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Two Sites of Azo Reduction in the Monooxygenase System

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SUMMARY

The mechanism of the azo reduction of sulfonazo III and amaranth by the rat hepatic monooxygenase system was studied. Air strongly inhibited (>95%) the enzymatic reduction of both azo compounds; a 100% CO atmosphere inhibited amaranth reduction (>90%) but only slightly inhibited sulfonazo III reduction (13%). The addition of 50 μM sulfonazo III to microsomal incubations stimulated oxygen consumption, NADPH oxidation, and adrenochrome formation, whereas 100 μM amaranth did not. The reduction potentials of these two azo compounds were also very different (amaranth, $E = -0.620$ V; sulfonazo III, $E = -0.265$ V versus normal hydrogen electrode). The organic mercurial mersalyl converted cytochrome P-450 to cytochrome P-420 (68%) and markedly decreased NADPH-cytochrome P-450(c) reductase activity (97%) in microsomal preparations, presumably by inactivating or destroying functional sulfhydryl groups important for the catalytic activity of these enzymes. GSH was used to restore, and $NADP^+$ to protect, the activities of the monooxygenase components from the effects of mersalyl. The data indicate that inactivation of NADPH-cytochrome P-450(c) reductase inhibits sulfonazo III and amaranth reduction, whereas inactivation of cytochrome P-450 inhibits only amaranth reduction. Furthermore, the reduction of sulfonazo III by purified microsomal NADPH-cytochrome P-450(c) reductase was significantly faster than the rate of reduction of amaranth. These studies demonstrate that two distinct sites of azo reduction exist in the monooxygenase system and that not all azo compounds are reduced by cytochrome P-450.

Azo compounds are important chemicals that are widely used in the pharmaceutical, food, and cosmetic industries. Under anaerobic conditions, the major route of metabolism is reductive cleavage of the azo linkage. In the liver this reaction is catalyzed by NADPH- and NADH-dependent components of the microsomal monooxygenase system (1, 2), although a cytosolic enzyme, presumably DT-diaphorase, can reduce a limited number of azo compounds (3, 4). Three microsomal enzymes catalyze hepatic azo reduction: 1) NADPH-cytochrome P-450(c) reductase (1, 5, 6); 2) a CO-sensitive component, presumably cytochrome P-450 (6, 7); and 3) an undefined CO-insensitive component, which is inducible by 3-methylcholanthrene (6). More recently, cytochrome b_5, and possibly NADH-cytochrome b_5 reductase, has been reported to participate in the reduction of azo compounds (3, 7). The enzyme responsible for azo reduction appears to depend on the substrate. The azo dye sulfonazo III is reduced initially by a one-electron transfer, forming an azo free radical. This reaction is catalyzed by a CO-insensitive component of rat liver microsomes (8), probably NADPH-cytochrome P-450(c) reductase (5). Fujita and Peisach (7, 9, 10) and Mallet *et al.* (11) have suggested that the azo dye amaranth is reduced by cytochrome P-450. Studies in our laboratory suggested that these two azo dyes are reduced by different components of the microsomal electron chain.

In the present study the organic mercurial mersalyl was used to differentially destroy the functional sulfhydryl groups of NADPH-cytochrome P-450(c) reductase and cytochrome P-450 (12, 13). This allowed us to examine the relative participation of these two pathways in the hepatic microsomal reduction of sulfonazo III and amaranth and to suggest that the involvement of cytochrome P-450 and its reductase may depend on the reduction potential of the respective azo substrate. Furthermore, the reduction of these two azo compounds by purified microsomal NADPH cytochrome P-450 reductase was compared.

Methods

Hepatic microsomes were prepared from 160–180-g fed male CD rats (Charles River, Wilmington, MA). The livers were homogenized in 3 volumes of 150 mM KCl/50 mM Tris·HCl, pH 7.4. The homogenates were centrifuged at 10,000 × *g* for 15 min, the pellets were discarded, and the supernatants were centrifuged at 165,000 × *g* for 40 min. The microsomal pellet was resuspended in KCl/Tris·HCl buffer and the protein was determined by the method of Sutherland *et al.* (14).

Oxygen uptake was determined with a Clark electrode (YSI-5331, Yellow Springs Instrument Co., Yellow Springs, OH) in a water-jacketed glass vessel containing 150 mM KCl/50 mM Tris/5 mM $MgCl_2$ (pH 7.4 at 37°). Microsomes were added through an injection port to give a final concentration of 1 mg/ml. The reaction was initiated by the addition of NADPH to give a final concentration of 130 μM, and the rate was measured from the initial slope. When substrates were included, they were added before the microsomes.

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The rate of oxidation of NADPH was determined at 37° in an Aminco DW2 spectrophotometer in the dual-wavelength mode. In all assays, the KCl/Tris·HCl/$MgCl_2$ buffer was warmed to 37° in a 1-cm path length cuvette. A suspension of microsomal protein (1 mg/ml final concentration) and stock solutions of sulfonazo III or amaranth were added. The reaction was initiated by the addition of NADPH to give a final concentration of 130 μM, and the difference in absorbance between 340 and 390 nm was followed for 2 min. The rate of oxidation of NADPH was determined from the initial slope using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

The adrenochrome assay for superoxide was a modification of the method of Misra and Fridovich (15), as previously described (16). NADPH-cytochrome P-450(c) reductase activity was determined from the initial change in absorbance at 550 nm (ϵ = 18.7 mM^{-1} cm^{-1}) in incubations containing horse heart cytochrome *c* (77 μM) from Sigma Chemical Co. (St. Louis, MO) and microsomal protein (20 μg/ml) (17). The reaction was initiated by the addition of NADPH to give a final concentration of 390 μM. Cytochrome P-450 was determined by the method of Omura and Sato (18). Azoreductase activity was determined in an Aminco anaerobic cuvette containing microsomes (1 mg/ml) and amaranth (100 μM) or sulfonazo III (50 μM). Buffer and substrate were deoxygenated by bubbling N_2 or CO through the solution for 5 min. A solution of NADPH (390 μM final concentration) was placed into the plunger assembly. N_2 or CO gas was allowed to flow through the top of the plunger assembly and the top of the cuvette for another minute. The cuvette was sealed and warmed for 1 min at 37°. The reaction was initiated by depressing the plunger. The reactions were monitored kinetically in an Aminco DW2 spectrophotometer in the split beam mode. The loss of amaranth absorbance was determined at 515 nm using ϵ = 4.86 mM^{-1} cm^{-1}; the loss of sulfonazo III absorbance was measured at 573 nm using ϵ = 19.9 mM^{-1} cm^{-1}.

Electrochemical measurements were made with a three-electrode Princeton Applied Research model 170 instrument with platinum working and auxiliary electrodes. The $E_{1/2}$ of amaranth or sulfonazo III (0.5 mM) was measured at 25° versus a saturated calomel reference electrode in KCl/Tris·HCl buffer, pH 7.8, and reported relative to the normal hydrogen electrode. Cyclic voltametric current-potential curves were obtained at a scan rate of 200 mV/sec with a current of 1 mA and were recorded on an X-Y recorder. The phase-sensitive AC voltammograms were measured in phase with the input alternating potential. Scans were performed from 0 to −1.50 V under a nitrogen atmosphere.

NADPH-cytochrome P-450 reductase was purified by the A-25 method of Dignam and Strobel (19). The enzyme was brought to homogeneity by the affinity chromatography method of Yasukochi and Masters (20). This preparation had a specific activity of 33 μM/min/mg of protein at 25°.

Results

Effect of mersalyl on the activity of NADPH-cytochrome P-450(*C*) reductase. Previous studies have shown that organic mercurials, like mersalyl, are potent inhibitors of the microsomal monooxygenase system (12, 13). Mersalyl inhibits microsomal drug biotransformations by converting cytochrome P-450 to cytochrome P-420 (21, 22) and by inactivating essential sulfhydryl groups of NADPH-cytochrome P-450(*C*) reductase (23, 24). The addition of mersalyl to a microsomal incubation resulted in almost complete loss of NADPH-cytochrome *c* reductase activity (Table 1). NADPH-cytochrome P-450(c) reductase in its purified state (24) or in a microsomal preparation (12) can be protected against mercurials by the prior addition of $NADP^+$. In this study, the addition of $NADP^+$ (1.6 mM) to a microsomal preparation before mersalyl treatment protected 69% of the reductase activity against inactivation (Table 1). $NADP^+$ itself did not significantly alter the activity of NADPH-cytochrome P-450(*C*) reductase when reduced by 390 μM NADPH. In agreement with previous studies, the addition of GSH (24) after mersalyl treatment completely restored the ability of the enzyme to reduce cytochrome *c*. GSH did not affect the cytochrome *c* reductase activity in the absence of mersalyl (Table 1).

Effect of mersalyl on the content of cytochrome P-450. As previously shown by others (13, 24), treatment with mersalyl inactivates cytochrome P-450 by converting it to cytochrome P-420 (Table 1). The loss of cytochrome P-450 resulted in a corresponding increase in cytochrome P-420 (data not shown). The addition of $NADP^+$ before mersalyl treatment did not protect the cytochrome from inactivation by the organic mercurial. On the other hand, the addition of GSH at a concentration 20 times greater than that of the mercurial converted about 27% of the cytochrome P-420 to cytochrome P-450 (Table 1). This percentage of recovery agrees with previous data (13).

Effect of $NADP^+$ pretreatment on the mersalyl inhibition of sulfonazo III and amaranth. The anaerobic reduction of sulfonazo III by 1 mg/ml of microsomal protein (Table 2) proceeded at a rate comparable to that of cytochrome *c* reduction (Table 1). Addition of mersalyl to the microsomal incubation resulted in a 97% inhibition of the rate of sulfonazo III reduction. This nearly complete inhibition is similar to that seen with cytochrome *c* reduction (Table 1). The addition of $NADP^+$, before the addition of the organic mercurial, protected 75% of the sulfonazo III reductase activity (Table 2). Again, this agrees with the effect of mersalyl on the $NADP^+$-protected rate of cytochrome *c* reduction (Table 1). As with cytochrome *c* reduction, $NADP^+$ did little to alter the rate of sulfonazo III reduction (Table 2).

The anaerobic reduction rate of amaranth by rat hepatic microsomes is almost 2 orders of magnitude less than that of sulfonazo III (Table 2). The addition of mersalyl completely inhibited the reduction of amaranth (Table 2). In contrast to sulfonazo III, the addition of $NADP^+$ before mersalyl treatment did little to protect against the mersalyl inhibition of microsomal amaranth reduction (Table 2). Again, the addition of $NADP^+$ alone did not interfere significantly with the microsomal reduction of amaranth.

Effect of GSH on mersalyl inhibition of sulfonazo III and amaranth reduction. The addition of GSH completely restores the sulfonazo III reduction activity of mersalyl-treated microsomal protein (Table 3), as was the case with NADPH-cytochrome *c* reductase activity (Table 1). GSH alone had no effect on the reduction of sulfonazo III (Table 3). Mersalyl also completely inhibits amaranth reduction (Table 3). The total loss of this activity occurs despite only a 60% loss in the total cytochrome P-450 content (Table 1). This high degree of inhibition probably reflects the 97% inhibition of NADPH-cytochrome P-450(c) reductase activity (Table 1) because, without the active flavoprotein, NADPH cannot reduce cytochrome P-450. Amaranth reduction is therefore inhibited by mersalyl at two sites. The addition of GSH to mersalyl-treated microsomes restored amaranth reduction to 61% of the rate found with untreated microsomes (Table 3). This result contrasts with the complete restoration of activity found with sulfonazo III and cytochrome *c* reduction after similar treatment. However, the GSH restoration of amaranth reductase activity after organic mercurial inactivation agrees well with the regeneration of cytochrome P-450 by reduced glutathione (Table 1). We suggest

TABLE 1

Effect of NADP+, mersalyl acid, and GSH pretreatment on NADPH-cytochrome P-450(c) reductase activity and cytochrome P-450 content in rat liver microsomes

The rate of reduction of 77 μM cytochrome *c* by 20 μg of microsomal protein per ml was determined after the addition of 390 μM NADPH in a buffer of 150 mM KCl, 50 mM Tris·HCl, and 5 mM $MgCl_2$ (pH 7.4 at 37°). Two minutes after mersalyl addition (80 nmol/mg of protein), cytochrome *c* reductase activity was determined. $NADP^+$ (80 nmol/mg of microsomal protein) was added to the incubation medium 3 min before the addition of the mersalyl. GSH was added 3 min after the mersalyl. Hepatic microsomes (2 mg of protein/ml) were treated with mersalyl at a final concentration of 80 nmol/mg of microsomal protein. The cytochrome concentration was calculated from the difference spectra recorded 8 min after the addition of the mersalyl to the microsomes. $NADP^+$ was added to the microsomes 3 min before the mersalyl. When glutathione was included in the incubation, it was added after the mersalyl and the CO difference spectra were recorded after an additional 5 min wait. Results are expressed as mean ± standard error of triplicate determinations. The data presented are from one experiment, which was typical of three different experiments.

$NADP^+$ (80 nmol / mg of protein)	Mersalyl acid (80 mol / mg of protein)	GSH (0.5 mM)	NADPH-Cytochrome P-450(c) Reductase		Cytochrome P-450	
			nmol / min-mg of protein	% control	nmol / mg of protein	% control
−	−	−	220 ± 11	100	0.93 ± 0.02	100
−	+	−	6 ± 1	3	0.30 ± 0.03	32
+	−	−	215 ± 12	98	0.90 ± 0.02	97
+	+	−	152 ± 10	69	0.35 ± 0.04	38
−	−	+	227 ± 12	103	0.96 ± 0.02	103
−	+	+	216 ± 10	98	0.55 ± 0.02	59

TABLE 2

Effect of $NADP^+$ pretreatment on the inhibition of microsomal sulfonazo III and amaranth reduction by mersalyl acid

Results are expressed as mean ± standard error of triplicate determinations. The data presented are from one experiment, which was typical of three different experiments.

$NADP^+$ (80 nmol / mg of protein)	Mersalyl acid (80 nmol / mg of protein)	Sulfonazo III reduction		Amaranth reduction	
		nmol / min-mg of protein	% control	nmol / min-mg of protein	% control
−	−	214 ± 8	100	2.82 ± 0.07	100
−	+	6.4 ± 0.4	3	0	0
+	+	160 ± 6	75	0.37 ± 0.01	13
+	−	201 ± 10	94	2.74 ± 0.06	97

TABLE 3

Effect of GSH on the inhibition of microsomal sulfonazo III and amaranth reduction by mersalyl acid

Results are expressed as mean ± standard error of triplicate determinations. The data presented are from one experiment, which was typical of three different experiments.

GSH (0.5 mM)	Mersalyl acid (80 nmol / mg of protein)	Sulfonazo III reduction		Amaranth reduction	
		nmol / min-mg of protein	% control	nmol / min-mg of protein	% control
−	−	214 ± 8	100	3.8 ± 0.1	100
−	+	6.4 ± 0.4	3	0	0
+	+	212 ± 1	99	2.3 ± 0.1	61
+	−	216 ± 12	101	4.3 ± 0.1	113

TABLE 4

Effect of different atmospheric conditions on the microsomal reduction of sulfonazo III and amaranth

Results are expressed as mean ± standard error of triplicate determinations. The data presented are from one experiment, which was typical of three different experiments.

Atmosphere	Sulfonazo III reduction	Amaranth reduction
	nmol / min-mg of protein	
N_2	216 ± 9	2.4 ± 0.1
Air	6.3 ± 0.2	0.06 ± 0.01
CO	189 ± 6	0.23 ± 0.1
N_2 (heat-inactivated microsomes)	6.3 ± 0.2	0.06 ± 0.01
N_2 (no microsomes)[a]	5.2 ± 0.1[b]	0.06 ± 0.01

[a] Microsomes were replaced by buffer.
[b] NADPH alone slowly reduces sulfonazo III (16).

that microsomal reduction of amaranth is primarily a cytochrome P-450-mediated reaction that is stimulated slightly by GSH (Table 3). Of course, the NADPH-cytochrome P-450(c) reductase must also be restored in order to reduce the cytochrome P-450.

Effect of different atmospheric conditions on the reduction of sulfonazo III and amaranth. The microsomal reduction of both sulfonazo III and amaranth was inhibited almost completely in air (Table 4). In agreement with Fujita and Peisach (9), carbon monoxide inhibits the microsomal amaranth reduction by about 90%, suggesting the involvement of cytochrome P-450 (Table 4). In contrast, sulfonazo III reduction is inhibited only 13% by a 100% CO atmosphere (Table 4). This small effect of CO suggests that a cytochrome P-450-dependent pathway is not the major route of sulfonazo III reduction. Other than the effect of CO, the relative rate of reduction is the most notable difference between microsomal amaranth and sulfonazo III reduction and probably reflects the generally much smaller turnover number of cytochrome P-450 relative to NADPH-cytochrome P-450(c) reductase.

Reduction of sulfonazo III and amaranth by purified microsomal NADPH-cytochrome P-450 reductase. If sul-

fonazo III is reduced primarily by NADPH-cytochrome P-450 reductase and amaranth is reduced by cytochrome P-450, then there should be a dramatic difference in the rates of reduction of these two compounds by purified reductase. As seen in Table 5, the rate of enzymatic reduction of sulfonazo III by purified NADPH-cytochrome P-450 reductase was almost 48 times faster than the rate of reduction of amaranth under similar conditions.

Effect of amaranth and sulfonazo III on oxygen consumption, NADPH oxidation, and superoxide formation. Flavoproteins, especially NADPH-cytochrome P-450(c) reductase, transfer a single electron to many different types of aromatic compounds such as nitroaromatic drugs (25, 26) to form anion free radicals of the parent drugs; the anion radical then reduces oxygen to form superoxide. Such a reaction sequence stimulates oxygen consumption, superoxide formation, and the oxidation of NADPH. Addition of 50 μM sulfonazo III to a microsomal incubation resulted in a 10-fold increase in the rates of NADPH-supported oxygen consumption, superoxide formation, and NADPH oxidation (Table 6). These data clearly show that the rapid rate of sulfonazo III reduction observed under nitrogen is not merely competitively inhibited by oxygen but that reduction occurs through an anion radical intermediate (8), which is air-oxidized, resulting in a futile redox cycle (16).

In contrast, the addition of 100 μM amaranth to microsomal incubations did not stimulate the NADPH-supported uptake of oxygen, superoxide formation, or the oxidation of NADPH (Table 6). These results are consistent with oxygen being an alternate electron acceptor for cytochrome P-450 (7) rather than inhibiting amaranth reduction through a futile cycle.

Reduction potentials of amaranth and sulfonazo III. The ease with which a substrate can be reduced by NADPH-cytochrome P-450(c) reductase or cytochrome P-450 is expected to be a reflection of their reduction potentials. Studies with nitroaromatic drugs have shown that compounds with higher (less negative) reduction potentials are reduced more readily by xanthine oxidase or reduced flavins (27). This would also appear to be the case with sulfonazo III ($E^1_{/2}$ = −0.265 V) and amaranth ($E^1_{/2}$ = −0.620 V). Although the voltammograms do not necessarily measure the thermodynamic redox potential, the 0.355 V difference is highly significant. In line with these reduction potentials, the rate of reduction of sulfonazo III is almost 2 orders of magnitude faster than that of amaranth (Table 4).

Repetitive scanning of the reduction of sulfonazo III. We have demonstrated with ESR that sulfonazo III is reduced to an azo free radical in anaerobic microsomal incubations containing NADPH (8). In air, sulfonazo III is reduced nonenzymatically via a two-electron pathway by high concentrations of NADPH (16), as indicated by the loss of absorbance at 575 nm (Fig. 1A). When microsomal incubations were made anaerobic, the reduction of sulfonazo III occurred enzymatically, and the transient azo free radical was observed at 689 nm (Fig. 1B). The absolute optical spectrum of the sulfonazo III radical is shown in Fig. 2. Spectrophotometric pulse radiolysis studies demonstrated that the azobenzene free radical has a similar absorption band in the 550–600 nm region (28). In agreement with Fujita and Peisach (29), repetitive scanning of microsomal incubations containing amaranth did not produce a corresponding transient species. Similar anaerobic microsomal incubations gave no ESR spectra that could be attributed definitively

TABLE 5

Reduction of sulfonazo III and amaranth by purified microsomal NADPH-cytochrome P-450(c) reductase from untreated male rats

The reduction was determined in a stoppered cuvette. The incubation mixture consisted of 3 ml of KCl/Tris/$MgCl_2$ (150 mM/50 mM/5 mM; pH 7.4 at 37°), substrate (50 μM), and NADPH-cytochrome P-450 reductase (5 μg/ml). The solution was flushed with nitrogen. The reaction was initiated by the addition of 100 μM NADPH. Values are the average of four incubations ± standard error.

Substrate	Reduction	Ratio
	nmol / min-mg of protein	
Sulfonazo III	15.4 ± 1.3	47.7
Amaranth	0.323 ± 0.010	

TABLE 6

Effect of amaranth and sulfonazo III on NADPH oxidation, oxygen uptake, and superoxide formation by rat hepatic microsomes

Results are expressed as mean ± standard error of triplicate determinations. The data presented are from one experiment, which was typical of three different experiments.

Sulfonazo III (50 μM)	Amaranth (100 μM)	NADPH oxidation	Oxygen uptake	Superoxide formation
		nmol of NADPH / min-mg of protein	nmol of oxygen / min-mg of protein	nmol of adrenochrome / min-mg of protein
−	−	8.3 ± 0.2	12.1 ± 0.2	2.6 ± 0.2
+	−	89.2 ± 0.8	122.6 ± 1.2	24.1 ± 0.9
−	+	8.6 ± 0.2	13.2 ± 0.5	2.8 ± 0.4

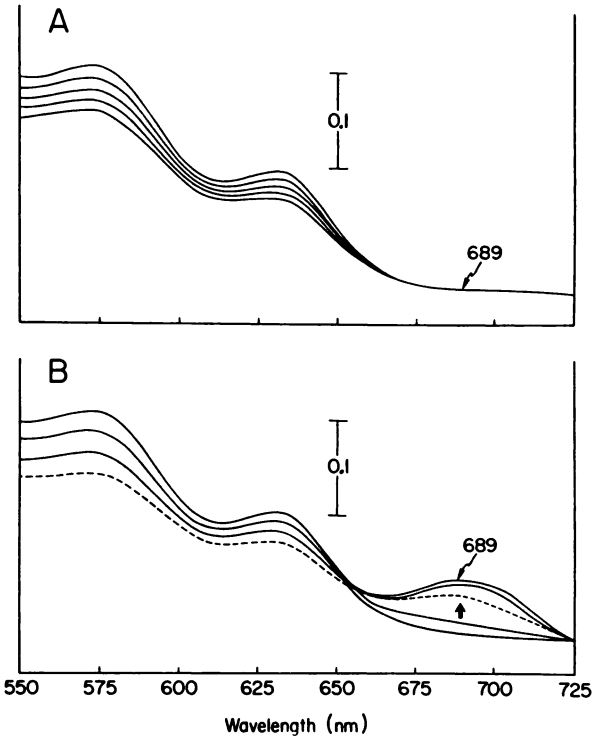

Fig. 1. Repetitive scanning of sulfonazo III disappearance in rat hepatic microsomal incubations under aerobic (A) and anaerobic (B) conditions. In A, the sample and reference cuvette contained 50 μM sulfonazo III and 100 μg/ml of microsomal protein in 150 mM KCl/50 mM Tris/5 mM $MgCl_2$ buffer (pH 7.4 at 37°). The sample cuvette contained 8.0 mM NADPH, whereas the reference cuvette did not contain any NADPH. In B, the contents of each cuvette were the same as in A, except the sample cuvette contained 0.4 mM NADPH while the reference cuvette did not contain any NADPH. The scanning speed was 50 nm/sec. The *dashed line* indicates the final scan in the figure and shows that the radical (absorbance maximum at 689 nm) had already reached its maximum or steady state concentration and had started to decay.

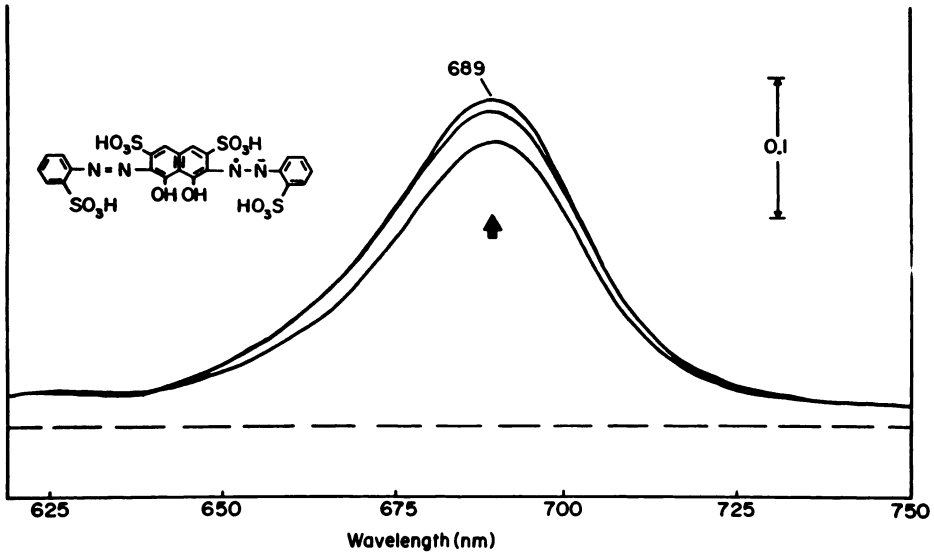

Fig. 2. The visible absolute spectrum of the sulfonazo III free radical. The sample cuvette was anaerobic and contained microsomal protein (100 μg/ml), whereas the reference cuvette was aerobic and contained 20 times as much NADPH (8.0 mM versus 0.4 mM). Both cuvettes contained 50 μM sulfonazo III and 150 mM KCl/50 mM Tris/5 mM $MgCl_2$ buffer (pH 7.4 at 37°). The reactions were started by the simultaneous addition of NADPH and scanned at 50 nm/sec. In order to obtain this spectrum, the rate of sulfonazo III disappearance at 631 nm (a secondary peak) was equalized in the sample and reference cuvettes by adjusting the microsomal protein concentration. This eliminated absorbance changes due to sulfonazo III disappearance and allowed the absolute spectrum of the radical to be obtained.

to an azo free radical metabolite of amaranth. This negative result cannot rule out the possibility that the amaranth free radical is formed, because the concentration of the radical may be below the sensitivity of the spectrometer.

Square root dependence of radical concentration. In the absence of other electron acceptors or donors, anion radicals and their conjugate acids usually decay by disproportionation, which is a second-order process. Kinetic considerations require that when a chemical species disappears by a second-order process, its steady state concentration will be proportional to the square root of its rate of formation. If the rate of formation is linear in enzyme concentration, then the steady state radical concentration will be proportional to the square root of the enzyme concentration (26). As shown in fig. 3, this is the case for the sulfonazo III free radical, whether the steady state concentration of the radical is monitored by visible (Fig. 3A) or ESR spectroscopy (Fig. 3B). This square root relationship suggests that the transient species at 689 nm is, in fact, the azo free radical. The pH dependence of the optical spectrum of the related azobenzene anion free radical indicates that the radical observed at pH 7.4 is the protonated anion free radical (28).

$$C_6H_5\dot{N}HNC_6H_5 \xrightarrow{pK\ 13.7} C_6H_5\bar{N}\dot{N}C_6H_5 + H^+$$

The disproportionation of this hydrazyl-type free radical would form the parent azo compound and the corresponding hydrazine.

Discussion

Azo compounds are reductively cleaved by a variety of biological systems (16, 26, 29, 30); the hepatic microsomal enzyme system is one of the more active (31). It has been suspected for a long time that the hepatic microsomal metabolism of azo compounds involved at least two different known pathways, one depending primarily on cytochrome P-450 and the other utilizing a flavoprotein to transfer reducing equivalents to the parent compound (5, 6). In this study we have unambiguously demonstrated the existence of both pathways by selectively inhibiting functional sulfhydryl groups of the microsomal electron transport chain, which are necessary for the reduction of the azo substrates amaranth and sulfonazo III.

In agreement with earlier studies (7, 9, 11), the microsomal reduction of amaranth is mediated primarily by cytochrome P-450. Although an absence of air inhibition of this azo reduction has been reported (32), we have found that the microsomal reduction of amaranth is strongly inhibited by air, in agreement with the reports of Fujita and Peisach (7, 29). On the other hand, sulfonazo III is reduced primarily by the flavoprotein NADPH-cytochrome P-450(c) reductase. Because the reduction rate of azo compounds by unbound flavins (30, 33) is proportional to the reduction potential of the azo compound (34), reduction by the hepatic microsomal enzyme system may follow a similar pattern. Furthermore, it is possible that azo compounds with high reduction potentials, such as sulfonazo III, will be reduced primarily by the flavoprotein NADPH-cytochrome P-450(c) reductase, whereas other azo substrates, whose redox potentials are more negative, will be reduced by cytochrome P-450, but certainly, then, binding of the azo substrates to cytochrome P-450 must also play a role. The reduction of amaranth by purified NADPH-cytochrome P-450(c) reductase but not by microsomal NADPH-cytochrome P-450(c) reductase is surprising and may be due to the presence of an alternative electron acceptor in microsomes, cytochrome P-450.

Neoprontosil was used extensively in earlier studies on the mechanism of microsomal azo reduction. Hernandez *et al.* (5, 6) showed that the enzyme system responsible for the reduction of this drug in rat liver preparations copurified with the flavoprotein NADPH-cytochrome P-450(c) reductase. This azoreductase activity was CO-insensitive and inhibited strongly by oxygen. This enzyme was not entirely responsible for all the azoreductase activity, inasmuch as a CO atmosphere or the treatment of microsomal preparations with steapsin decreased the microsomal azoreductase activity. In addition, a good correlation existed between phenobarbital-induced, CO-sensitive neoprotonsil reductase activity and total cytochrome P-450 content (6) as has been demonstrated for amaranth reductase activity (9). It is unclear whether the reduction potential of neoprotonsil or some other property of its chemical structure allows reduction by both cytochrome P-450 and its reductase. In contrast, sulfonazo III and amaranth are reduced by only one of these enzymes.

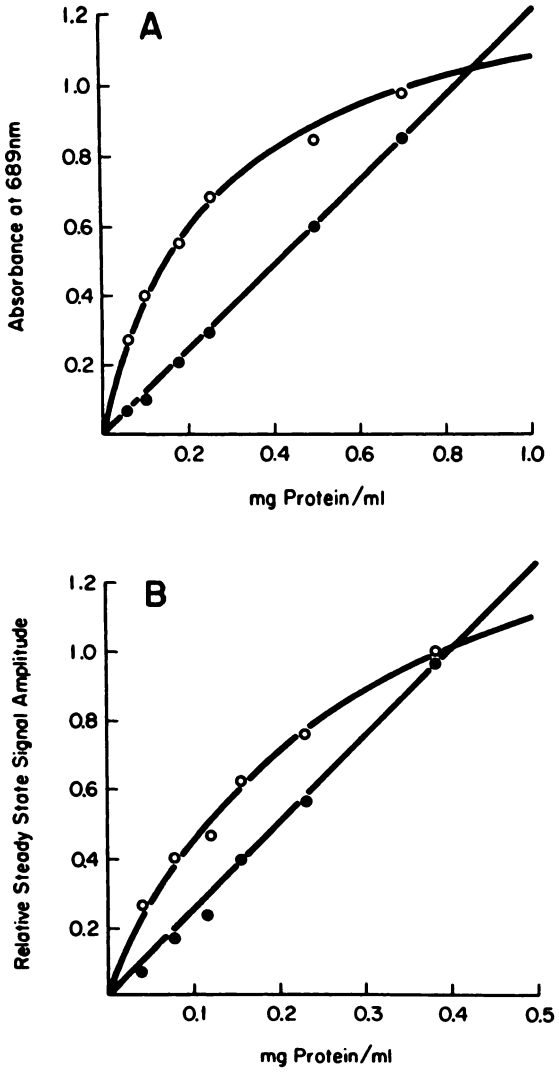

Fig. 3. The relative steady state concentration of the sulfonazo III anion radical (○) and the square of the relative steady state concentration (●) versus the microsomal protein concentration of rat hepatic microsomes. A, The relative steady state radical concentrations were determined by visible spectroscopy at 37° with an Aminco-Chance DW2 spectrophotometer in the split beam mode. Anaerobic incubations contained 50 μM sulfonazo III, an NADPH-generating system, and various concentrations of microsomal protein. The reaction was initiated by depressing the plunger assembly of an anaerobic cuvette which contained a buffer solution of 1 mg of NADPH. The steady state radical concentration was determined at 689 nm. B, Steady state concentrations were determined by ESR spectroscopy at 25°. The anaerobic reaction was started by adding 1 mg of NADPH to 3 ml of a solution containing 500 μM sulfonazo III, 5.5 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and various concentrations of microsomal protein. The solution was then aspirated into the ESR cavity and the steady state radical concentration was determined by repetitive scans every 30 sec. The ESR instrumental conditions were: nominal microwave power, 5 mW; and modulation amplitude, 20 G.

The hepatic microsomal azoreductase system has been shown to be remarkably nonspecific (16, 30). The reduction potential of the substrate may be the primary factor in determining both the rate and site of azo reduction. In conclusion, our data indicate that no azo compound is a "typical" azo substrate for rat hepatic microsomal reduction and that the dominant role of cytochrome P-450 in amaranth reduction cannot be generalized to all azo compounds, as has previously been suggested (7, 9, 10, 29).

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References

1. Mueller, G. C., and J. A. Miller. The reduction cleavage of 4-dimethylaminoazobenzene by rat liver: the intracellular distribution of the enzyme system and its requirement for triphosphopyridine nucleotide. *J. Biol. Chem.* **180**:1125–1136 (1949).
2. Fouts, J. R., J. J. Kamm, and B. B. Brodie. Enzymatic reduction of prontosil and other azo dyes. *J. Pharmacol. Exp. Ther.* **120**:291–300 (1957).
3. Autrup, H., and G. P. Warwick. Some characteristics of two azoreductase systems in rat liver: relevance to the activity of 2-[4′-di(2″-bromopropyl)-aminophenylazo] benzoic acid (CB10-252), a compound possessing latent cytotoxic activity. *Chem. Biol. Interact.* **11**:329–342 (1975).
4. Huang, M.-T., G. T. Miwa, N. Cronhein, and A. Y. H. Lu. Rat liver cytosolic azoreductase. *J. Biol. Chem.* **254**:11223–11227 (1979).
5. Hernandez, P. H., J. R. Gillette, and P. Mazel. Studies on the mechanism of action of mammalian hepatic azoreductase. I. *Biochem. Pharmacol.* **16**:1859–1875 (1967).
6. Hernandez, P. H., P. Mazel, and J. R. Gillette. Studies on the mechanism of action of mammalian hepatic azoreductase. II. *Biochem. Pharmacol.* **16**:1877–1888 (1967).
7. Fujita, S., and J. Peisach. Electron transfer between liver microsomal cytochrome b_5 and cytochrome P-450 in the azo reductase reaction. *Biochem. Biophys. Res. Commun.* **78**:328–335 (1977).
8. Mason, R. P., F. J. Peterson, and J. L. Holtzman. The formation of an azo anion free radical metabolite during the microsomal azo reduction of sulfonazo. III. *Biochem. Biophys. Res. Commun.* **75**:532–540 (1977).
9. Fujita, S., and J. Peisach. Liver microsomal cytochromes P-450 and azoreductase activity. *J. Biol. Chem.* **253**:4512–4513 (1978).
10. Fujita, S., Y. Okada, and J. Peisach. Inhibition of azoreductase activity by antibodies against cytochromes P-450 and P-448. *Biochem. Biophys. Res. Commun.* **102**:492–498 (1981).
11. Mallett, A. K., L. J. King, and R. Walker. Solubilisation, purification and reconstitution of hepatic microsomal azoreductase activity. *Biochem. Pharmacol.* **34**:337–342 (1985).
12. Franklin, M. R., and R. W. Estabrook. On the inhibitory action of mersalyl on microsomal drug oxidation: a rigid organization of the electron transport chain. *Arch. Biochem. Biophys.* **143**:318–329 (1971).
13. Franklin, M. R. The incomplete conversion of hepatic cytochrome P-450 to P-420 by mercurials. *Mol. Pharmacol.* **8**:711–721 (1972).
14. Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olsen. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. *J. Biol. Chem.* **180**:825–837 (1949).
15. Misra, H. P., and I. Fridovich. The generation of superoxide radical during the autoxidation of ferredoxins. *J. Biol. Chem.* **246**:6886–6890 (1971).
16. Mason, R. P., F. J. Peterson, and J. L. Holtzman. Inhibition of azoreductase by oxygen. *Mol. Pharmacol.* **14**:665–671 (1978).
17. Phillips, A. H., and R. G. Langdon. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *J. Biol. Chem.* **237**:2652–2660 (1962).
18. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
19. Dignam, J. D., and H. W. Strobel. NADPH-cytochrome P-450 reductase from rat liver: purification by affinity chromatography and characterization. *Biochemistry* **16**:1116–1123 (1977).
20. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
21. Netter, K. J., and S. Jenner. Über die Rolle von Sulfhydrylgruppen bei der mikrosomalen Arzneimitteldemethylierung. *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* **255**:120–132 (1966).
22. Orrenius, S. On the mechanism of drug hydroxylation in rat liver microsomes. *J. Cell Biol.* **26**:713–723 (1965).
23. Murakami, K., and H. S. Mason. An electron spin resonance study of microsomal Fex *J. Biol. Chem.* **242**:1102–1110 (1967).
24. Williams, C. H., Jr., and H. Kamin. Microsomal triphosphorpyridine nucleotide-cytochrome *c* reductase of liver. *J. Biol. Chem.* **237**:587–595 (1962).
25. Peterson, F. J., and J. L. Holtzman. Drug metabolism in the liver: a perspective. In: *Extrahepatic Metabolism of Drugs and Other Foreign Compounds* (T. Gram, ed.). Spectrum Publishing, New York, 1–107 (1980).
26. Mason, R. P. Free radical intermediates in the metabolism of toxic chemicals, in *Free Radicals in Biology* (W. A. Pryor, ed.), Vol. V. Academic Press, New York, 161–222 (1982).
27. Clarke, E. D., P. Wardman, and K. H. Goulding. Anaerobic reduction of nitroimidazoles by reduced flavin mononucleotide and by xanthine oxidase. *Biochem. Pharmacol.* **29**:2684–2687 (1980).

28. Neta, P., and H. Levanon. Spectrophotometric study of the radicals produced by the reduction of *syn*- and *anti*-azobenzene. *J. Phys. Chem.* **81**:2288–2292 (1977).
29. Fujita, S., and J. Peisach. The stimulation of microsomal azoreduction by flavins. *Biochim. Biophys. Acta* **719**:178–189 (1982).
30. Gillette, J. In *Handbook of Experimental Pharmacology Concepts in Biochemical Pharmacology* (B. B. Brodie and J. R. Gillette, eds.), Part 2. Springer-Verlag, New York, 349–361 (1971).
31. Walker, R. The metabolism of azo compounds: A review of the literature. *Food Cosmet. Toxicol.* **8**:659–676 (1970).
32. Martin, C. N., and J. C. Kennelly. Rat liver microsomal azoreductase activity of four azo dyes derived from benzidine, 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine. *Carcinogenesis (Lond.)* **2**:307–312 (1981).
33. Gingell, R., and R. Walker. Mechanisms of azoreduction by *Streptococcus faecalis*. II. The role of soluble flavins. *Xenobiotica* **1**:231–239 (1971).
34. Dubin, P., and K. L. Wright. Reduction of azo food dyes in cultures of *Proteus vulgaris*. *Xenobiotica* **5**:563–571 (1975).

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