Biochemical Pharmacology, Vol. 34, No. 18, pp. 3259–3264, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

STUDIES ON MICROSOMAL AZOREDUCTION

N,N-DIMETHYL-4-AMINOAZOBENZENE (DAB) AND ITS DERIVATIVES

WALTER G. LEVINE

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

(*Received* 24 *September* 1984; *accepted* 30 *January* 1985)

Abstract—The azoreduction of *N,N*-dimethyl-4-aminoazobenzene (DAB) and *N*-methyl-4-aminoazobenzene (MAB) by rat liver microsomes was investigated. It was shown that measurement of azoreduction of DAB and structurally related azo dyes by the conventional method of substrate disappearance required an anaerobic environment since N-demethylated and ring-hydroxylated metabolites formed aerobically interfered with the assay system, producing quantitatively inaccurate results. Oxygen partially, but not totally, inhibited azoreduction of DAB. Glutathione (GSH) inhibited the azoreduction of DAB but stimulated the azoreduction of MAB. Dithiothreitol also stimulated azoreduction of MAB but had little effect on azoreduction of DAB. Para-hydroxymercuribenzoate (PHMB) and *N*-ethylmaleimide (NEM) blocked titratable microsomal thiol groups and inhibited azoreduction of MAB. However, the inhibitory action of NEM was weak with DAB azoreduction although PHMB was a potent inhibitor. These findings suggest that microsomal azoreduction of DAB and MAB may proceed via different mechanisms, possibly through different species of cytochrome P-450 which have selective dependence upon the sulfhydryl environment.

Reduction of azo linkage in drugs and other foreign chemicals has been known for many years. Within the liver, several sources of azoreductase have been reported. Early studies by Hernandez *et al.* [1, 2] indicated that azoreductase activity in microsomes is partially CO-insensitive and they showed that reduction of neoprontosil and neotetrazolium is catalyzed by NADPH-cytochrome *c* reductase purified from rat liver microsomes. In their purification procedure, half the azoreductase activity was lost during the initial microsomal solubilization in steapsin, although NADPH-cytochrome *c* reductase activity was retained. Furthermore, azoreduction was inhibited by carbon monoxide prior to, but not after, solubilization. This implied that azoreductase activity was also associated with cytochrome P-450 which would be destroyed by their method of solubilization. Fujita and Peisach [3, 4] subsequently showed that reduction of amaranth by rat liver microsomes is almost completely inhibited by carbon monoxide and is therefore attributable exclusively to cytochrome P-450. Antibodies against phenobarbital-induced cytochrome P-450 and against methylcholanthrene-induced cytochrome P-448 almost totally inhibit amaranth reduction by microsomes from phenobarbital- and methylcholanthrene-induced mice respectively [5]. Obviously, substrate selectivity is a major factor in considering mechanisms for microsomal azoreductases. Microsomal reduction of either neoprontosil or amaranth is accelerated by added FMN or FAD but the stimulation is unaffected by carbon monoxide, suggesting that the site of interaction may be NADPH-cytochrome *c* reductase rather than cytochrome P-450 [4, 6].

A rather different type of azoreductase activity is found in rat liver cytosol [7]. The soluble enzyme is induced by 3-methylcholanthrene, as is microsomal azoreductase, exhibits far more limited substrate specificity, is inhibited by dicumarol and is insensitive to carbon monoxide. Its few known substrates include methyl red [7], resorufin [8] and a number of quinones but not *N,N*-dimethyl-4-aminoazobenzene (DAB). It has been shown to be identical to DT-diaphorase [7].

Reduction of DAB by rat liver has been known for nearly 40 years [9]. Early studies [10] showed a need for NADPH. In view of current knowledge, this implied a role for cytochrome P-450, although the hemoprotein was unknown at the time. Matsumato and Terayama [11] observed differences in the rate of reduction of DAB and its mono- and didemethylated metabolites, *N*-methyl-4-aminoazobenzene (MAB) and 4-aminoazobenzene (AB). Reduction of DAB is induced in mice by 3-methylcholanthrene and phenobarbital [5] and in rats by phenobarbital [12].

A problem in studying azoreductase is that inhibition by oxygen is seen for some but not all substrates [13]. Hernandez *et al.* [1] speculated that oxygen sensitivity may be attributable to reoxidation of the hydrazo intermediate. In the case of sulfonazo III, Mason *et al.* [14] showed that the initial one electron reduction product, a free radical, is rapidly reoxidized by oxygen. Thus, during the reaction, there is no apparent loss of substrate despite considerable oxygen utilization and conversion to superoxide [14]. Reduction of DAB has been studied under aerobic [9, 15–18] and anaerobic [15, 18, 19] conditions. Some investigators report lack of inhibition by oxygen [15, 18]. This may imply that DAB reduction does not proceed via a free radical, as does sulfonazo III or that the free radical is not readily

reoxidized by oxygen. Carbon monoxide may not inhibit DAB reduction while reports on induction by phenobarbital and 3-methylcholanthrene are conflicting [15–17].

It is apparent that the mechanism of DAB reduction by hepatic microsomes requires further investigation. In contrast to the abundance of knowledge concerning oxidative pathways, relatively little is known of azoreduction mechanisms. The present study deals with problems of DAB reduction in relation to its oxidative pathways of metabolism, N-demethylation and ring-hydroxylation. Studies in this laboratory have indicated that these pathways are selectively catalyzed by isozymes of cytochrome P-450 based on induction and inhibition of activity [20] and catalysis by purified reconstituted cytochrome P-450 systems [21]. Regulation of these activities by thiols such as glutathione (GSH) and dithiothreitol (DTT) has been observed [22], and the question has been raised as to the physiologic role of GSH in this regard. Such considerations have now been applied to azoreduction of DAB and its monodemethylated derivative, MAB. This study considers the effect of thiols, such as GSH and DTT, as well as microsomal SH groups in the reduction of these azo dyes. Also considered are technical problems in the measurement of azo dye reduction in aerobic versus anaerobic environment in view of the multiple pathways catalyzed by rat liver microsomes.

METHODS

Animals. Male Wistar rats, 180–240 g, were housed under conditions of controlled lighting (12 hr on, 12 hr off) and given free access to food and water. Rats were decapitated between 9:00 a.m. and 11:00 a.m. Livers were removed and perfused with ice-cold 0.15 M KCl and 10 mM Tris buffer, pH 7.4. They were homogenized in 3 vol. of the same medium and centrifuged for 10 min at 10,000 *g*. Microsomes were prepared by centrifuging the 10,000 *g* supernatant fraction for 1 hr at 100,000 *g*. The pellet was washed twice and suspended in the same medium so that 1 ml contained the microsomes from 1 g of liver. If immediately frozen and stored in liquid nitrogen (77 K), enzymic activity and response to GSH were maintained for at least 6 weeks.

Chemicals. The following sources were used: DAB, Pfaltz & Bauer (Stamford, CT); AB, Eastman Kodak (Rochester, NY); and $NADP^+$, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, GSH, DTT, Sigma Chemical Co. (St. Louis, MO). ^{14}C-Labeled DAB was purchased from the New England Nuclear Corp. (Boston, MA) and was shown to be 96–98% radiochemically pure by thin-layer chromatography. MAB was a gift of Dr. Fred Kadlubar, National Center for Toxicological Research. Other chemicals were of the highest purity commercially available.

DAB and MAB reduction. The reaction mixture was identical to that previously described for aerobic reactions [23], except that 0.1 mM NADH was added. One ml of incubation medium contained 50 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, 10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.5 mM NADP, 0.1 mM NADH, 1 to 1.5 mg microsomal protein, and 0.1 mM DAB or MAB. Other reactants were added as indicated below. All reactants except microsomes were added to the main chamber of a Thunberg tube and the microsomes were placed in the side arm. Both sides were then closed with a septum rubber stopper, and the tube was gassed for 10 min with argon unless otherwise indicated. The microsomes were then tipped into the main compartment, and the tube was incubated at 37° for 10 min. The reaction was stopped by the addition of 1 ml of 20% trichloroacetic acid in acetone–ethanol–water (1:1:2). The mixture was centrifuged, and the absorbance of the dye in the supernatant material was determined at the appropriate wavelength (see Table 2). Incubations were performed in triplicate. All experiments were repeated one to three times. The experiments for Table 3 involved gassing with nitrogen. No quantitative or qualitative differences were detectable when comparing experiments performed in argon versus nitrogen atmospheres.

Aerobic metabolism of DAB. This was performed in air using ^{14}C-labeled DAB in the same reaction mixture described for reduction experiments. After a 10-min incubation, [^{14}C]DAB and its metabolites were extracted into 5 ml of ether. After evaporation, the residue was dissolved in 40 μl of acetone and subjected to thin-layer chromatography on Gelman ITLC-SA sheets using methanol-ethyl acetate-toluene (1:1:12) as solvent. After development, the chromatogram was sprayed with 0.1 N HCl to visualize the azo dyes and 0.025% dimethylaminocinnamaldehyde in ethanol to visualize the primary amine reduction products. The relative migration of each of the compounds is indicated in Table 1. Spots corresponding to cochromatographed reference standards were cut out and counted in a liquid scintillation spectrometer.

Titration of microsomal SH groups. 5,5'-Dithiobis(nitrobenzoic acid) (DTNB, Ellman's reagent) reacts non-specifically with thiol groups to yield

Table 1. Separation of DAB and its oxidized and reduced metabolites by thin-layer chromatography*

Compound	Apparent R_f†
DAB	0.86
MAB	0.78
AB	0.65
4'-OH-DAB	0.50
4'-OH-MAB	0.44
4'-OH-AB	0.35
N,N-Dimethyl-*p*-phenylenediamine	0.30
N-Methyl-*p*-phenylenediamine	0.25
p-Phenylenediamine	0.17

* Extracted compounds were spotted on Gelman ITLC-SA sheets which were developed with methanol–ethyl acetate–toluene (1:1:12), dried, and sprayed with 0.1 N HCl followed by 0.025% 4-dimethylaminocinnamaldehyde in ethanol.

† If care is taken not to overload the chromatogram, clean separation of each compound is obtained.

nitromercaptobenzoate which has an intense absorption maximum at 412 nm [24]. To determine total "available" SH groups, microsomes were incubated at room temperature with 1 mM DTNB in 0.1 M Tris buffer, pH 8.2, and the increase in absorption was monitored at 412 nm in a Gilford recording spectrophotometer.

RESULTS

It was first determined whether measurement of microsomal azoreduction of DAB was valid under aerobic conditions. DAB azoreduction is usually measured by the decrease in absorbance of the protonated form of the dye at 520 nm, the absorption maximum for DAB [9, 15, 19, 25]. In previous aerobic studies [9, 15–18], however, no consideration was made for the possible change in absorbance due to conversion to the N-demethylated and ring-hydroxylated metabolites (MAB, AB, 4′-OH-DAB) which form aerobically. To test the possible influence of these metabolites on the azoreduction assay, the absorbance of each was measured in 10% trichloroacetic acid in acetone–ethanol–water (1:1:4), concentrations which would be present during analysis of microsome-catalyzed dye disappearance (see Methods). As seen in Table 2, the absorbance of DAB and of its aerobic metabolites were quite different at 520 nm. This would lead to quantitatively inaccurate results if DAB reduction were measured aerobically in the conventional manner. In support of this, DAB metabolism was performed aerobically following which extracted substrate and metabolites, oxidized and reduced, were subjected to thin-layer chromatography using a system which separates each of these compounds (Table 1). The results are indicated in Table 3. Values for the three reduced products of DAB, MAB and AB (*N*,*N*-dimethyl-*p*-phenylenediamine, *N*-methyl-*p*-phenylenediamine and *p*-phenylenediamine respectively) have been combined since each azo dye would be present during the reaction and thus subject to reduction. The combined figure thus reflects total azoreduction. It is apparent that azoreduction was depressed in the presence of oxygen but not completely. Thus, 100% oxygen inhibited the formation of reduced products by only 50% relative to the anaerobic system. It is unlikely that this inhibition is attributable simply to shunting of the substrate (DAB) to oxidative pathways since far less DAB was consumed anaerobically than aerobically. Were "shunting" a valid explanation, substrate consumption should have been approximately the same, aerobically and anaerobically. It is also apparent from Table 3 that, as expected, considerable amounts of N-demethylated (MAB and AB) and ring-hydroxylated (4′-OH-DAB) products form aerobically. Reference to Table 2 reveals that formation of these products would itself give an erroneous impression as to loss of substrate in view of the change in A_{520}. Thus, using the commonly applied substrate disappearance method in an aerobic system of azoreduction, the calculated rate would be the algebraic sum of true loss of substrate plus formation of oxidized products. Since each of the oxidized products has a lower absorbance at 520 nm than does DAB, an erroneously high rate of substrate loss would be obtained. It was concluded that specific measurement of DAB reduction could not be carried out aerobically in this manner. Therefore, all further determinations were carried out anaerobically. The efficiency of the gassing procedure is indicated in Table 3. The aerobic pathways, N-demethylation (AB formation) and ring-hydroxylation (4′-OH-DAB formation), were inhibited 96 and 85%, respectively, in an anaerobic atmosphere.

Table 2. Spectral characteristics of protonated forms of DAB and its aerobic metabolites

Compound*	A_{520}	True spectral peak (μm)
DAB	2.018	520
MAB	1.705	510
AB	0.703	500
4′-OH-DAB	0.951	555

* Each compound was contained in 10% trichloroacetic acid in acetone–ethanol–water (1:1:4). Final dye concentration was 50 μM.

GSH and certain synthetic thiols markedly affect aerobic pathways of microsomal metabolism of DAB [22]. GSH and DTT were also found to affect azoreduction of DAB and MAB but in different ways (Fig. 1). GSH at low concentrations inhibited DAB reduction but stimulated MAB reduction. Although the degree of stimulation of MAB reduction was not large (32% at 3 mM), it is apparent that GSH affects DAB and MAB reduction differently. This is in contrast to the aerobic N-demethylation and ring-

Table 3. Formation of oxidized and reduced products of DAB–Effect of oxygen*

Conditions†	Substrate or products present after 10-min incubation (nmoles)				
	DAB	MAB	AB	4′-OH-DAB	Reduced products
Anaerobic	80.8	1.5	0.4	0.4	10.6
Air	60.4	12.2	9.2	2.7	8.9
100% Oxygen	64.9	8.5	8.6	1.7	5.6

* [^{14}C]DAB (100 nmoles) was incubated at 37° with microsomes as described in Methods. After 10 min, remaining substrate and products were extracted into ether, separated by thin-layer chromatography, and quantified by counting areas corresponding to co-chromatographed reference standards.

† All reactions were performed in anaerobic tubes. Gassing with oxygen or nitrogen for 10 min was done as indicated prior to starting the reaction.

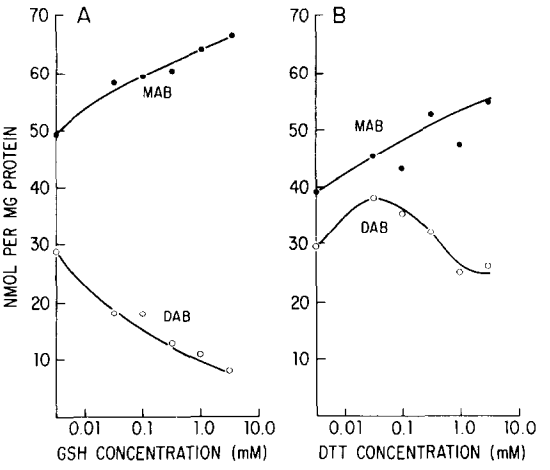

Fig. 1. Effects of GSH and DTT on the microsomal azoreduction of DAB and MAB. Each of the dyes was incubated for 10 min at 37° with rat liver microsomes, various concentration of the thiols, an NADPH-generating system, and buffer in a total volume of 1 ml. The mixture was contained in a Thunberg tube which was thoroughly gassed with argon. The reaction was started by tipping in the microsomes from the side arm. The reaction was stopped by addition of 20% trichloroacetic acid in acetone–ethanol–water (1:1:2), and absorbance of the remaining dye was determined after centrifugation. Experiments were performed in triplicate, and each point is the mean. The ranges for the three values never varied more than 20% of the mean.

hydroxylation of DAB and the N-demethylation of MAB, each of which are markedly stimulated by GSH [22]. DTT had a very slight effect on DAB reduction which, although highly reproducible, was considered to be of little significance. On the other hand, DTT stimulated MAB reduction as does GSH.

In view of the effects of GSH and DTT on microsomal azoreduction of DAB, as well as on its N-demethylation and ring-hydroxylation reported previously [22], the involvement of microsomal SH groups in azoreduction was considered. Azoreduction of DAB was measured in the presence of *p*-hydroxymercuribenzoate (PHMB) and *N*-ethylmaleimide (NEM), each of which alkyates SH groups.

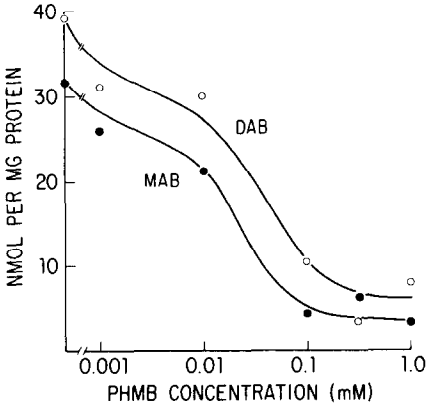

Fig. 2. Effect of PHMB on the azoreduction of DAB and MAB. Conditions were those described in the legend of Fig. 1.

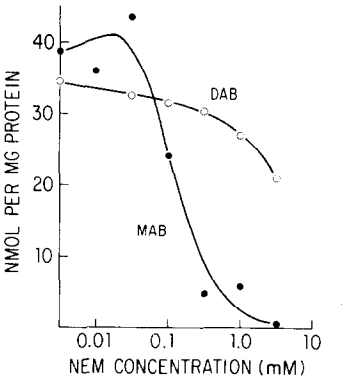

Fig. 3. Effect of NEM on the azoreduction of DAB and MAB. Conditions were those described in the legend of Fig. 1.

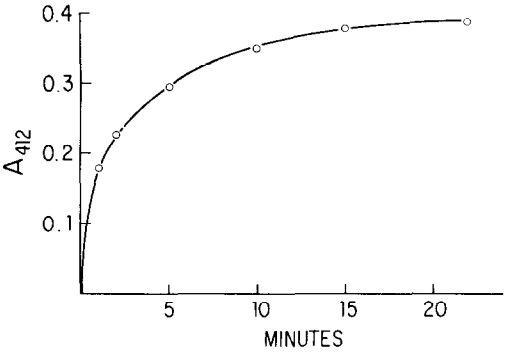

Fig. 4. Titration of microsomal SH groups by DTNB (Ellman's reagent). Approximately 1 mg of microsomal protein was contained in 1 ml of 0.1 M Tris buffer, pH 8.2. DTNB (final concentration = 0.1 M) was added, and A_{412} was monitored in a Gilford recording spectrophotometer.

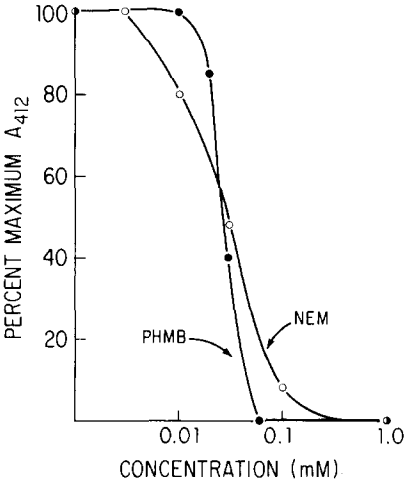

Fig. 5. Titration of microsomal SH groups by DTNB (Ellman's reagent) in the present of various concentrations of PHMB and NEM. Conditions were as described in the legend of Fig. 4.

Relatively low concentrations of PHMB were required to inhibit reduction of both DAB and MAB, and the pattern of inhibition was similar for both substrates (Fig. 2). This is consistent with the finding of Autrup and Warwick [15] who observed that PHMB inhibited DAB reduction with a 9000 *g* supernatant fraction of rat liver [15]. NEM, on the other hand was a very potent inhibitor of MAB reduction, but only weakly inhibited DAB reduction (Fig. 3). In fact, meaningful inhibition of DAB reduction was only seen at 1 mM or more of NEM, where reaction of NEM with the amino group of lysine may be seen. This suggested diverse thiol-dependent azoreduction mechanisms for the two substrates; however, it was important to determine if PHMB and NEM were indeed acting through alkylation of microsomal SH groups. Therefore, titration of microsomal SH groups was carried out with DTNB (Ellman's reagent) which reacts non-specifically with protein as well as low molecular weight thiols [24]. The increase in absorbance at 412 nm during incubation of microsomes with 1 mM DTNB at pH 8.2 is illustrated in Fig. 4. It is apparent that some microsomal SH groups are readily available to DTNB and reacted within 2 min while others reacted somewhat more slowly. Maximal color was generally obtained within 20–30 min. The effects of PHMB and NEM on the titration of microsomal SH groups were then determined (Fig. 5). Each of these agents blocked the reaction with DTNB at concentrations, commensurate with those which affected azoreductase activity (Figs. 2 and 3). The one exception was that NEM blocked the reaction of microsomal SH with DTNB (Fig. 5) at concentrations which exhibited little inhibition of azoreduction of DAB, although MAB was inhibited markedly (Fig. 1).

DISCUSSION

Measurement of microsomal azoreduction of DAB is commonly performed by monitoring loss of substrate through decrease in A_{520}. To be quantitatively accurate, both oxidative and reductive pathways must be taken into account. Aerobic pathways, N-demethylation and ring hydroxylation, lead to products which have a lower absorbance at 520 nm in their protonated form than does DAB (Table 2). For example, if DAB were entirely N-demethylated to AB, the decrease in A_{520} would imply that approximately two-thirds of the dye had been reduced, although none had occurred. The finding of no difference in DAB reduction rates aerobically or anaerobically by Autrup and Warwick [15], De-Araujo *et al.* [17] and Elliott [18] is not consistent with our results (Table 2) where substrate and the aerobic and anaerobic products were determined individually. It is apparent that *true* azoreduction, measured by formation of reduced products separated from oxidized products, proceeds slightly more slowly in the presence of air than anaerobically. However, azoreduction, measured by specific loss of substrate, proceeds more rapidly in air. The difference is attributable to formation of N-demethylated and ring-hydroxylated products. Since these products absorb somewhat differently from DAB at 520 nm (Table 2), it is apparent that an anaerobic environment is essential to the accurate measurement of azoreduction of DAB and related azo dyes by substrate disappearance.

Regulation of enzymic and other cellular activities by thiols is long established [26]. This may involve the sulfhydryl status of the enzyme itself or the environment in which activity is measured. Ferrochelatase is totally inactivated by alkylation of a single sulfhydryl group by NEM [27]. On the other hand, microsomal glutathione *S*-transferase is activated by NEM [28], although cytosolic glutathione *S*-transferase is inhibited [29]. Microsomal NADPH-cytochrome *c* reductase is inhibited 95% by modification of three of its six thiol groups by DTNB [30] and by PCMB [31]. Specificity of the agent used to modify sulfhydryl groups is of considerable importance since DTNB inhibits benzo[*a*]pyrene hydroxylation by purified cytochrome P-448, whereas other sulfhydryl reagents do not [32]. Furthermore, NEM and PCMB each react with cytochrome $P\text{-}450_{cam}$ although enzymic activity is unaffected [33, 34]. At the present time it appears likely that one or more thiol groups in microsomes are required for azoreductase activity, but the exact nature of this requirement is not yet established. PHMB was a far more effective inhibitor of DAB azoreduction than was NEM. This may imply that essential thiol groups are less accessible to NEM than to PHMB. Alternatively, thiol groups may not be essential for the azoreduction of DAB, and PHMB, may inhibit by a totally different mechanism. Both alkylating agents readily inhibited the azoreduction of MAB, suggesting that DAB and MAB are reduced by different mechanisms, possibly by selective species of cytochrome P-450 with dissimilar dependence on viable thiol groups. The evidence supports the conclusion that microsomal thiol groups are essential for MAB azoreduction. Autrup and Warwick [15] also reported inhibition of DAB reduction by PHMB, but found that greater than 0.1 mM was required for inhibition, fully an order of magnitude more than that reported here. This may be due to their use of a 9000 *g* supernatant fraction rather than washed microsomes. These investigators also found that dicumarol inhibited DAB reduction but concentrations as high as 1 mM were required. This is consistent with the finding of Huang *et al.* [7] that cytosolic azoreductase is inhibited by as little as 0.1 μM dicumarol but the enzyme does not reduce DAB.

Previous evidence [3–5] implies that nearly all of the azoreduction of amaranth in rat liver microsomes is attributable to cytochrome P-450. This is based on almost complete inhibition by carbon monoxide and by the inhibitory action of antibodies to cytochromes P-450 and P-448. Autrup and Warwick [15] reported partial inhibition of DAB reduction by carbon monoxide, whereas De-Araujo *et al.* [17] reported none. The latter authors also found no inhibition by SKF 525-A which is unexpected if reduction is catalyzed by cytochrome P-450 and is inconsistent with preliminary findings in this laboratory* that DAB reduction is strongly inhibited by SKF 525-A and

* C. Schwartz, H. Raza and W. G. Levine, unpublished findings.

dichlorophenoxyethylamine (DPEA). On the other hand, SKF 525-A and DPEA stimulate and inhibit, respectively, microsomal azoreduction of neoprontosil [6]. This again emphasizes substrate specificity in consideration of azoreduction mechanisms. Although reports on oxidative mechanisms for many cytochrome P-450-catalyzed reactions are frequently seen, there are relatively few studies on azoreduction mechanisms. Little is known as to the specificity of the various isozymes of cytochrome P-450 for azoreduction although other aspects of selective catalysis have been widely investigated [35, 36]. It is not known if individual isozymes of cytochrome P-450 exhibit similar azoreductase activity or whether there is partial substrate specificity as is seen for oxidative pathways. Previous reports from this laboratory suggest that there is some degree of cytochrome P-450 specificity for ring-hydroxylation, the first and the second N-demethylations of DAB [20–22]. This is based on studies with specific inducers and inhibitors, and purified reconstituted cytochrome P-450 systems. The results with GSH, together with those with PHMB and NEM, imply that azoreductions of DAB and MAB may proceed by different mechanisms. GSH stimulates oxidative pathways for DAB, ring-hydroxylation and N-demethylation [22]. At high concentrations of GSH, inhibition of N-demethylation is seen, probably due to conjugation of GSH with a reactive intermediate in the N-demethylation process [37]. It is unlikely that conjugation is involved in the stimulation of the oxidative reactions. In this study it is seen that GSH was a potent inhibitor of DAB reduction (Fig. 1). Inhibition is not due to shifting the substrate to GSH-stimulated pathways, N-demethylation and ring-hydroxylation, since these are inactive in an anaerobic environment. Glutathione may act as a free radical trap if the first reduction product of DAB is indeed a free radical, as reported for sulfonazo III by Mason *et al.* [14]. However, glutathione stimulates MAB reduction, implying a different mechanism of azoreduction for the secondary amine. If DAB reduction proceeds through a one electron reduction product, it would be predicted that MAB reduction would proceed in a similar manner. In view of the differences in response, this would argue against GSH inhibiting DAB reduction by acting as a free radical trap. An alternative possibility is that azoreductions of DAB and MAB are catalyzed by different isozymes of cytochrome P-450, mechanisms of which are not identical. The relatively weak inhibition of DAB reduction measured aerobically suggests that, if formation of a one electron reduction product is the first step in the azoreduction of DAB, it is not readily reoxidized by oxygen.

Acknowledgements—This study was supported in part by the following grants from the National Institutes of Health: CA-14231, CA-13330, AM-17702. Excellent technical assistance was provided by Steven Lee and Craig Schwartz.

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