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Interaction of Cytochrome P-450 with Antibodies Raised against a Solubilized P-450 Preparation from Hepatic Microsomes

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SUMMARY

Soluble cytochrome P-450 prepared from microsomes of rat liver or from livers of chick embryos was shown to be capable of eliciting an immunological response in rabbits. The γ-globulin fraction from sera of immunized rabbits reacted with soluble P-450 of the same species. Precipitating reactions were obtained when antibody and antigen were incubated in the fluid phase, as well as by agar diffusion techniques. Lack of cross-reactivity between species was demonstrated when antibody to rat cytochrome P-450 was allowed to react with soluble P-450 from chick embryo livers and when antibody to chick cytochrome P-450 was allowed to react with soluble P-450 from rat liver. The precipitate obtained upon reaction of antibody and antigen from the same species showed a typical CO difference spectrum with an absorption maximum at 450 nm and a type II difference spectrum with aniline. However, no type I difference spectrum could be obtained after the addition of sodium hexobarbital to the antibody-antigen complex, suggesting that the binding site of the immune complex may be at or near the type I binding site.

INTRODUCTION

Cytochrome P-450 has been found in man (1), animals (2–5), yeast, (6), and bacteria (7, 8). The cytochrome in these species is characterized by its carbon monoxide–hemoprotein complex, which has an absorption maximum at 450 nm. In mammalian liver, this cytochrome is the terminal oxidase in the "mixed-function" oxidation of drugs, carcinogens (9, 10), steroids (11), and fatty acids (12, 13). There are unexplained differences among various animal species in hepatic microsomal drug metabolism and in the duration of drug action (10). Such species variation could be due at least partly to qualitative differences in the hemoprotein P-450 or to differences in the rate-limiting components of the oxidase system. The use of immunoassay methods to investigate this cytochrome appears to hold some promise in clarifying the basis for these species differences. In addition, such methods afford the possibility of selective enzymatic blockade of hepatic drug metabolism, which could be quite informative from the experimental point of view. Previous workers have prepared antisera which react with purified and solubilized preparations of cytochrome b_5, NADH–cytochrome b_5 reductase (14), and NADPH–cytochrome c reductase (15) from rat liver.

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We have described (16) a procedure for the partial purification and solubilization of cytochrome P-450 from chick embryo liver microsomes, using protease VII for solubilization of cytochrome b_5 and NADPH–cytochrome *c* reductase and Lubrol WX for solubilization of cytochrome P-450. In the final solubilized P-450 preparation, cytochrome b_5 was absent and minimal amounts of NADH– and NADPH–cytochrome *c* reductases and cytochrome P-420 were present.

The present report describes an analysis of the reactions given by antisera raised against partially purified and solubilized cytochrome P-450 preparations obtained from the chick and the rat. Using these immune sera, immunological differences between the soluble preparations of cytochrome P-450 from rat and chick embryo livers have been demonstrated, and the spectral characteristics of the precipitate obtained upon reaction of the antisera with the solubilized P-450 preparations have been studied.

MATERIALS AND METHODS

Preparation of solubilized P-450 fraction from livers of phenobarbital-treated rats. Immature male Long-Evans rats (70–75 g) were fed a commercial diet and water ad libitum. Sodium phenobarbital in 0.9% NaCl solution was injected intraperitoneally at a dose of 37.5 mg/kg twice a day for 4 days. Hepatic microsomes were prepared, and the microsomal pellets were layered with 0.1 M K_2HPO_4–KH_2PO_4 buffer, pH 7.0, and stored at −20° until used.

Microsomal cytochrome P-450 was partially purified and solubilized by the method described previously (16) for chick embryo liver microsomes. The final P-450 preparation was dissolved in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, containing 25% glycerol, and stored at −20° until used. This preparation has been termed "soluble P-450," since no pellet was obtained and no cytochrome P-450 was lost, as determined spectrally, after centrifugation of the preparation for 120 min at 105,000 × *g* in 0.1 M sodium phosphate buffer, pH 7.0, containing 5% glycerol. The specific gravity of 5% glycerol in 0.1 M sodium phosphate buffer, determined with a hydrometer, was found to be equal to 1.03 at 4°.

Soluble cytochrome P-450 fractions were also prepared from microsomes of livers of chick embryos treated with allylisopropylacetamide or 17α-hydroxy-11-ketopregnanolone. AIA[4] or the steroid (1.5% solution, w/v), dissolved in propylene glycol in a volume of 0.2 ml, was injected into the yolk sacs of 17–19-day-old chick embryos through a small hole drilled into the blunt end of the eggs, which then were incubated at 37° for 24 hr.

In these solubilized fractions, neither cytochromes P-450 and b_5 nor hemoglobin could be detected by spectroscopic methods. Less than 5% contamination with NADPH– and NADH–cytochrome *c* reductase was found.

Immunological studies. Pairs of adult white rabbits were immunized with soluble cytochrome P-450 prepared from liver microsomes of PB-treated rats or from liver microsomes from AIA-treated chick embryos. Soluble P-450 (specific content, 2.65 nmoles/mg of protein), containing 10 mg of protein per milliliter of 0.1 M sodium phosphate buffer–25% glycerol, was emulsified with an equal volume of Freund's complete adjuvant (Difco). Subcutaneous injections were performed every 2 weeks for 3 months, and blood was taken 10 days after the last injection. The γ-globulin fraction of the antisera was obtained by two precipitations from half-saturated ammonium sulfate and dialyzed against 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0, for 24 hr. Control γ-globulin fractions were prepared in a similar manner from untreated rabbits. The γ-globulin fractions were suspended in 0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.0. Ouchterlony double-diffusion analysis (17) was performed at room temperature for 24 hr in 0.005% NaN_3 and 0.6% Agarose dissolved in 0.05 M KH_2PO_4–Na_2HPO_4 buffer, pH 7.2.

The reaction between the antibody fractions of the antisera and soluble cytochrome P-450 was studied in the following manner. Soluble P-450 fractions, when used

[4] The abbreviations used are: AIA, allylisopropylacetamide; PB, phenobarbital.

as antigens, were prepared from livers of AIA-treated chick embryos, 17α-hydroxy-11-ketopregnanolone-treated chick embryos, PB-treated rats, and untreated rats. Immunoglobulins isolated from rabbit antisera and used as antibody fractions were anti-AIA-treated chick embryo P-450 and anti-PB-treated rat P-450. To study precipitation reactions between antibody fractions and soluble cytochrome P-450, 1.0 ml of the solubilized P-450 preparation (protein concentration, 0.5–0.8 mg/ml, unless otherwise specified) was mixed with 1.0 ml of antibody fraction containing various amounts of protein. As a control, 1.0 ml of the γ-globulin fraction (protein concentration, 35–40 mg/ml) from nonimmunized rabbits was used. The antibody and antigen solutions were mixed and incubated at 36° for 60 min and stored overnight at 4°. The solutions were then centrifuged in a Spinco model L ultracentrifuge at 54,000 × *g* for 30 min, using a type 50 rotor. The top, turbid layer was carefully removed with a pipette and discarded, and the remaining supernatant fluid was collected and saved. The precipitates were suspended in 1.5–2.0 ml of 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, containing 25% glycerol. No precipitates were formed when γ-globulin fractions from untreated rabbits were added to each of the antigen preparations.

Analytical methods. Cytochrome P-450 and P-420 contents were determined by the method of Omura and Sato (18), using extinction coefficients of 91 mM^{-1} cm^{-1} between 450 and 490 nm for cytochrome P-450 in the starting microsomal fraction (18), 58 mM^{-1} cm^{-1} between 450 and 490 nm for soluble cytochrome P-450 (19), and 111 mM^{-1} cm^{-1} between 420 and 490 nm for cytochrome P-420 (18). For the determination of cytochrome b_5, the NADH reduced minus oxidized difference spectrum was measured between 424 and 409 nm, and the hemoprotein content was determined using an extinction coefficient of 185 mM^{-1} cm^{-1} (18). Protein was determined by the method of Lowry *et al.* (20), using bovine serum albumin as a standard. NADPH- and NADH–cytochrome *c* reductase activities were estimated by measuring the rate of reduction of cytochrome *c* as described previously (16).

The sodium hexobarbital- and aniline-induced difference spectra of cytochrome P-450 in the solubilized preparation and in the precipitate obtained after reaction between antigen (PB rat P-450 preparation) and antibody (anti-PB rat P-450) were determined at room temperature, using an Aminco-Chance dual-wavelength, split-beam spectrophotometer in the split-beam mode, as described previously (16).

The final concentration of sodium hexobarbital or aniline in the sample cuvette was 4 mM. This concentration was considered to be saturating, since it was at least 10 times the K_s (spectral binding constant) values reported previously by Schenkman *et al.* (21) for hexobarbital and aniline.

RESULTS

Preparation of solubilized cytochrome P-450 from livers of rats treated with PB. Rats were treated with PB at a dose of 37.5 mg/kg twice a day for 4 days. Figure 1 schematizes the procedure for obtaining the solubilized cytochrome P-450 preparation from rat hepatic microsomal fractions. The precipitate obtained with saturated $(NH_4)_2SO_4$ was dissolved in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7.0, containing 25% glycerol, and this preparation has been termed soluble P-450. About 30% of the cytochrome P-450, as determined spectroscopically, and about 34% of the protein were recovered in the soluble P-450 fraction. The CO difference spectrum of the solubilized P-450 preparation was similar to that obtained using microsomes. No peak at 420 nm was seen, indicating the absence of cytochrome P-420. The specific content of the solubilized P-450 preparation, 3.14 nmoles/mg of protein, was higher than that of P-450 in the starting microsomes, 2.04 nmoles/mg of protein. The soluble cytochrome P-450 preparation contained NADPH- and NADH–cytochrome *c* reductases, measured enzymatically by the method of Dallner *et al.* (22), in amounts of less than 5% of those present in the starting microsomes. The soluble cytochrome P-450 thus prepared was used as the antigen in the immunological studies.

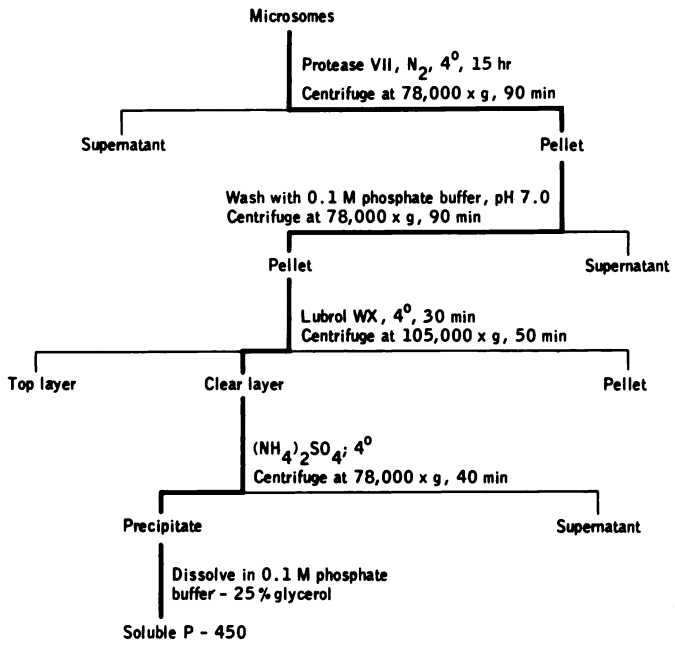

FIG. 1. *Schematic representation of procedure for preparation of soluble cytochrome P-450*
Microsomes were treated with protease VII (20 μg/mg of microsomal protein) to release cytochrome b_5 as described earlier (16). Following treatment with protease VII and centrifugation, the pellet obtained was treated with Lubrol WX (2 mg/mg of protein), and the solubilized P-450 preparation thus obtained was precipitated with saturated $(NH_4)_2SO_4$ at a final concentration of 50%.

Antigen-antibody reaction. Rabbits were immunized with solubilized cytochrome P-450 obtained from liver microsomes of PB-treated rats. Following immunization of the rabbits, γ-globulin fractions were prepared from their sera, and the ability of the antibody fraction to precipitate cytochrome P-450 was studied in fluid-phase experiments. Solubilized cytochrome P-450 from livers of PB-treated rats was incubated with various amounts of the antibody fraction. Following incubation and subsequent centrifugation, the amounts of cytochrome P-450 were determined in the precipitates and supernatant fractions. Increases in the amount of antibody in the mixture resulted in increases in the cytochrome P-450 content of the precipitate (Fig. 2). At the antigen to antibody fraction protein ratio of 1:95, nearly all the cytochrome P-450 was precipitated by the immunoglobulin fraction. In control experiments, when the γ-globulin fraction of nonimmune serum was used, no precipitation of soluble P-450 occurred.

The antigen-antibody reaction was also studied by immunizing rabbits with soluble cytochrome P-450 derived from livers of AIA-treated chick embryos or from PB-treated rats. The immunoglobulin fraction obtained from the antisera was incubated with the corresponding antigen or other soluble P-450 fractions. Following incubation, the reaction mixture was centrifuged and the cytochrome P-450 content in the supernatant fluid was determined. The data from these experiments are presented in Table 1. When anti-AIA chick P-450 was allowed to react with the P-450 antigen obtained from AIA-treated chick embryos and 17 α-hydroxy-11-ketopregnanolone-treated chick embryos, only about 3% and 15%, respectively, of the P-450 content of the antigen remained in the supernatant fraction. Chick embryos were treated with AIA or 17α-hydroxy-11-ketopregnanolone because these compounds have been shown (16) to be potent inducers of cytochrome P-450 in chick embryo liver. However, when PB-treated rat P-450 was allowed to react

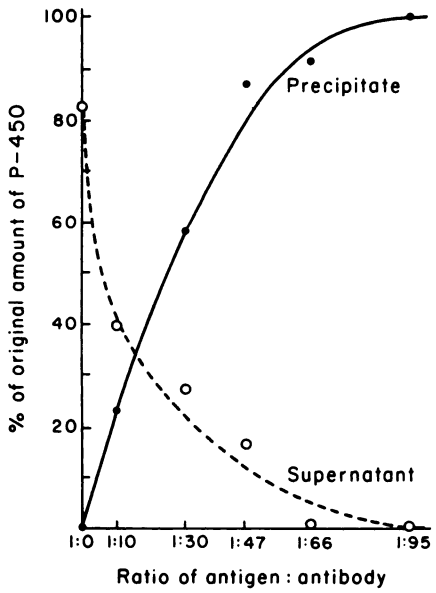

FIG. 2. *Quantitative precipitation reaction between anti-PB rat P-450 and soluble P-450 from PB-treated rats*

The antibody fraction from sera of rabbits immunized against cytochrome P-450 from PB-treated rats was obtained as described in MATERIALS AND METHODS. Various protein concentrations of the antibody fraction were prepared in a final volume of 1.0 ml. One milliliter of the soluble P-450, containing 0.79 mg of protein and 1.58 nmoles of cytochrome P-450 in 0.1 M sodium phosphate buffer–25% glycerol, was then added, and the mixture was incubated for 1 hr at 37° and stored overnight at 4°. Following centrifugation, the supernatant fractions and precipitates were assayed for cytochrome P-450 content, and the percentage values were calculated from the original P-450 content of the soluble P-450 preparation.

with anti-AIA chick P-450, over 95% of the cytochrome P-450 from PB-treated rats remained in the supernatant fraction (Table 1). Similarly, when the antibody to the soluble P-450 fraction from PB-treated rats was used, the P-450 content in the supernatant fraction was decreased by 90% upon reaction of the antibody with soluble P-450 from PB-treated rats. In contrast, less than 10% of the cytochrome P-450 content was precipitated out of solution when anti-PB rat P-450 was allowed to react with soluble P-450 from AIA-treated chicks (Table 1).

Antigen-antibody studies by gel diffusion. The antigen-antibody reactions were also studied by double-diffusion experiments in agarose, using the Ouchterlony double-diffusion analysis technique (17). Figure 3a shows the precipitation reactions obtained upon reaction of rat cytochrome P-450 with various amounts of the antibody fraction obtained from sera of rabbits immunized to rat P-450. Close inspection of the precipitin lines revealed the presence of three bands: two relatively faint lines were present on either side of a strong central band. No reactivity was observed between the γ-globulin fraction of sera from nonimmunized rabbits and soluble P-450 from rat liver (Fig. 3a). The outer faint line was also obtained when anti-rat cytochrome P-450 was

TABLE 1

Quantitative precipitation reactions between various soluble cytochrome P-450 preparations and antibodies to rat liver and chick embryo liver P-450

Soluble cytochrome P-450 preparations from AIA-treated or 17α-hydroxy-11-ketopregnanolone-treated chick embryo livers and from PB-treated rat livers were prepared as described previously (16). Antibody fractions were prepared as described in MATERIALS AND METHODS. One milliliter of the soluble P-450, containing 1.5–2.6 nmoles of P-450 (0.5–1.0 mg of protein), was incubated with 1.0 ml of antibody fraction (50–78 mg of protein) at the antigen to antibody fraction ratios indicated. Following incubation at 37° for 1 hr and storage overnight at 4°, the contents of the incubation flasks were centrifuged at 54,000 × *g* for 30 min. The cytochrome P-450 content of the supernatant fluid was then determined and expressed as a percentage of the P-450 content in the supernatant obtained when the antigen was incubated with the γ-globulin fraction from nonimmunized rabbits.

Antibody and soluble P-450 preparation	Antigen to antibody fraction protein ratio	P-450 content of supernatant
		% control
Anti-AIA chick P-450		
AIA chick P-450	1:156	3.3
17α-Hydroxy-11-keto-pregnanolone chick P-450	1:156	15.6
PB rat P-450	1:112	95.2
Anti-PB rat P-450		
PB rat P-450	1:51	10.4
AIA chick P-450	1:112	99.2

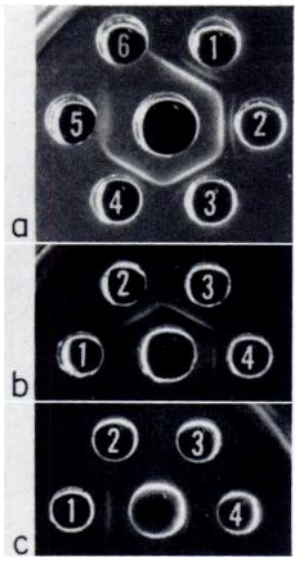

FIG. 3. *Double-diffusion pattern of antibody interaction with soluble cytochrome P-450 preparation in Agarose*

a. Soluble P-450 (3.28 nmoles/mg of protein) from PB-treated rats, containing 160 μg of protein, was placed in the center well. In the outer wells was placed anti-PB-treated rat P-450 containing the following amounts of protein: *1*, 15.0 mg; *2*, 10.5 mg; *3*, 7.5 mg; *4*, 4.5 mg; *5*, 1.5 mg. Well *6* contained the γ-globulin fraction (7.1 mg of protein) from nonimmunized rabbits.

b. The center well contained anti-PB-treated rat P-450 (4 mg of protein). The outer wells contained the following: *1*, soluble P-450 (6.04 nmoles/mg of protein) from livers of AIA-treated chick embryos; *2*, soluble P-450 (1.88 nmoles/mg of protein) from livers of untreated rats; *3*, soluble P-450 from livers of PB-treated rats; *4*, γ-globulin fraction from sera of untreated rats. The soluble P-450 preparations used contained about 200 μg of protein.

c. The center well contained anti-AIA-treated chick embryo liver P-450 (4 mg of protein). The outer wells contained the same antigens as in Fig. 3b, in the same sequence. Double-diffusion analyses were carried out in 0.6% Agarose and 0.005% NaN_3 dissolved in 0.05 M sodium phosphate buffer, pH 7.0, at room temperature for 24 hr.

allowed to react with the γ-globulin fraction from sera of untreated rats, as shown in Fig. 3b. This line could be removed by incubating the soluble P-450 with serum from untreated rats prior to reaction with the antibody fraction, but was not studied further. The nature of the antigens in the remaining two bands is the subject of further investigation.

Figure 3b also shows the Ouchterlony diffusion analysis of anti-PB rat P-450 upon reaction with various antigens. Precipitation lines were obtained only when control rat P-450 or PB-treated rat P-450 reacted with anti-PB rat P-450. Cytochrome P-450 from AIA-treated chick embryos did not cause a precipitation reaction with anti-PB rat P-450. Similarly, no cross-reaction was ob-

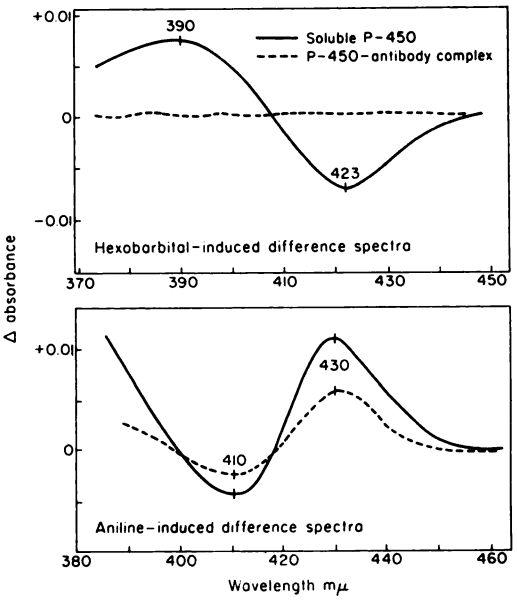

FIG. 4. *Substrate-induced difference spectra of soluble cytochrome P-450 and soluble P-450–antibody complex*

The protein concentration and specific content of soluble P-450 from PB-treated rats were 2 mg/ml and 1.26 nmoles/mg of protein, respectively. To prepare the P-450–antibody complex, the soluble P-450 preparation, containing 6 mg of protein in 3 ml of sodium phosphate buffer, was incubated with anti-PB rat P-450 (200 mg of protein per 3 ml) at 37° for 1 hr. The incubation flask was then stored overnight at 4° and centrifuged at 54,000 × *g* for 30 min. The precipitate obtained was suspended in 0.1 M sodium phosphate buffer, pH 7.0, to give a final concentration of protein, derived from soluble P-450, of 2 mg/ml for determination of the sodium hexobarbital-induced and aniline-induced difference spectra. The final concentration of sodium hexobarbital or aniline in the sample cuvette was 4 mM.

served between rat P-450 and anti-chick P-450 (Fig. 3c).

Characterization of cytochrome P-450 precipitated by antibody. Following incubation of PB-treated rat cytochrome P-450 with anti-PB rat P-450 under the conditions described in MATERIALS AND METHODS, the precipitate obtained was resuspended in 0.1 M sodium phosphate buffer, pH 7.0. The suspension of the precipitate showed a typical CO difference spectrum when reduced with sodium dithionite and complexed with CO, with an absorption maximum at 450 nm. No peak at 420 nm was detected in this preparation. Figure 4 shows the difference spectra obtained when either sodium hexobarbital, a type I substrate, or aniline, a type II substrate, was added to the soluble P-450 preparation and to the antibody–P-450 complex. The soluble P-450 preparation gave a type I difference spectrum with sodium hexobarbital and a type II difference spectrum with aniline. However, the suspension of P-450 precipitated after incubation with antibody did not show a type I binding difference spectrum with hexobarbital, but was capable of producing a type II difference spectrum with aniline. When soluble cytochrome P-450 was incubated with control γ-globulin under the same experimental conditions as those for incubation with the antibody, no loss of the type I binding spectrum occurred.

DISCUSSION

We have already described (16) a method for the partial purification and solubilization of cytochrome P-450 from chick embryo liver microsomes. In the present report, liver microsomes from PB-treated rats were used as the starting material for the purification and solubilization of cytochrome P-450. The soluble P-450 preparation was devoid of cytochrome P-420, as measured by the CO difference spectrum. Recently Mannering (19) has reported an extinction coefficient of 58 mM^{-1} cm^{-1} between 450 and 490 nm for the CO difference spectrum of soluble cytochrome P-450 derived from PB-treated rat liver microsomes. Using this extinction coefficient for the soluble P-450 and an extinction coefficient of 91 mM^{-1} cm^{-1} (18) for the membrane-bound P-450 in the starting microsomes, the specific content of the soluble P-450 preparation, measured as nanomoles of P-450 per milligram of protein, was about 1.5 times that of the starting microsomes.

We have shown that rabbits can form antibody to cytochrome P-450 when soluble P-450 from rats or chicks is used as the source of antigen. Addition of increasing amounts of anti-rat P-450 to soluble P-450 resulted in the precipitation of increasing amounts of soluble P-450 (Fig. 2). Similarly, using the gel diffusion technique, when antigen was allowed to react with antiserum against the same species, one strong and two weak precipitation lines were obtained on the Ouchterlony plates (Fig. 3a). One of the weak lines appeared to be nonspecific for cytochrome P-450, because this line was also obtained upon reaction of anti-rat P-450 with the γ-globulin fraction of sera prepared from untreated rats (Fig. 3b). Which of the remaining lines is specific for cytochrome P-450 is now under investigation.

Studies presented in this paper also show that the microsomal cytochrome P-450 prepared from chick embryo liver was not immunologically similar to that obtained from rat liver. Both the fluid-phase studies (Table 1) and the gel diffusion studies (Fig. 3b and c) showed a lack of cross-reaction between anti-chick P-450 and rat P-450 and between anti-rat P-450 and soluble P-450 from chick embryos. While the lack of cross-reaction indicates relative immunological differences between the P-450 preparations from different species, these soluble P-450 preparations showed similar CO difference spectra and abilities to bind type I and type II substrates. Species differences regarding these parameters might be revealed in the affinities of the P-450 for particular substrates in drug metabolism studies. These studies are presently in progress. The possibility must be kept in mind, however, that binding sites vicinal to the heme moiety of the P-450 may be structurally similar in all species, and that the immunological differences noted here relate only to structural characteristics of the remainder of the P-450 apoproteins.

The precipitate obtained when the antibody fraction to cytochrome P-450 was allowed to react with P-450 from the same species gave a typical CO difference spectrum and a type II difference spectrum with aniline, but displayed no type I difference spectrum with sodium hexobarbital (Fig. 4). This suggests that the site of binding of the antibody fraction to soluble P-450 may be at or near the type I substrate-binding site. Another explanation for the failure of the P-450–antibody complex to show a type I binding difference spectrum might be that anti–P-450 precipitates P-450 without the phospholipid which is a component of P-450 and may be required for the type I binding site (23). In addition, the incubation of soluble cytochrome P-450 with antibody results in no conversion of P-450 to P-420, since cytochrome P-420 was absent from the resuspended precipitate of the mixture after reaction with antibody.

It is probable that the soluble cytochrome P-450 preparation contains proteins other than the hemoprotein P-450, or that the preparation may represent a membrane fraction or its subunit. With these possibilities in mind, antibodies may be formed in part or entirely to the non-cytochrome moiety of the soluble P-450 preparation. However, the fluid-phase experiments and substrate-binding studies indicate that the antibody(ies) formed can precipitate cytochrome P-450, and although the antigenic site may not involve the cytochrome moiety, it is near enough for the antibody-antigen complex to mask the type I binding site.

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