N_2-CO)	Cytochrome P-450‡	N_2-CO/ P-450
	Nitrogen atmosphere		CO atmosphere				
Microsomes							
3-Methylcholanthrene	264 ± 8·0	+ 35·5 ($P < 0·001$)§	172 ± 10·1	+ 60·0 ($P < 0·01$)	92	1·75 ± 0·06	52·6
Corn oil control	195 ± 7·7		108 ± 6·0		87	0·64 ± 0·03	136·0
Solubilized microsomal protein							
3-Methylcholanthrene	72 ± 0·9	± 5·5 ($P > 0·3$)	69 ± 4·0	− 4·2 ($P > 0·1$)			
Corn oil control	69 ± 3·9		72 ± 6·0				

* Rats were pretreated with 3-MC as described under Materials and Methods.

† Expressed as mμmole of sulfanilamide/30 min/mg protein.

‡ Expressed as mμmole/mg of microsomal protein.

§ $P < 0·001$ for (N_2-CO) differences and for the difference between cytochrome P-450 from 3-MC-treated animals and controls. The results represent the means ± S.E. obtained from 4 separate groups of 5 pooled livers per group.

microsomes with steapsin, the inductive effect of phenobarbital was still observed compared to the saline controls, and was not inhibited by carbon monoxide, indicating that the increased azoreductase activity of this fraction corresponded to stimulation of NADPH–cytochrome c reductase. This interpretation is further supported by the finding that carbon monoxide does not affect the azoreductase activity of the solubilized preparation (Table 2).

When intact microsomes were incubated under a carbon monoxide atmosphere, the inductive effect of phenobarbital was no longer seen, even though NADPH–cytochrome c reductase was 76 per cent higher than controls (Table 4). This finding would imply that in microsomes the reduction of azo dyes occurs to a large extent through cytochrome P-450 and that NADPH–cytochrome c reductase, although necessary for directly or indirectly reducing cytochrome P-450, may not be the controlling step in the reduction of azo compounds. An analysis of the ratios (Table 4) further indicates that the inductive effect of phenobarbital may be entirely attributed to the increased formation of cytochrome P-450.

In contrast to phenobarbital, 3-MC, in addition to stimulating azoreductase activity by increasing the formation of cytochrome P-450, stimulated an additional pathway. This interpretation is based on the finding that a significant percentage of the 3-MC-induced activity of azoreductase was unaffected by carbon monoxide, but was sensitive to steapsin solubilization. This interpretation is supported by an analysis of the ratios (Table 6) which indicate that the increased microsomal azoreductase activity is not proportional to the increased cytochrome P-450. That cytochrome P-450 may not be rate limiting in microsomal metabolism is also implied from the studies of Sladek and Mannering[11] who found that 3-MC pretreatment increased cytochrome P-450 levels without increasing the *N*-demethylation of ethylmorphine. Dallner *et al.*[12] also observed that in liver microsomes from developing rats, low detoxication activity could not be explained by rate limitation at the level of the flavoprotein or cytochrome P-450. Sladek and Mannering,[11] from their studies, concluded that 3-MC pretreatment results in the formation of a new hemoprotein.

Other studies[9, 13] indicate that cytochrome P-450 is not required for microsomal metabolism of all drugs since sulfoxidation of diaminodiphenyl sulfide and the *N*-hydroxylation of aniline and *N*-ethylaniline are not inhibited by CO. It therefore seems apparent that the levels of cytochrome P-450 may be markedly increased without a concomitant increase in microsomal activity for some substrates.

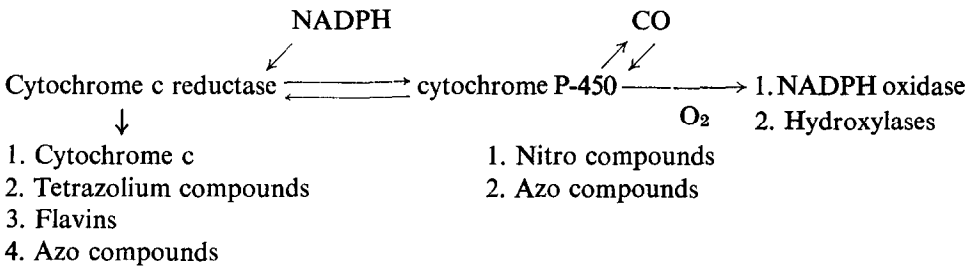
Additional evidence for the above interpretation stems from the finding that although 3-MC pretreatment increases azoreductase activity, it does not stimulate NADPH–cytochrome c reductase (Table 5). Therefore, 3-MC must stimulate azoreductase either by increasing cytochrome P-450 or by some other pathway. Since the increased azoreductase activity is only partially blocked by carbon monoxide (Table 6) there must be an additional pathway or some other mechanism by which 3-MC exerts its effect.

Preliminary studies in this laboratory indicate that purified NADH–cytochrome b_5 reductase can transfer electrons from NADH to neoprontosil in the presence or absence of cytochrome b_5. However, it is unlikely that 3-MC stimulates the reduction of azo dyes by NADH–cytochrome b_5 reductase, either directly or through cytochrome b_5, since neither NADH–cytochrome b_5 reductase nor cytochrome b_5 are induced by 3-MC.[4] In addition, although phenobarbital pretreatment stimulates both cytochrome

b_5 and NADH–cytochrome b_5 reductase,[14, 15] we could directly correlate the effects of phenobarbital with increased cytochrome P-450.

Other studies in this laboratory indicate that when an NADH-generating system is used, the reduction of neoprontosil proceeds at a rate 65 per cent of that observed when an NADPH-generating system is used. The rate of reaction in the presence of NADH was too fast to be attributed to NADPH–cytochrome c reductase, because Strittmatter and Velick[16] showed that NADPH–cytochrome c reductase reduces cytochrome c 20–30 times faster with NADPH than with NADH. Although cytochrome P-450 can be fully reduced by NADH,[17] the transfer of electrons from NADH to the azo compound via P-450 can account for only a fraction of the azoreductase activity observed, since carbon monoxide produces only a slight inhibition of azoreductase activity in the presence of NADH compared to NADPH. The effects of 3-MC on the NADH-dependent pathways are being investigated.

The current findings, coupled with earlier observations, therefore suggest the existence of a number of mechanisms for the reduction of azo compounds by liver microsomes.



Based on the above scheme, azo compounds may be reduced: (1) directly by NADPH–cytochrome c reductase, as indicated by azoreductase activity found by Kamm[18] and by Hernandez *et al.*[1] in a high purified NADPH–cytochrome c reductase preparation; (2) via carbon monoxide-binding pigment (P-450), as indicated in the current and earlier finding[19] that carbon monoxide partially blocks azoreductase activity in microsomes but does not affect NADPH–cytochrome c reductase; (3) via a 3-MC inducible pathway which is insensitive to carbon monoxide but sensitive to solubilization; (4) via other pathways, i.e. NADH-dependent cytochrome b_5 reductase or a NADH pathway not previously described.

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