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Evidence against multiple forms of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in rat liver microsomes

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The mixed-function oxidase system of the liver endoplasmic reticulum (microsomes) catalyzes the hydroxylation of a wide variety of lipophilic compounds including steroids, fatty acids, drugs, pesticides, carcinogens and other xenobiotics [1, 2]. An intriguing property of this electron transport system is its apparent non-specificity with regard to the substrates it hydroxylates. This property has led to the hypothesis that multiple mixed-function oxidase activities are present in liver microsomes [3–7], and currently there is much interest in the mechanism by which multiple activities might be attained. The two known enzymatic components of the system present in rat liver microsomes are a flavoprotein, NADPH-cytochrome c reductase and the terminal, substrate-binding oxidase, cytochrome P-450 [1–3]. Any mechanism which might be proposed for attaining different hydroxylation activities would have to include multiplicity at the level of the cytochrome component since this binds the substrate, and indeed there is spectral [1, 2], catalytic [8–10], and SDS-polyacrylamide gel electrophoretic [11, 12] evidence which is consistent with this proposal. It is not known, however, if the multiple hydroxylation activities are due solely to the existence of multiple forms of the cytochrome component, or if completely different electron transport chains are present in microsomes and different forms of NADPH-cytochrome c reductase might also be present in this membrane. To investigate this question, the properties of the NADPH-cytochrome c reductase enzymes present in the liver microsomes of control rats might be compared with those present in the liver microsomes from rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC), two compounds known to induce different hydroxylation activities in the microsomes [1, 2]. Since methods have not yet been developed by which the reductase enzyme can be isolated from rat liver microsomes without the use of proteases [13], however, such comparisons have been difficult to perform.

Kuriyama *et al.* [14] have shown that the trypsin-solubilized NADPH-cytochrome c reductase enzymes from the liver microsomes of control and PB-pretreated rats behave similarly during chromatography on Sephadex G-100 and on DEAE-cellulose. In addition, they appear immunologically identical on the basis of Ouchterlony double diffusion analysis against antibody prepared to the trypsin-solubilized reductase from the microsomes of PB-pretreated rats. These observations cannot be considered proof that the reductase enzymes present in these microsomes are the same, however, since these studies were performed on protease-solubilized enzymes and proteases undoubtedly modify the catalytic properties of the reductase, as suggested by the inability of such enzymes to reconstitute microsomal hydroxylations [15, 16].

Antibody prepared to protease-solubilized NADPH-cytochrome c reductase enzymes present in the liver microsomes of PB-pretreated rats has also been shown to inhibit, to the same extent, the reductase enzymes present in the microsomes from control and PB-pretreated rats [14, 17] and in the microsomes from 3-MC-pretreated rats [17]. While this property too suggests that the reductases found in all three types of microsomes are immunologically similar, it also cannot be taken as proof that they are the same, since a similar antibody is capable of inhibiting the reductase from adrenal microsomes, and the enzymes from liver and adrenal microsomes are not immunologically identical on the basis of Ouchterlony double diffusion analysis [18].

More recently, Lu *et al.* [9] have attempted to study the existence of multiple forms of the reductase by using partially purified detergent-solubilized NADPH-cytochrome c reductase fractions from the liver microsomes isolated from PB- and 3-MC-pretreated rats to reconstitute hydroxylation activity with partially purified cytochrome P-450 fractions also isolated from these liver microsomes. The results of these studies suggested that, for benzphetamine *N*-demethylation, the reductase fractions play a role in the ability of the reconstituted system to metabolize this compound. This suggests that different reductases may be present in the liver microsomes isolated from 3-MC- and PB-pretreated rats and emphasizes the necessity of pursuing studies on the physical properties of the "native" reductases present in these different microsomes.

We have recently described a technique which could be used to determine the molecular weight of the "native" reductase present in the microsomes isolated from PB-pretreated rats [19], and in this communication describe the use of this technique for the comparison of the molecular weights of the NADPH-cytochrome c reductase enzymes present in the rat liver microsomes from control and PB- or 3-MC-pretreated rats. This method employs the use of an antibody prepared to a protease (bromelain)-solubilized fragment of NADPH-cytochrome c reductase enzyme to immunoprecipitate the "native" form of this enzyme from sodium deoxycholate-solubilized ^{125}I-labeled microsomes. The molecular weight of the "native" form of the reductase enzyme was then determined by electrophoresing the immunoprecipitate on sodium dodecyl sulfate (SDS)-polyacrylamide gels and scanning the gels for ^{125}I to determine the migration position of the "native" reductase on the

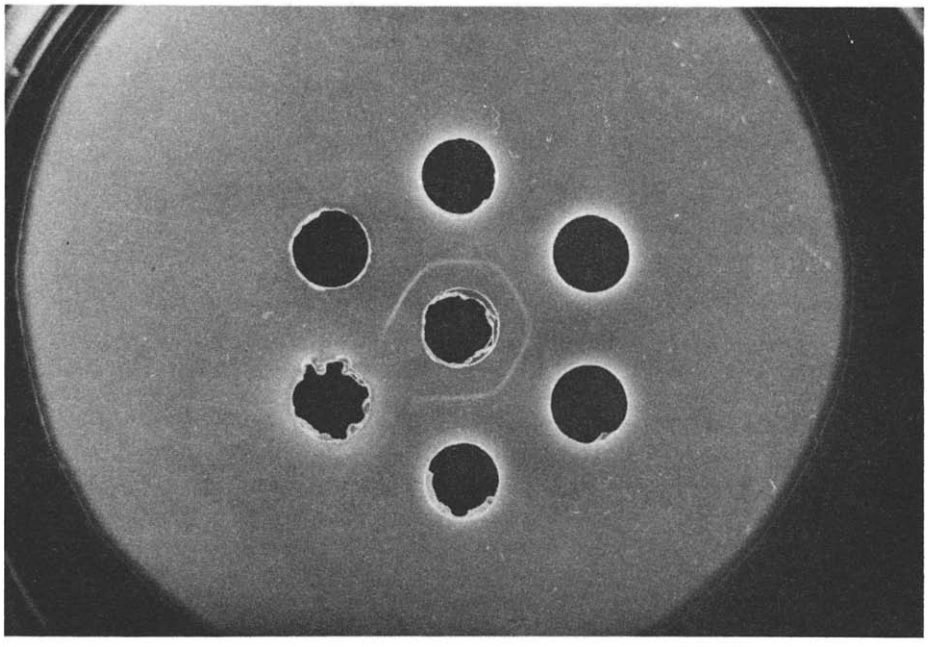

Fig. 1. Ouchterlony double diffusion analysis of the precipitin reactions between antibody to bromelain-solubilized NADPH-cytochrome c reductase, the bromelain reductase, and detergent-solubilized liver microsomal proteins from control and PB- or 3-MC-pretreated rats. The center well contains anti-NAPDH-cytochrome c reductase IgG (120 μg protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Wells 1 and 4 contain 180 μg microsomal protein from PB-pretreated rats (6 mg/ml in 0·015 M sodium phosphate buffer, pH 8·0 containing 2% sodium deoxycholate). Well 2 contains 360 μg microsomal protein from 3-MC-pretreated rats (12 mg/ml in phosphate buffer containing 4% sodium deoxycholate). Well 3 contains 360 μg microsomal protein from control rats (12 mg/ml in phosphate buffer containing 4% sodium deoxycholate). Well 6 contains 3 μg bromelain-solubilized NADPH-cytochrome c reductase (0·2 mg/ml in phosphate buffer). Well 5 contains 0·015 M sodium phosphate buffer, pH 8·0 containing 4% sodium deoxycholate.

gels. Since this antibody will cross-react with the reductases present in the liver microsomes isolated from control and 3-MC-pretreated rats, we now report the use of this immunoprecipitation technique to compare the molecular weights of the reductases from the liver microsomes of control and PB- or 3-MC-pretreated rats.

For these studies, liver microsomes were isolated [3] from male Sprague–Dawley rats (Spartan Research Animals, Inc.) weighing between 75 and 100 *g*. These rats were treated with either PB (50 mg/kg) by daily i.p. injections 5 days prior to sacrifice or with 3-MC (20 mg/kg in corn oil) by i.p. injections 36 and 24 hr prior to sacrifice. Ouchterlony double diffusion analysis [20] was performed in 1% agar dissolved in 0·015 M sodium phosphate, pH 8·0 containing 0·1% sodium deoxycholate and 0·02% sodium azide. The preparation of antibody to bromelain-solubilized NADPH-cytochrome c reductase and the use of this antibody for immunoprecipitation of the reductase from sodium deoxycholate-solubilized rat liver microsomal proteins has been previously described [19]. NAPDH-ferricyanide reductase activity was assayed by the previously published method [19]. The 1% SDS-polyacrylamide gel technique of Fairbanks *et al.* [21] was utilized in these studies with the modifications as described earlier [19]. After electrophoresis, gels were stained with Coomassie blue and the protein profiles visualized by scanning the gels at 550 nm using a Gilford spectrophotometer.

As can be seen in Fig. 1, on the basis of the precipitin reactions occurring during Ouchterlony double diffusion analysis, the antibody prepared to the bromelain-solubi-

Table 1. NADPH-ferricyanide reductase activities in the immunoprecipitates formed between antibody to the bromelain-solubilized reductase and detergent-solubilized liver microsomal proteins from control and PB- or 3-MC-pretreated rats*

Fraction	Total μmoles ferricyanide reduced/min		
	PB	3-MC	Control
Microsomes	1·6	0·9	0·8
Sodium deoxycholate-solubilized microsomes	1·6	0·9	0·8
Immunoprecipitate	0·4	0·2	0·2
Supernatant from immunoprecipitate	> 0·01	> 0·01	> 0·01

* Liver microsomes (4 mg protein) from control and PB- or 3-MC-pretreated rats were solubilized in sodium deoxycholate, centrifuged, and antibody was added to the supernatants to form immunoprecipitates. After washing, the immunoprecipitates were suspended in 0·05 M Tris-HCl (pH 7·5 at 25°) and 10 mM EDTA. One aliquot of each fraction was assayed for NADPH-ferricyanide reductase activity in order to determine the total activity in each fraction.

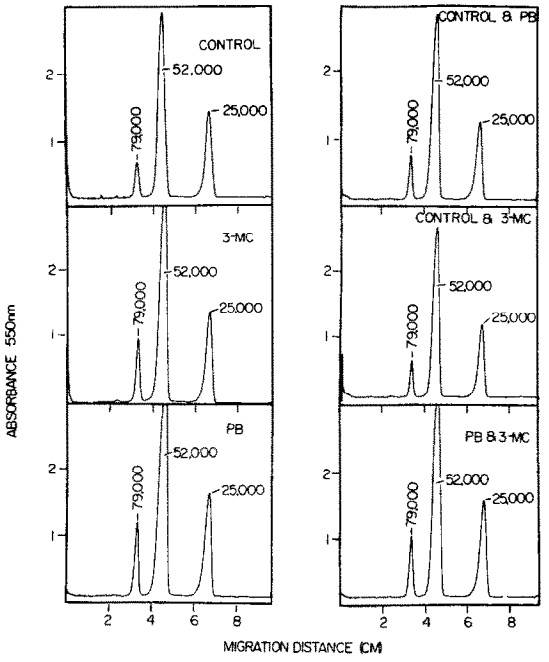

Fig. 2. Comparison of the SDS-polyacrylamide gel electrophoresis protein profiles of the immunoprecipitates formed between antibody prepared to purified bromelain-solubilized NADPH-cytochrome c reductase and detergent-solubilized liver microsomes from control and PB- or 3-MC-pretreated rats. The scans on the left-hand side of this figure compare the profiles obtained for immunoprecipitates formed between reductase antibody and detergent-solubilized liver microsomes from control and PB- or 3-MC-pretreated rats, while the scans on the right-hand side compare the profiles obtained when mixtures of these samples were electrophoresed on a single gel. The molecular weight values for the protein components present in each immunoprecipitate are based on R_f values and were determined independently for each gel.

lized NADPH-cytochrome c reductase isolated from the rat liver microsomes of PB-pretreated rats would cross-react with a single component present in the sodium deoxycholate-solubilized microsomes from control and PB- or 3-MC-pretreated rats. Furthermore, since the precipitin lines formed with each sample fused, the component being immunoprecipitated from each sample was immunochemically identical. To identify the component immunoprecipitated from each sample as the reductase enzyme, an immunoprecipitate formed between the antibody and each type of sodium deoxycholate-solubilized rat liver microsome was assayed for NADPH-ferricyanide reductase activity (Table 1). It has previously been observed that antibody to NADPH-cytochrome c reductase will inhibit the activity of this enzyme in microsomes [14, 17, 18, 22], and we have reported that, when the antibody is added to sodium deoxycholate-solubilized microsomal proteins, the ability of this enzyme to reduce cytochrome c is inhibited much more than its ability to reduce ferricyanide [19]. Therefore, the latter activity was assayed in this experiment. It can be seen that addition of the antibody to detergent-solubilized microsomes inhibited about 75 per cent of the ferricyanide reductase activity; however, all of the residual activity was found in the immunoprecipitate. This demonstrates that in each case NADPH-cytochrome c reductase was the microsomal protein being immunoprecipitated by the antibody and, in each case, that all the reductase enzyme had been immunoprecipitated by the antibody.

The protein components present in each immunoprecipitate were then examined by SDS-polyacrylamide gel electrophoresis. The protein profiles obtained for each immunoprecipitate are shown in Fig. 2 and are similar to that previously described for the immunoprecipitate formed between the antibody and sodium deoxycholate-solubilized microsomal proteins from PB-pretreated rats [19]. In each case, the immunoprecipitate contained three protein constituents, one of molecular weight 79,000, one of molecular weight 52,000 and a third of molecular weight 25,000. The 52,000 and 25,000 dalton components have the molecular weights expected for the heavy and light chains of IgG [23] and are, therefore, probably derived from the antibody. The 79,000 dalton component present in the immunoprecipitate formed between the reductase antibody and sodium deoxycholate-solubilized liver microsomes from PB-pretreated rats has been previously identified as the reductase enzyme [19]. It is, therefore, likely that the component of identical molecular weight present in the immunoprecipitate formed with sodium deoxycholate-solubilized microsomes from control and 3-MC-pretreated rats is the reductase enzyme present in these microsomes. From this experiment, it appears that the "native" reductases from each type of microsomes have identical molecular weights. Even in mixing experiments in which combinations of two different immunoprecipitates were electrophoresed in a single gel, no difference could be detected in the molecular weights of the "native" reductases (Fig. 2, right-hand scans).

Thus, on the basis of the experiments we report in this communication, it appears that the "native" NADPH-cytochrome c reductase enzymes present in the liver microsomes from control and PB- or 3-MC-pretreated rats are identical both on the basis of their antigenic similarity and their apparent molecular weights on SDS-polyacrylamide gels. This conclusion concurs with that of Kuriyama *et al.* [14] who suggested on the basis of the immunological and chromatographic similarity of the trypsin-solubilized reductase from the liver microsomes of control and PB-pretreated rats that the reductases present in these rat liver microsomes were the same. It is also consistent with the observations that antibody to the trypsin-solubilized reductase from PB-pretreated rats inhibits the reductase activity to the same degree in the liver microsomes from control and PB- or 3-MC-pretreated rats [17]. The one anomalous finding, however, is that by Lu *et al.* [9] in which it has been shown that the "native" NADPH-cytochrome c reductases partially purified from detergent-solubilized microsomes from PB- and 3-MC-pretreated rats appear to have a role in the ability of reconstituted microsomal hydroxylation systems to metabolize benzphetamine. Certainly this would not be expected if the reductase from the two types of microsomes were identical. Since the reductase preparations used in those studies were not pure, however, the observations made by Lu *et al.* [9] may have resulted from a contaminant in the preparations. Alternatively, the "native" reductases in the three different types of microsomes may differ in some very subtle manner which cannot be detected by studying their immunochemical properties or apparent molecular weights on SDS-polyacrylamide gels. Clearly, it will be necessary to isolate the "native" reductase from the three types of microsomes to differentiate between these possibilities.

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Hepatic drug metabolism in retinol-deficient rats

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Oxidative drug and steroid metabolism by hepatic microsomes requires NADPH and molecular oxygen and utilizes cytochrome P-450 as the terminal oxidase [1]. Among the factors affecting the activity of hepatic oxidative enzymes is the nutritional status of the animal. For example, protein deficiency in growing rats has been shown to lower hepatic content of cytochrome P-450 and reduce the rates of ethylmorphine and aniline metabolism [2]. Starvation also influences hepatic drug metabolism [2–5], the specific effects deficiency in growing rats has been shown to lower hepatic animal and the hepatic substrate employed. For example, Gram *et al.* [4] found starvation of male rats to produce increases in hepatic microsomal concentration of cytochrome P-450 and in the rates of ethylmorphine demethylation and aniline hydroxylation. Little change in drug oxidation was seen in starved females. Fasted guinea pigs [5], on the other hand, metabolized aniline but not aminopyrine faster than controls, and had normal hepatic levels of cytochrome P-450.

Some vitamins also seem to have an important role in the regulation of hepatic oxidative metabolism. Patel and Pawar [6] reported that chronic riboflavin deficiency in male and female rats resulted in a decline in hepatic cytochrome P-450 concentration and the metabolism of several drug substrates. Administration of riboflavin to deficient animals reversed the effects of vitamin deficiency. Vitamin C deficiency also lowers the rate of hepatic drug oxidation [5, 7]. Hepatic microsomes obtained from guinea pigs on vitamin C-deficient diets for 21 days contained less cytochrome P-450 and NADPH cytochrome P-450 reductase activity than those obtained from controls, resulting in diminished metabolism of aniline, aminopyrine and *p*-nitroanisole. Each of these effects was reversed by ascorbic acid treatment *in vivo*. Recent studies [8] indicate that α-tocopherol also plays a role in the regulation of drug metabolism. Because of the apparent importance of some vitamins for the maintenance of normal hepatic oxidative metabolism, we have now examined the effects of retinol deficiency on these enzymes in rats.

Disease-free, weanling male albino Wistar rats were obtained from Hilltop Farms, Scottdale, Pa. Animals were caged individually under standardized conditions of light

Table 1. Effects of retinol deficiency on hepatic oxidative metabolism in rats*

Group	Body wt (g)	Liver wt (g)	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Drug metabolism (nmoles/min g liver)	
					Ethylmorphine	Aniline
Control	345 ± 12 (12)	11·7 ± 0·6 (12)	24·9 ± 1·1 (12)	0·98 ± 0·08 (12)	746·7 ± 75·3 (6)	43·3 ± 3·5 (6)
Retinol-deficient	247 ± 11 (12)†	7·3 ± 0·3 (12)†	19·3 ± 0·7 (12)†	0·56 ± 0·02 (12)†	346·6 ± 66·9 (8)†	25·1 ± 4·2 (8)†
Pair-fed	320 ± 10 (4)‡	10·2 ± 0·5 (4)‡	25·7 ± 1·9 (4)‡	0·91 ± 0·10 (4)‡	673·8 ± 83·2 (4)‡	39·7 ± 5·1 (4)‡
Retinol-deficient + retinol	325 ± 13 (5)†	10·8 ± 0·7 (5)†	26·3 ± 1·8 (5)†	0·90 ± 0·07 (5)†	709·3 ± 69·4 (5)†	41·3 ± 3·9 (5)†

* Values are expressed as mean ± S.E.; number of observations per group is indicated in parentheses.
† $P < 0.05$ (vs control group).
‡ $P < 0.05$ (vs retinol-deficient group).