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CYTOSOLIC FACTORS THAT ALTER THE METABOLISM OF *N,N*-DIMETHYL-4-AMINOAZOBENZENE BY RAT LIVER MICROSOMES*

WALTER G. LEVINE† and STEVEN B. LEE

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

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Abstract—*N,N*-Dimethyl-4-aminoazobenzene (DAB), an azo dye carcinogen, is *N*-demethylated and 4′-hydroxylated by rat liver microsomes. Addition of hepatic cytosol to the microsomal system stimulated both pathways. This occurred in the presence of added NADPH or an NADPH-generating system. Cytosol was effective only when present prior to addition of substrate; no stimulation was seen when added after the reaction had begun. This suggested a direct effect on the microsomes rather than a chemical interaction with one or more metabolic intermediates of DAB. The degree of stimulation was somewhat different when using microsomes from phenobarbital- or β-naphthoflavone-treated animals, implying a selectivity of the cytosolic effect for various isozymes of cytochrome P-450. Some loss of stimulatory activity occurred with dialysis. Activity was restored by adding back glutathione (GSH) which can stimulate DAB metabolism even in the absence of cytosol. DAB metabolism is also stimulated by EDTA. Although both EDTA and cytosol inhibit lipid peroxidation, cytosol stimulated DAB metabolism even in the presence of EDTA. Therefore, suppression of lipid peroxidation does not explain satisfactorily the cytosolic effect. Separation of cytosolic proteins by gel filtration revealed a factor which inhibits *N*-demethylation but not 4′-hydroxylation of DAB. Heating at 100° partially inactivated the stimulatory activity. However, inhibitory activity was less susceptible to heat inactivation than was stimulatory activity. These results indicate that, in the whole cell, microsomal metabolism of xenobiotics is regulated to an appreciable extent by macromolecular cytosolic substances.

It has long been established that the metabolism of numerous xenobiotics can be carried out in the presence of washed hepatic microsomes, oxygen, and NADPH or a suitable NADPH-generating system. However, addition of hepatic cytosol to this system often enhances metabolic rates. Explanations for this effect vary. Enhanced generation of NADPH was suggested in the case of oxidation of benzo[*a*]pyrene [1] and *N*-demethylation of aminopyrine and ethylmorphine [2–4]. In both instances, addition of sufficient NADPH sharply increased metabolic rates, and added cytosol was, then, no longer effective. A cytosolic protein found in the Z-fraction enhances aminopyrine *N*-demethylation but not aniline hydroxylation [5]. Microsomal dechlorination of chloroethanes and chloropropanes is stimulated by cytosol [6] although there is some doubt as to the participation of cytochrome P-450 in this reaction. It has also been proposed that cytosolic enhancement of microsomal activity is due to suppression of lipid peroxidation [3, 5, 7, 8] which otherwise would lead to inhibition of cytochrome P-450 activity [3, 7–10]. The cytosolic factors have been characterized as heat stable [11], heat labile [2], and non-dialyzable [2]. Recently, it has been suggested that specific cytosolic proteins act as carriers for benzo[*a*]pyrene, thus enhancing its microsomal oxidation [12, 13]. These may include ligandin and other glutathione *S*-transferases, each of which exhibits binding properties for numerous xenobiotics [14].

N,N-Dimethyl-4-aminoazobenzene (DAB), an azo dye, is metabolized by way of *N*-demethylation and ring-hydroxylation, and the conjugated products are excreted in the bile [15]. Recent evidence indicates that the two pathways are preferentially catalyzed by different isozymes of cytochrome P-450 [16]. This selectivity may also apply to the two *N*-demethylation steps. Our earlier studies indicated a considerable difference between rates of metabolism by washed microsomes and by 10,000 *g* supernatant fractions. We have investigated this difference and found a marked stimulation of the microsomal metabolism of DAB by cytosol. The two major pathways are not affected equally.

MATERIALS AND METHODS

Preparation of liver fractions. Male Wistar rats (200–225 g) were decapitated, and the livers were perfused and homogenized in 3 vol. of cold 0.15 M KCl, 0.01 M Tris buffer (pH 7.4). The homogenate was centrifuged for 10 min at 10,000 *g*. The supernatant material was centrifuged for 1 hr at 100,000 *g*. The cytosol was carefully removed, avoiding floating lipid particles, and the pellet was resuspended in buffer and again centrifuged. The pellet was resuspended in buffer so that the final suspension contained microsomes from 1 g liver/ml (approximately 15 mg protein/ml). They were used immedi-

* A preliminary report of those findings was made at the 1982 meeting of the Federation of American Societies for Experimental Biology [*Fedn Proc.* **41**, 1730 (1982)].

† Author to whom correspondence should be sent.

ately or stored in liquid nitrogen with no perceptible loss of activity for 6 weeks. The cytosol was either used as such or dialyzed against buffer for 24 hr.

DAB metabolism. Microsomes were incubated at 37° with 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4), 10 mM $MgCl_2$, 0.5 mM NADP, 5 mM glucose-6-phosphate, and 1 unit glucose-6-phosphate dehydrogenase, in a total volume of 1.0 ml. In some experiments, 1 mM NADPH was added, and NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were omitted. The isocitrate system could not be used for NADPH generation due to contamination of the isocitrate dehydrogenase preparation (Sigma) with EDTA, as described below. Where indicated, various amounts of cytosol were added. The reaction was started by the addition of radiolabeled DAB in 10 μl of a methanolic solution and incubation was continued for 10 min. Substrate and metabolites were extracted once with ether (95% recovery of radioactivity) and quantitated by thin-layer chromatography as previously described [17].

Gel filtration. Freshly prepared cytosol was concentrated by ultrafiltration to approximately one-fourth its original volume, dialyzed against 0.01 M sodium phosphate (pH 7.4) in 10% glycerol, and passed through a 2.5 × 95 cm Bio-gel P-150 column. The flow rate was 16 ml/hr. Fractions (3 ml) were collected, and absorbance at 280 nm was determined. The fractions within each of the three major protein peaks were combined, concentrated, dialyzed versus 10 mM Tris buffer (pH 7.4), and tested for their effect on the microsomal metabolism of DAB.

Lipid peroxidation. Microsomes were incubated for 30 min at 37° with 50 mM HEPES buffer (pH 7.4), 0.5 mM NADP, 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 0.1 mM ferric chloride and 4 mM ADP in a total volume of 1.0 ml. Additions of cytosol and GSH were made as indicated in Results. The reaction was stopped by the addition of 2 ml of a solution containing 15% trichloroacetic acid, 0.25 N hydrochloric acid, 0.375% thiobarbituric acid, and 0.001% butylated hydroxyanisole to prevent spontaneous lipid peroxidation. The mixture was heated at 90° for 30 min, cooled, and centrifuged for 20 min at 3000 *g*. Absorbance at 532 nm was determined for the supernatant material. The quantity of malondialdehyde formed was calculated from the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Protein. Determinations were done by the method of Lowry *et al.* [18].

Treatment of animals. Where indicated, rats were injected i.p. with phenobarbital (PB), 75 mg/kg daily for 4 days, or β-naphthoflavone (BNF), 40 mg/kg daily for 3 days; controls received saline. Microsomes were prepared 24 hr after the last injection. To deplete GSH, rats were injected i.p. with diethylmaleate, 3.3 mmoles/kg; 30–60 min later, hepatic GSH was 10% of control levels.

Chemicals. The following sources were used: DAB, Pfaltz & Bauer (Stamford, CT); glucose-6-phosphate, glucose-6-phosphate dehydrogenase, GSH, NADP and NADPH, Sigma Chemical Co. (St. Louis, MO); and [^{14}C]DAB, New England Nuclear Corp. (Boston, MA). The radiolabeled DAB was shown to be 96–98% pure by thin-layer chromatography.

Statistics. Where indicated, results were evaluated by Student's *t*-test. $P < 0.05$ was taken as an indication of significant difference.

RESULTS

In each of the figures are indicated the nmoles of substrate (DAB) used up and the nmoles of *N*-demethylated (AB) and 4'-hydroxylated (4'-OH-DAB) products formed in 10 min. The monodemethylated product, MAB, was also present in significant amounts during the reaction but, since its rate of formation and metabolism varied with reaction conditions, it was not possible to calculate accurately its rate of formation. Therefore, in this study, *N*-demethylation was taken as the amount of AB formed from DAB in 10 min. The rates of formation of 4'-OH-DAB and AB far exceeded their rates of metabolism under these experimental conditions, and these metabolites accumulated during the reaction. Therefore enzyme activity was expressed as the amount AB or 4'-OH-DAB formed in 10 min. In Fig. 1, it can be seen that addition of cytosol to microsomes enhanced DAB disappearance as well as AB and 4'-OH-DAB formation. No metabolism of DAB was seen in the absence of microsomes, with or without cytosol, and activity was totally dependent on the presence of NADPH or an NADPH-generating system. Addition of cytosol to microsomes stimulated DAB metabolism when either NADPH or an NADPH-generating system was used (Fig. 2). Thus, stimulation was not attributable to enhanced NADPH generation as speculated in the past [4]. Maximal enhancement of *N*-demethylation (AB formation) was usually observed at low cytosolic concentrations while inhibition occasionally was seen at high concentrations, suggesting the presence of inhibitory as well as stimulatory factors in the cytosol. Stimulation of 4'-hydroxylation exceeded that for *N*-demethylation and was seen at all concentrations of cytosol. It was necessary that cytosol be present at zero time for stimulation to be seen (Fig. 3). Addition 3 or 6 min after the reaction was started with DAB in some cases may actually have led to depressed *N*-demethylation. Dialysis of cytosol resulted in partial loss of stimulatory activity (Fig. 4) which was readily restored by addition of GSH which is normally found in whole cytosal (Fig. 5). Similarly, cytosol prepared from rats treated with diethylmaleate to deplete hepatic GSH had diminished ability to stimulate microsomal metabolism of DAB (Table 1). In a parallel experiment, cytosol was shown to reverse iron-dependent microsomal lipid peroxidation (Fig. 6). This ability was sharply diminished after treatment with diethylmaleate or dialysis of cytosol. However, the effect was restored upon addition of GSH. In the microsomal lipid peroxidation system in Fig. 6, the rate of malondialdehyde formation in the absence of added cytosal was 50 nmoles per 30 min per mg of microsomal protein. In the presence of GSH-depleted or dialyzed cytosol (5 mg protein) the rates were 39 and 44 respectively. Upon addition of 3 mM GSH to either of these cytosolic preparations,

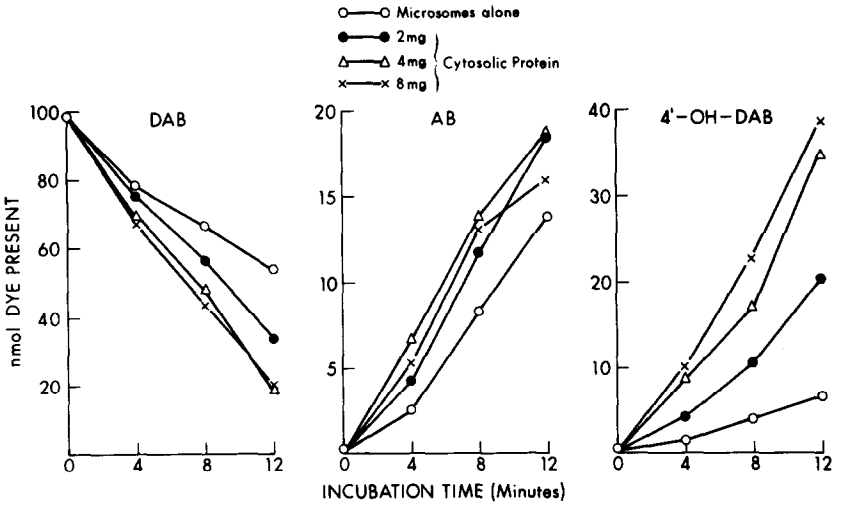

Fig. 1. Time course of microsomal metabolism of DAB in the presence of various quantities of cytosol. Incubations were carried out for 4, 8 or 12 min, and DAB, AB and 4′-OH-DAB were analyzed as described in Materials and Methods. Each point is the mean of triplicate experiments.

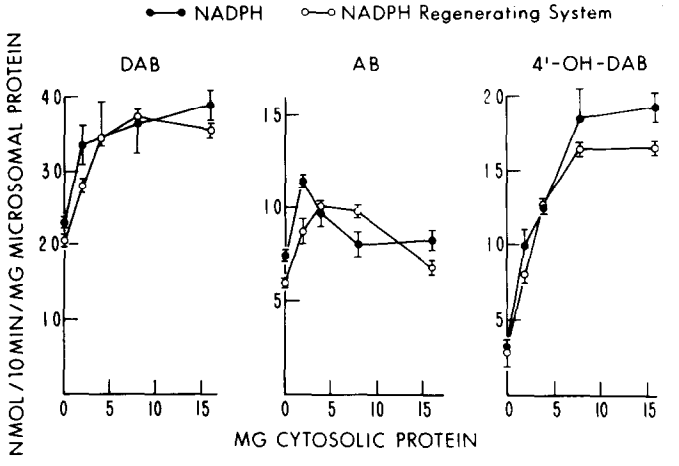

Fig. 2. Effect of cytosol on the microsomal metabolism of DAB in the presence of added NADPH or an NADPH-generating system. Each point represents the mean ± S.E. of triplicate experiments that were carried out for 10 min.

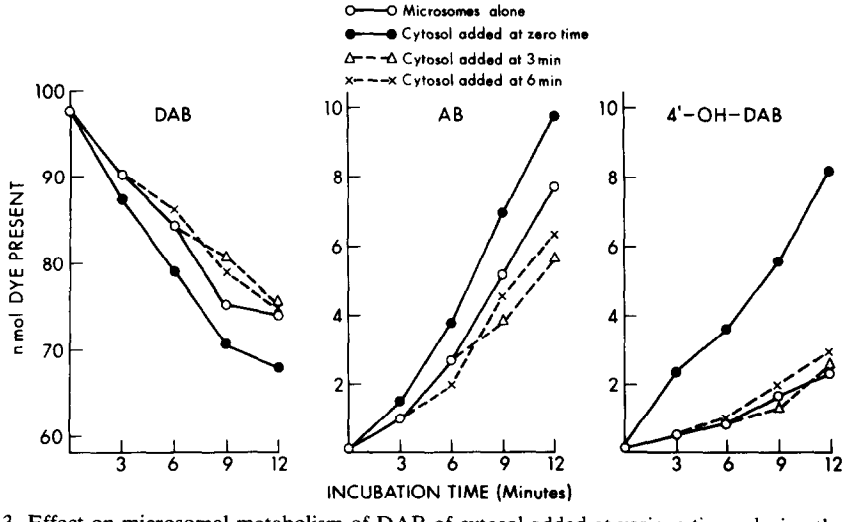

Fig. 3. Effect on microsomal metabolism of DAB of cytosol added at various times during the course of the reaction. Cytosol (0.2 ml) was added prior to or at 3 or 6 min after the reaction was started by the addition of DAB. Analysis of DAB and its metabolites was carried out at 3, 6, 9 or 12 min. Each point is the mean of triplicate experiments.

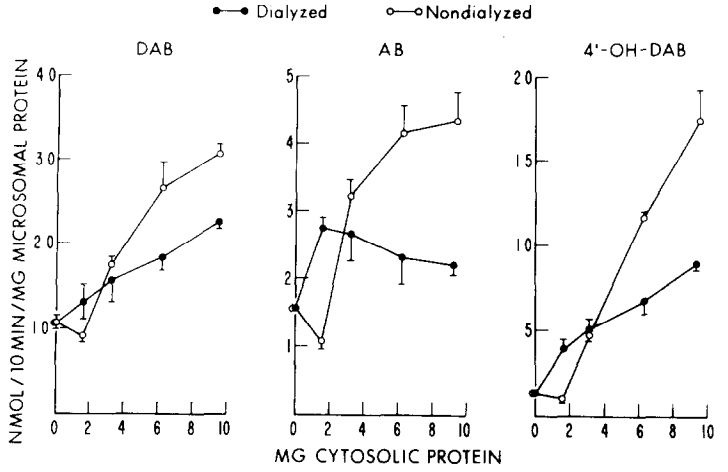

Fig. 4. Effect of dialyzed and non-dialyzed cytosol on the microsomal metabolism of DAB. Each point represents the mean ± S.E. of triplicate experiments which were carried out for 10 min.

the rate was 3, approximately that seen in the presence of whole cytosol (Fig. 6). The specific effects of GSH on microsomal metabolism of DAB are reported elsewhere [19].

To determine if the cytosolic effects were due entirely to suppression of lipid peroxidation, cytosol was added in the presence of EDTA, which alone totally suppresses microsomal lipid peroxidation [20, 21]. In the absence of cytosol, EDTA stimulated both *N*-demethylation and 4'-hydroxylation (Fig. 7), but cytosol was stimulatory both in the presence and absence of EDTA (Fig. 7), demonstrating that the effect was not limited to suppression of lipid peroxidation.

The fact that EDTA stimulated DAB metabolism helped to explain an earlier unpublished observation in this laboratory, that generation of NADPH using an isocitrate system led to more rapid DAB metabolism compared to using a glucose-6-phosphate system. Warner and Neims [22] had reported the presence of EDTA in Sigma Type IV isocitrate dehydrogenase. When DAB metabolism was measured with added NADPH or an NADPH-generating system containing glucose-6-phosphate, the addition of one unit of Sigma Type IV isocitrate dehydrogenase, the amount ordinarily used in such experiments, increased activity to an extent equal to that with EDTA (see Fig. 7). Isocitrate itself had no effect. This confirmed a previous report that isocitrate dehydrogenase (Sigma Type IV) stimulated microsomal *N*-demethylation of ethylmorphine as did EDTA [8]. Further investigation showed that the isocitrate dehydrogenase preparation from Boehringer–Mannheim did not have this stimulatory activity.

In view of our previous finding that induction of cytochrome P-450 had selective effects on the two pathways of DAB metabolism [16], the effect of cytosol was determined on microsomes from PB- and BNF-treated rats. Cytosolic stimulation of both pathways was seen in each case (Table 2). Microsomes from BNF-treated rats contain a species of cytochrome P-450 which exhibited a very low ratio of 4-hydroxylation/*N*-demethylation for DAB (Table 2 and Ref. 16). The low rate of 4'-hydroxylation was affected only slightly by cytosol in contrast to the

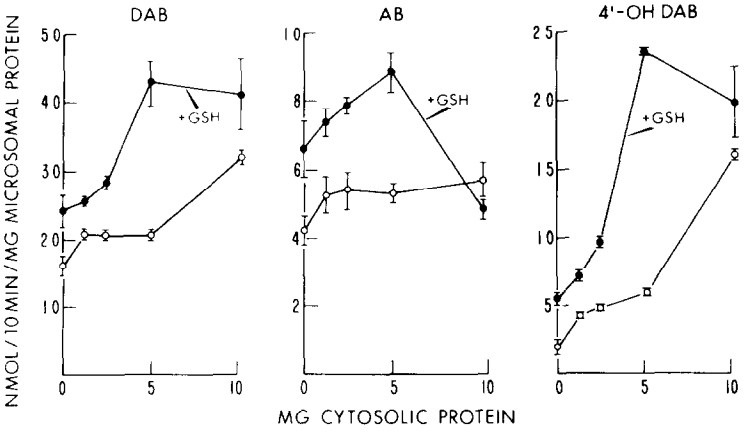

Fig. 5. Restoration of stimulatory effect of dialyzed cytosol by addition of GSH. DAB metabolism was measured in the presence of various quantities of cytosol with and without 3 mM GSH. Each point represents the mean ± S.E. of triplicate experiments carried out for 10 min.

Table 1. Effect of control and GSH-depleted cytosol on microsomal metabolism of DAB

	Rate of substrate (DAB) disappearance or product (AB, 4′-OH-DAB) formation* [nmoles·(10 min)$^{-1}$·(mg microsomal protein)$^{-1}$]		
	No cytosol	Control cytosol	GSH-depleted cytosol†
DAB	19.0 ± 0.8	27.2 ± 1.2‡	20.1 ± 0.2
AB	4.1 ± 0.4	8.6 ± 0.3‡	5.1 ± 0.1
4′-OH-DAB	0.60 ± 0.20	5.9 ± 0.5‡	4.3 ± 0.1‡

* Each value is the mean ± S.E.M. of triplicate experiments.
† GSH-depleted cytosol was prepared from rats 1 hr after injection of diethylmaleate, 3.3 mmoles/kg.
‡ Each value differs significantly from the corresponding value obtained in the absence of added cytosol ($P < 0.05$).

marked stimulation of 4′-hydroxylation in control and PB microsomes (Table 2). AB formation in BNF microsomes was stimulated slightly more than in control microsomes but to a somewhat greater extent in PB microsomes.

It was determined that simulation by cytosol was not due to a nonspecific effect of protein since bovine serum albumin was without effect when present in concentrations equal to that of added cytosol (Fig. 8). Heating the cytosol at 100° for 10 min destroyed some, but not all, of the stimulatory activity (Fig. 9). In particular, stimulation of *N*-demethylation was far less after heating, and significant inhibition was seen at high concentrations of heated cytosol.

Preliminary experiments were carried out to isolate the cytosolic factor(s). Cytosol passed through a column of Bio-gel P-150 yielded three major protein fractions (Fig. 10). Fraction I, which appeared near the void volume, stimulated both *N*-demethylation and 4′-hydroxylation. Fraction III, which contained lower molecular weight proteins (10,000–15,000), inhibited *N*-demethylation but had no effect on 4′-hydroxylation. This may explain the biphasic response to added cytosol frequently observed for *N*-demethylation but not 4′-hydroxylation (Figs. 2, 4, 5, and 7).

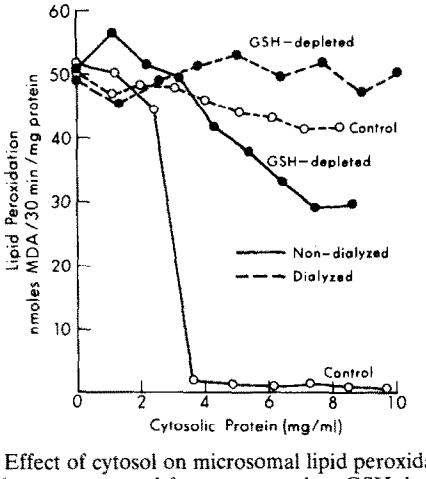

Fig. 6. Effect of cytosol on microsomal lipid peroxidation. Cytosols were prepared from untreated or GSH-depleted animals and were used either directly or after dialysis. Lipid peroxidation was measured by the formation of malondialdehyde (MDA). Each point is the mean of triplicate experiments.

DISCUSSION

In accord with previous findings for other substrates [1, 2, 4, 5, 7, 8], rat liver cytosol significantly stimulated both the *N*-demethylation and 4′-hydroxylation of DAB by microsomes. This was seen in PB- and BNF-induced as well as control microsomes, although the degree of stimulation varied from one system to another. After BNF treatment, 4′-hydroxylation of DAB proceeded relatively slowly [16] and responsed weakly to added cytosol (Table 2), in contrast to the striking cytosolic stimulation of 4′-hydroxylation catalyzed by control and PB-induced microsomes. The slow rate of 4′-hydroxylation is probably not attributable to residual non-induced cytochrome since the reaction also proceeds slowly in the presence of a purified reconstituted cytochrome P-448 system prepared from rats induced with 3-methylcholanthrene [16]. Although *N*-demethylation of DAB was strongly induced by BNF, it too responded somewhat less than was seen with microsomes from PB-treated rats. These observations suggest a specific interaction between cytosol and selective isozymes of cytochrome P-450. Thus, as the nature of the cytochromes changes with induction, so the response to cytosol also changes.

Despite numerous studies, the mechanism of the microsome–cytosol interaction is not clear. In view of the lack of response to bovine serum albumin (Fig. 8), it can be concluded that not all proteins exhibit this effect and that there is some degree of specificity for cytosolic proteins. Enhancement of NADPH generation was once proposed for a system deficient in isocitrate dehydrogenase [4]. This certainly is not applicable to the present study since cytosolic stimulation is seen with an NADPH-generating system as well as with added NADPH (Fig. 2). Suppression of lipid peroxidation may contribute to the effect since EDTA stimulates DAB metabolism and totally suppresses microsomal lipid peroxidation, possibly through chelation of iron [10, 20, 21]. However, lipid peroxidation would be expected to depress cytochrome P-450 activity during the incubation period, resulting in a gradual reduction in metabolic rate. This is seen for the microsomal *N*-demethylation of ethylmorphine where the grad-

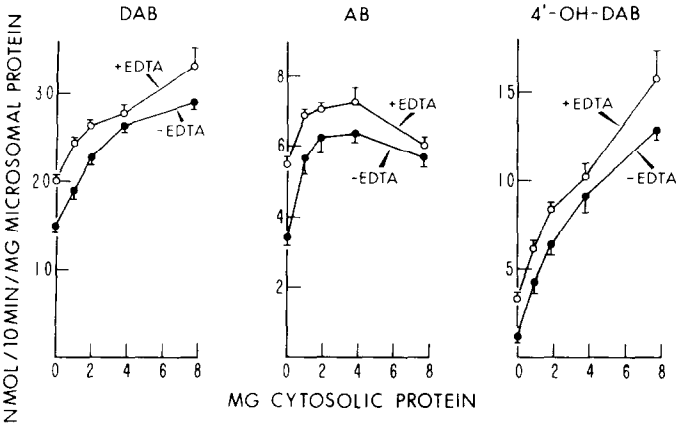

Fig. 7. Effect of EDTA on the cytosolic stimulation of DAB metabolism by microsomes. DAB metabolism was measured in the presence of various quantities of cytosol with and without 0.1 mM EDTA. Each point represents the mean ± S.E. of triplicate experiments carried out for 10 min.

ual loss of activity is blocked by EDTA [3]. As seen in Fig. 1, microsomal *N*-demethylation and 4'-hydroxylation of DAB did not diminish during the course of the reaction, in the absence and presence of cytosol. If lipid peroxidation were gradually inactivating cytochrome P-450 during the course of the reaction, the inhibition of lipid peroxidation by cytosol added at any time during the reaction should enhance the metabolic rate. Since enhancement was seen only when cytosol was present at zero time, it is likely that cytosol acts in another manner. Furthermore, deferoxamine, which completely abolishes lipid peroxidation, has no effect on the rate of DAB metabolism (unpublished observations).

It is apparent from these studies that both dialyzable and non-dialyzable factors contribute to the cytosolic effect. Of the dialyzable substances, GSH is a likely contributor to the stimulatory effect. It is present in high concentrations (5–8 mM) in liver cytosol, it restores the activity lost upon dialysis, and it stimulates both pathways of DAB metabolism, even in the absence of cytosol [19]. GSH also suppresses microsomal lipid peroxidation [23], but such a role in the enhancement of DAB metabolism is not established at the present time.

Heating cytosol at 100° did not entirely destroy stimulatory activity (Fig. 9), which suggests that a non-enzymic mechanism may contribute to the cytosolic response. Both heat-labile [2, 12] and heat-stable [11] cytosolic factors have been reported to stimulate microsomal *N*-demethylation activity. A heat-stable factor was found in the supernatant material after centrifuging heat-coagulated cytosolic proteins [11]. Thus, it is unlikely to be a non-specific effect of precipitated protein. At high concentrations, heated cytosol was more inhibitory to *N*-demethylation of DAB than was non-heated cytosol (Fig. 9). It is inferred that one or more heat-stable inhibitory factors specifically block *N*-demethylation but not 4'-hydroxylation of DAB. This may relate

Table 2. Effect of cytosol on the metabolism of DAB by control, PB-induced and BNF-induced microsomes

	Rate of substrate (DAB) disappearance or product (AB, 4'-OH-DAB) formation* [nmoles·(10 min)$^{-1}$·mg microsomal protein)$^{-1}$]	
	−Cytosol	+Cytosol (% increase)
Controls		
DAB	22.0 ± 1.7	34.6 ± 1.7† (57%)
AB	9.3 ± 0.9	15.4 ± 0.9† (66%)
4'-OH-DAB	3.0 ± 0.6	10.6 ± 0.7† (253%)
PB-induced		
DAB	14.0 ± 0.1	33.7 ± 1.0† (141%)
AB	4.0 ± 0.1	13.8 ± 0.3† (245%)
4'-OH-DAB	1.1 ± 0.4	12.3 ± 0.6† (1018%)
BNF-induced		
DAB	97.9 ± 0.4	117.2 ± 2.6 (20%)
AB	18.4 ± 1.9	33.3 ± 3.0† (81%)
4'-OH-DAB	2.5 ± 0.01	3.7 ± 0.0† (48%)

* Each value is the mean ± S.E.M. of triplicate experiments.

† Values differ significantly ($P < 0.05$) from those obtained in the absence of added cytosol.

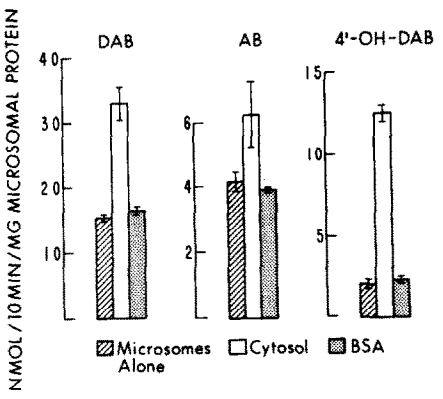

Fig. 8. Comparison of effects of cytosol and bovine serum albumin (BSA) on the microsomal metabolism of DAB. Both the cytosol and BSA added contained 5 mg of protein. Each point represents the mean ± S.E. of triplicate experiments carried out for 10 min.

to catalysis of these reactions by different isozymes of cytochrome P-450 [16] and their selective sensitivity to cytosolic factors.

Recent studies indicate that specific cytosolic proteins, including at least one isozyme of GSH *S*-transferase, bind and, in effect, solubilize benzo[*a*]pyrene, enhancing its microsomal metabolism [12, 13]. It was proposed that cytosolic proteins with hydrophobic binding sites act as carriers for the polycyclic hydrocarbon to the microsomal site of cytochrome P-450. Extrapolation of such mechanisms to the present study is difficult. If the cytosolic factors did act as carriers of DAB to the microsomal site of cytochrome P-450, then the stimulatory effect should be apparent even when cytosol is added after the reaction has begun. However, this is not the case (Fig. 3). Once the metabolism of DAB had commenced, it was refractory to the addition of cytosol. In addition, the solubilizing effect of cytosolic proteins may not be applicable since we have also seen a similar

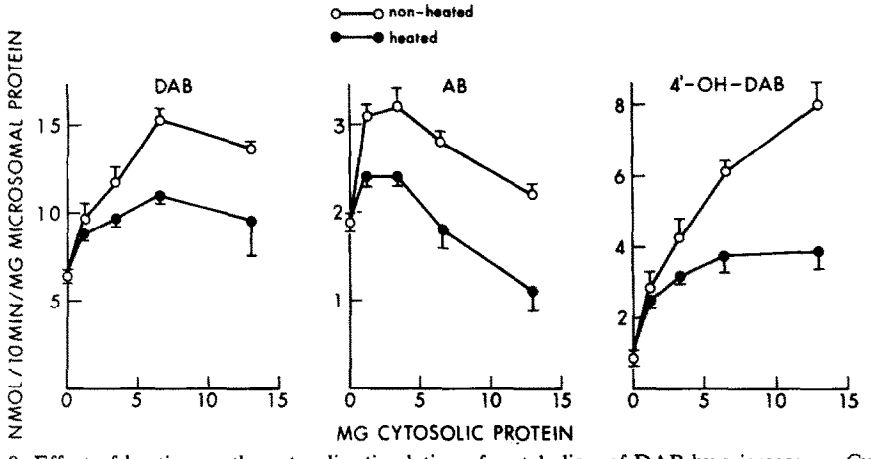

Fig. 9. Effect of heating on the cytosolic stimulation of metabolism of DAB by microsomes. Cytosol was placed in boiling water for 10 min. Each point represents the mean ± S.E. of triplicate experiments carried out for 10 min.

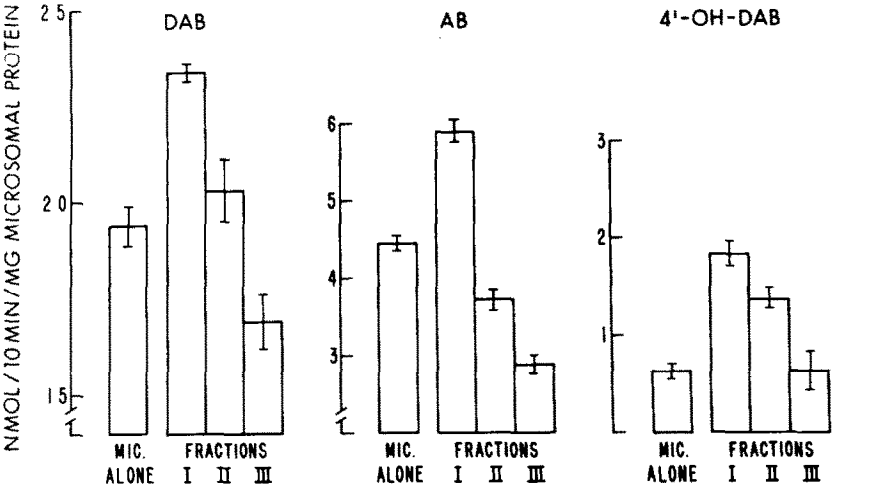

Fig. 10. Effects of three cytosolic protein fractions on the metabolism of DAB by microsomes. Partial separation was effected on a 2.5 × 95 cm Bio-gel P-150 column equilibrated with 10 mM sodium phosphate (pH 7.4) in 10% glycerol. Samples from each of the three protein peaks were pooled, concentrated, and dialyzed versus 10 mM Tris buffer (pH 7.4). In each experiment 5 mg of protein was added. Each value is the mean ± S.E. of triplicate experiments.

stimulation of the *N*-demethylation of aminopyrine, a water-soluble substrate (unpublished observations), confirming other reports [2, 5].

Another possibility which warrants further investigation is that DAB may bind to microsomes in a manner which is less than optimal for maximal rates of metabolism. Such binding may be optimized only by cytosolic factors present at the time of addition of DAB to microsomes. Once binding to microsomes has occurred, no influence by cytosol is possible. Stimulation of ethylmorphine demethylation by EDTA decreases the apparent K_m for this substrate and ostensibly increases its binding [3]. On the other hand, cytosol increases both the rate of microsomal demethylation of ethylmorphine and its K_m, suggesting weaker binding [2].

In conclusion, dialyzable and non-dialyzable cytosolic factors influence the microsomal metabolism of DAB. The dialyzable factors include GSH. The non-dialyzable factors are probably proteins, some of which are heat stable. The mechanism by which these proteins stimulate metabolism is not entirely explained by suppression of lipid peroxidation. It is speculated that a specific interaction of cytosolic factors with one or more isozymes of cytochrome P-450 accounts for the enhanced metabolism of DAB. This possibility will be considered in future investigations.

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