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Immunochemical Studies on the Role of Reduced Nicotinamide Adenine Dinucleotide Phosphate—Cytochrome c (P-450) Reductase in Drug Oxidation

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SUMMARY

Rabbit hyperimmune serum prepared to purified rat liver NADPH–cytochrome *c* rereductase (NADPH:cytochrome *c* oxidoreductase, EC 1.6.2.3) specifically inhibits the activity of this enzyme; thus, NADH–cytochrome *c* reductase was not affected. Measurement of the effects of the hyperimmune serum to NADPH–cytochrome *c* reductase on drug oxidation indicated that aminopyrine *N*-demethylation and aniline hydroxylation by rat liver microsomes from phenobarbital-treated animals, as well as benzpyrene hydroxylation by microsomes from 3-methylcholanthrene-treated animals, were inhibited. In addition, the activity of NADPH–cytochrome *c* reductase of normal, phenobarbital-treated, and 3-methylcholanthrene-treated rats was inhibited to the same extent by the γ-globulin fraction prepared from hyperimmune serum. Liver NADPH cytochrome–P-450 reductase of animals treated with phenobarbital was also inhibited by the antireductase globulin. The results indicate that the NADPH–cytochrome P-450 reductases involved in drug oxidation by liver microsomes from normal, phenobarbital-treated, and 3-methylcholanthrene-treated animals are similar.

INTRODUCTION

NADPH–Cytochrome *c* reductase (NADPH:cytochrome *c* oxidoreductase, EC-1.6.2.3) is believed to function *in vivo* as a component of the hepatic microsomal mixed-function oxidase system (1). This is indicated by the relatively synchronous and equivalent induction of both NADPH–cytochrome *c* reductase and mixed-function oxidase activity following treatment of rats with phenobarbital (2). Recent evidence suggests that this enzyme may also serve as the reductase for cytochrome P-450 (3–5). The findings that (a) approximately the same amounts of NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase activities were retained following steapsin treatment of liver microsomes (6) and (b) antibody to NADPH–cytochrome *c* reductase inhibited ω-hydroxylation of fatty acids in rat liver microsomes (7) also indicate that this enzyme may serve as P-450 reductase in the mixed-function oxidase system.

In order to test this hypothesis, rabbit antiserum to rat liver NADPH–cytochrome *c* reductase was prepared and was used to study its effect on the drug oxidases of rat

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liver microsomes. The results indicate that (*a*) NADPH–cytochrome *c* reductase, aminopyrine *N*-demethylation, aniline hydroxylation, and benzpyrene hydroxylation were inhibited by the hyperimmune serum to NADPH–cytochrome *c* reductase, (b) NADPH–cytochrome *c* reductase from the livers of normal rats, as well as those treated with either phenobarbital or 3-methylcholanthrene, was equally inhibited by antireductase globulin, and (c) NADPH–cytochrome P-450 reductase was inhibited by antireductase globulin.

MATERIALS AND METHODS

Materials. Aminopyrine was purchased from Matheson, Coleman, and Bell, and benzpyrene from Aldrich Chemical Company. [3,4-^{3}H]Benzpyrene (800 mCi/mmole) was obtained from Schwarz BioResearch, Inc. Phenobarbital sodium was obtained from Merck and Company, and 3-methylcholanthrene was purchased from Calbiochem. Aniline was obtained from Merck, and was distilled under vacuum before use. All other chemicals were of reagent grade, and glass-distilled water was used.

Enzyme assays. Male Sprague-Dawley rats (200–250 g) were employed in all experiments. Hepatic microsomes were prepared as described by Omura *et al.* (8). Trypsin digests were prepared by incubating liver microsomes in 0.1 M potassium phosphate buffer, pH 7.4, with trypsin (6 mg/10 g of liver, wet weight) dissolved in 1.0 mM HCl for 20 hr at 4°.

NADPH–cytochrome *c* reductase was purified approximately 400-fold from the washed microsomes and assayed according to the method of Omura and Takesue (9); the specific activity of the purified enzyme was 24 μmoles/min/mg of protein, and the preparation contained no NADH–cytochrome *c* reductase or NADH–cytochrome b_5 reductase activity. The difference in final specific activity between the enzyme preparation described in this report and that of Omura and Takesue (9) (37 μmoles/min/mg of protein) was due to the preparation of cytochrome *c* employed. Our assay employed horse heart cytochrome *c*, whereas Omura and Takesue used purified yeast cytochrome *c* (10).

Aminopyrine *N*-demethylation and aniline hydroxylation were measured by the methods of Schenkman *et al.* (11), and NADPH–cytochrome P-450 reductase by the procedure of Schenkman and Cinti (5).

Benzpyrene hydroxylation was determined by a modification of the method of Silverman and Talalay (12). The assay mixture contained 0.4 mM NADP, 8 mM isocitrate, 15 μg of isocitrate dehydrogenase (Sigma type IV, 7.3 μmoles/min/mg of protein), 10 nCi of [3,4-^{3}H]benzpyrene, 80 μM benzpyrene, and 20 mM potassium phosphate buffer, pH 7.4. The mixture was incubated for 10 min to generate maximal NADPH, and the assay then was initiated by the addition of microsomes (0.4 mg of protein) in 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 1.0 ml. The remainder of the assay was carried out as previously described (12).

Protein was determined by the method of Lowry *et al.*(13).

Preparation of antiserum. A male albino rabbit was immunized against 400-fold purified NADPH–cytochrome *c* reductase. The animal received one subcutaneous injection of 2 mg of enzyme mixed with Freund's complete adjuvant (Calbiochem). Three weeks after the initial injection, an intramuscular injection of 1 mg of the enzyme was given in 0.9% NaCl and repeated twice more at weekly intervals. One week after the final injection, blood was obtained from the animal by cardiac puncture. Blood was similarly collected from nonimmunized rabbits to serve as a control in the enzymatic assays. Serum was obtained by centrifugation of the clotted blood. The γ-globulin fraction of the serum was partially purified by precipitation at 33% saturation with ammonium sulfate, followed by dialysis (14).

Ouchterlony plates were purchased from Hyland Laboratories for determination of the double-diffusion patterns of the precipitin reactions.

Administration of drugs. Phenobarbital sodium was dissolved in 0.9% NaCl and injected at a dose of 100 mg/kg once daily

for 3 days. Methylcholanthrene was administered in corn oil (Mazola) at a level of 20 mg/kg once daily for 3 days.

RESULTS

The results of titration with rabbit antiserum of the activities of microsomal-bound and 400-fold purified NADPH–cytochrome *c* reductase are presented in Table 1. Both enzyme preparations were markedly inhibited by the hyperimmune serum; the purified enzyme was affected to a greater extent than was the membrane-bound preparation from untreated rats. This phenomenon occurred despite the presence of approximately 40 times less enzyme protein in the assay mixture for the microsomal enzyme. The difference in sensitivity to antiserum may be the result of either (a) a nonspecific interaction between antibody and nonenzymatic components of the membrane or (b) an impure antibody having reactivity with more than one component of the microsomes. It appears that the latter explanation is the less likely, since the enzyme preparation employed possessed a high specific activity and apparently was not a mixed antigen, in that it gave essentially one major precipitin bond upon reaction with hyperimmune serum (Fig. 1). Identical quantities of normal rabbit serum and microsome-adsorbed antiserum, employed as controls, were without effect on the activity of NADPH–cytochrome *c* reductase.

Evidence for the role of NADPH–cytochrome *c* reductase in drug oxidation is presented in Table 2. Both aminopyrine *N*-demethylation and aniline hydroxylation by microsomal preparations from livers of rats treated with phenobarbital were inhibited by the antiserum to NADPH–cytochrome *c* reductase. The extent of inhibition of enzymatic activity was a function of the concentration of protein; thus addition of larger amounts of microsomes from barbiturate-treated animals

TABLE 1

Effect of NADPH–cytochrome c reductase antiserum on membrane-bound and purified NADPH–cytochrome c reductase

Results represent the averages of duplicate determinations.

Antiserum	NADPH–cytochrome *c* reductase[a]	
	Microsomal (%)	Purified (%)
μl	*nmoles/min/mg protein*	
	52.7 (100)	23,600 (100)
10	40.1 (76)	14,300 (60)
25	29.2 (55)	8,270 (35)
50	20.8 (39)	4,230 (18)
100	10.6 (20)	1,475 (6)
100[b]		23,800 (101)

[a] The concentration of microsomal protein in the assay mixture was 0.132 mg/ml; that of purified enzyme, 0.005 mg/ml; and that of antiserum, 4 mg/ml of assay mixture.

[b] Adsorbed antiserum was prepared by incubation of antiserum with an equal volume of microsomes (0.2 mg of protein per milliliter) for 15 min at 25°, followed by centrifugation at 100,000 × *g* for 30 min. The remaining supernatant fluid was then added to the assay of the purified enzyme.

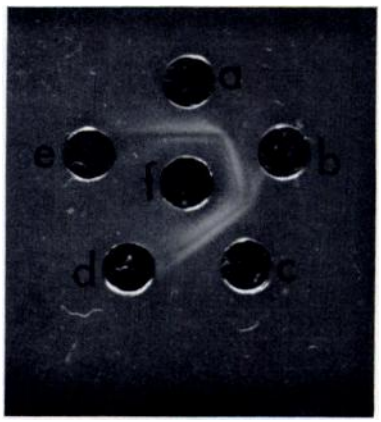

FIG. 1. *Ouchterlony double-diffusion patterns of rat liver NADPH–cytochrome c reductase*

The plate was developed for 12 hr at 25° and then for 2 days at 4°. Well *a*, purified enzyme (2.4 mg of protein per milliliter; specific activity, 24 μmoles/min/mg of protein); well *b*, trypsin-digested extract of microsomes from untreated animals (27 mg of protein per milliliter; specific activity, 63 mμmoles/min/mg of protein); well *c*, trypsin-digested extract of microsomes from phenobarbital-treated animals (49 mg of protein per milliliter; specific activity, 130 mμmoles/min/mg of protein); well *d*, normal rabbit γ-globulin (10 mg of protein per milliliter) from untreated animals; well *e*, 0.9% NaCl; well *f*, rabbit anti reductase γ-globulin (12 mg of protein per mill liter).

TABLE 2

Effect of NADPH–cytochrome c reductase antiserum on microsomal drug-metabolizing enzymes and NADH–cytochrome c reductase

Results are the means ± standard errors of determinations on three rats. Normal serum or antiserum (0.1 ml) was incubated for 5 min at 25° with 0.1 ml of microsomal suspension before addition of the assay mixture. The protein concentration of normal serum or antiserum in the assay was 4 mg/ml.

Enzyme	Microsomal protein	Normal serum	Antiserum
	mg/assay	*nmoles/min/mg microsomal protein*	
Aminopyrine demethylase[a]	0.2	24.4 ± 2.0	0
	0.8	25.7 ± 3.0	16.0 ± 2.1
Aniline hydroxylase[a]	0.2	3.69 ± 0.30	0
	0.8	4.13 ± 0.58	1.91 ± 0.16
Benzpyrene hydroxylase[b]	0.2	3.78 ± 0.21	0
NADH–cytochrome *c* reductase[c]	0.025	1260 ± 35	1150 ± 30
NADH–cytochrome b_5 reductase[c]	0.025	76 ± 5	70 ± 3

[a] Rats were treated once daily with 100 mg/kg of phenobarbital for 3 consecutive days. Activity is expressed as nanomoles of formaldehyde produced per minute per milligram of protein for aminopyrine demethylase, and as nanomoles of *p*-aminophenol produced per minute per milligram of protein for aniline hydroxylase.

[b] Rats were treated once daily with 20 mg/kg of 3-methylcholanthrene for 3 consecutive days.

[c] Untreated rats.

resulted in a lesser degree of inhibition by the hyperimmune serum. Benzpyrene hydroxylation by liver microsomes prepared from 3-methylcholanthrene-treated animals was also markedly decreased by the hyperimmune serum. The enzyme activities (Table 2), assayed after treatment with either phenobarbital or 3-methylcholanthrene, were about 4 times higher than those of microsomes from untreated rats. Of particular importance was the lack of effect of hyperimmune serum to NADPH–cytochrome *c* reductase on microsomal NADH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase, a finding indicative of the specificity of the antibody preparation.

In later experiments a partially purified (approximately 10-fold) γ-globulin fraction prepared from the hyperimmune serum to NADPH–cytochrome *c* reductase was employed. Ouchterlony double-diffusion patterns of the interaction between antibody and various enzyme preparations (Fig. 1) indicated that the precipitin reaction was relatively specific for NADPH–cytochrome *c* reductase. A major precipitin band and a minor reaction component were obtained with both the pure and crude enzymes, while no antigen-antibody reaction was detected with γ-globulin from a nonimmunized animal. However, the purified enzyme migrated electrophoretically as a homogeneous protein on polyacrylamide gel (9) and was at least as pure as preparations of NADPH–cytochrome *c* reductase reported previously (10, 15, 16). Thus, it is unlikely that the enzyme was not a relatively pure antigen; the minor precipitin line that appeared on double-diffusion analysis might well have been due to dissociation of the enzyme to yield a subunit which migrated faster than the holoenzyme. Evidence that NADPH–cytochrome *c* reductase is composed of subunits has been reported by Ichikawa and Yamano (17).

As previously shown with hyperimmune serum, the antireductase globulin inhibited microsomal NADPH–cytochrome *c* reductase of normal liver (Fig. 2), while γ-globulin from a nonimmunized rabbit had no effect. In addition, enzyme preparations from the liver microsomes of rats treated with phenobarbital and with 3-methylcholanthrene were affected to a similar degree. It should be noted that 3-methylcholanthrene did not induce NADPH–cytochrome *c* reductase, causing instead a slight reduction in specific

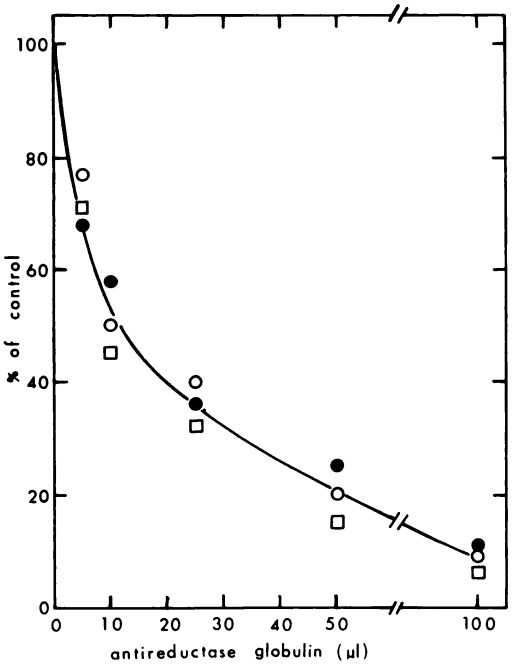

FIG. 2. *Effect of anti-NADPH-cytochrome c reductase globulin on microsomal NADPH-cytochrome c reductase activities from normal, phenobarbital-treated, and 3-methylcholanthrene-treated rats*

Experimental conditions were the same as described in Table 1. The concentration of microsomal protein was 0.08 mg in a total volume of 1.0 ml, and that of antireductase globulin was 12 mg of protein per milliliter. ○, untreated rats; ●, phenobarbital-treated rats; □, 3-methylcholanthrene-treated rats. The specific activities of NADPH-cytochrome *c* reductase were: untreated rats, 42.0; phenobarbital-treated rats, 84.2; 3-methylcholanthrene-treated rats, 33.3 nmoles/min/mg of protein. Each point is the mean of duplicate determinations on each of two rats.

activity. A similar effect has been noted by Glaumann (18).

The activity of NADPH-cytochrome P-450 reductase of microsomal preparations from phenobarbital-treated rats was also inhibited by the globulin fraction from hyperimmune serum to NADPH-cytochrome *c* reductase (Fig. 3). The activity of NADPH-cytochrome P-450 reductase was inhibited to approximately the same extent as the metabolism of aminopyrine and aniline (Table 2), but only one-half as much as NADPH-cytochrome *c* reductase, at equivalent amounts of antibody and microsomal protein.

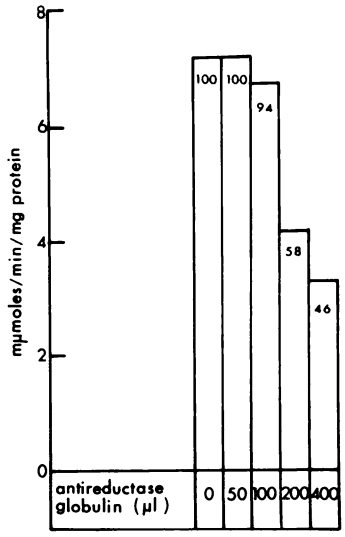

FIG. 3. *Effect of anti-NADPH-cytochrome c reductase globulin on P-450 reductase*

Microsomes were incubated with either globulin or antireductase globulin for 5 min at 37° before addition of 0.1 M potassium phosphate buffer, pH 7.5, to a final volume of 2.5 ml. Assays contained a total of 0.8 mg of microsomal protein. The reactions were initiated by the rapid addition of 20 µl of NADPH (final concentration, 0.36 mM), and reduction was measured by the increase in absorbance between 450 and 475 nm as described previously (5). Each value is the result of determinations using the microsomal fraction pooled from two rats treated once daily with 100 mg/kg of phenobarbital for 3 consecutive days.

DISCUSSION

Previous studies by Kuriyama *et al.* (10) have shown that the normal and phenobarbital-induced NADPH-cytochrome *c* reductase enzymes are immunologically identical in their reactivity with antibody prepared to normal cytochrome *c* reductase. This has been confirmed by our experiments. Lu *et al.* (19) have also reported that the ability of NADPH-cytochrome *c* reductase to function in the over-all hydroxylation of fatty acids in a partially purified, solubilized system was related to its ability to reduce cytochrome P-450. The data reported in this paper support the concept that NADPH-cytochrome *c* reductase has a

common role in aminopyrine *N*-demethylation, aniline hydroxylation, and benzpyrene hydroxylation. Inhibition of NADPH–cytochrome P-450 reductase activity by hyperimmune serum to NADPH–cytochrome *c* reductase also implies that the latter enzyme serves to transfer electrons to cytochrome P-450 in the microsomal mixed-function oxidase system. These effects were independent of the type of inducing agent employed (i.e., phenobarbital or methylcholanthrene), and the induced enzyme activities were equally sensitive to inhibition by antireductase serum. These findings suggest that NADPH–cytochrome *c* reductase enzymes of both normal and barbiturate- and hydrocarbon-induced microsomes are antigenically identical. The lesser sensitivity to the antibody of NADPH–cytochrome P-450 reductase from phenobarbital-treated rats as compared to NADPH–cytochrome *c* reductase is not entirely unexpected when one considers that the assay of the former enzyme activity relies on endogenous cytochrome P-450 while the latter activity utilizes an exogenous supply of cytochrome *c*. Close proximity between cytochrome P-450 and the reductase would be expected to lessen the opportunity for the antibody to interfere with the interaction between substrate and enzyme. In the case of NADPH–cytochrome *c* reductase, the antigen-antibody reaction conceivably prevents by steric hindrance the binding of the relatively large cytochrome *c* molecule to the active site of the enzyme, thereby producing relatively greater inhibition.

In view of the results, it is further hypothesized that the differences in induction between methylcholanthrene- and barbiturate-induced enzyme activity are predominantly the result of an alteration of the cytochrome P-450 hemoprotein (20–23) and are not due to any qualitative change in the structure of NADPH–cytochrome *c* reductase. However, the possibility still remains that an altered but antigenically similar form of the reductase is formed following administration of the different inducing agents. Additional immunochemical studies of purified NADPH–cytochrome *c* reductase from phenobarbital- and methylcholanthrene-treated animals may clarify this matter.

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