ANTIBODY SPECIFICITY FOR CYTOCHROME P450 IN THE MICROSOMAL ELECTRON TRANSPORT SYSTEM

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ABSTRACT

ANTIBODY SPECIFICITY FOR CYTOCHROME P450

IN THE MICROSOMAL ELECTRON TRANSPORT SYSTEM

By

Linda Christine Chaney

The objective of this research was twofold: 1) to present evidence for the specificity of anti-hemoprotein 3 for hemoprotein 3 and 2) to present evidence for the orientation of hemoprotein 3 within the microsomal membrane. The 44,000 dalton hemoprotein partially purified from microsomes isolated from phenobarbital pretreated rats was the antigen used to elicit an antibody response in rabbits. The antibody was isolated and its specificity initially examined by Dr. Welton using the technique of immunoprecipitation. The antibody appeared to be specific for hemoprotein 3.

By using several different immunologic techniques, evidence is given to support further the specificity of anti-hemoprotein 3 for hemoprotein 3 and to determine indirectly the orientation of the hemoprotein within the microsomal membrane. The results from Ouchterlony double diffusion analyses, agglutination assays and complement

fixation assays indicate that the antibody is specific for hemoprotein 3. The results from antibody inhibition of aminopyrine-N-demethylation imply that the active site of hemoprotein 3 is either buried within the microsomal membrane or protected on the exterior of the membrane by surrounding proteins.

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By

Linda Christine Chaney

A THESIS

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To My Parents

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Steven D. Aust, my graduate advisor, for his advice, encouragement and friendship throughout the phases of my work. I would also like to thank members of my committee, Drs. John C. Speck and Pamela J. Fraker, for their assistance and encouragement.

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LIST OF ABBREVIATIONS

BHT	butylated hydroxytoluene
CM	carboxymethyl
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetate
IgG	immunoglobulin G
3-MC	3-methylcholanthrene
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PB	phenobarbital
Reductase	NADPH-cytochrome c reductase
SDS	sodium dodecyl sulfate
SRBC	sheep red blood cells
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

The rat liver endoplasmic reticulum contains an electron transport system which utilizes both NADPH and O_2 in the hydroxylation of various lipophilic compounds(1-3). This microsomal mixed-function oxidase system detoxifies various lipophilic drugs, carcinogens, pesticides and xenobiotics by making them more water soluble and therefore, more easily excreted. Two unusual properties are characteristic of the mixed-function oxidase system: 1) it catalyzes a wide range of reactions that can generally be classified as hydroxylation reactions(4,5) and 2) this hydroxylation activity can be induced by <u>in vivo</u> treatment of animals with various lipophilic xenobiotics(3,6). Induction of enzymes by phenobarbital, a general inducer which increases hydroxylation activity towards most substrates, and 3-methylcholanthrene, a specific inducer which stimulates activity towards a particular substrate, has been studied(3).

Two microsomal proteins associated with the mixed-function oxidase electron transport chain are NADPH-cytochrome c reductase and cytochrome P450(1-3).

NADPH-cytochrome c reductase, first isolated and characterized from liver microsomes by Williams and Kamin(7), is a flavoprotein assayed by its ability to transfer electrons from NADPH to exogenous cytochrome c. Cytochrome P450, first discovered in liver microsomes by Klingenberg(8) and Garfinkel(9), is a hemoprotein. Its name is derived from a carbon monoxide difference spectrum of dithionite-reduced samples in which maximum absorbance occurs at 450 nm(10,11). The current hypothesized mechanism for involvement of both microsomal proteins in the mixed-function oxidase system is summarized in Figure 1(12).

The idea that a mixed-function oxidase system could metabolize a wide array of substrates without some specificity is baffling since most enzymes function with specific substrates. Perhaps specificity does exist and the system does conform to the concept of substrate specificity commonly found in other systems. If, for example, animals are pretreated with PB, then induction of several enzymes in the microsomal electron transport system occurs and the system metabolizes a wide range of substrates. On the other hand, induction of specific enzymes by pretreatment of animals with 3-MC occurs, and a specific substrate is metabolized. The differences observed in drug metabolism by these inducers may be due to multiple enzyme systems

Figure 1. **MECHANISM FOR THE INVOLVEMENT OF NADPH-CYTOCHROME C REDUCTASE AND CYTOCHROME P450 IN THE MIXED-FUNCTION OXIDASE SYSTEM**

The schematic, taken from Gillette(12), shows the current hypothesized mechanism for the involvement of NADPH-cytochrome c reductase and cytochrome P450 in the mixed-function oxidase system.

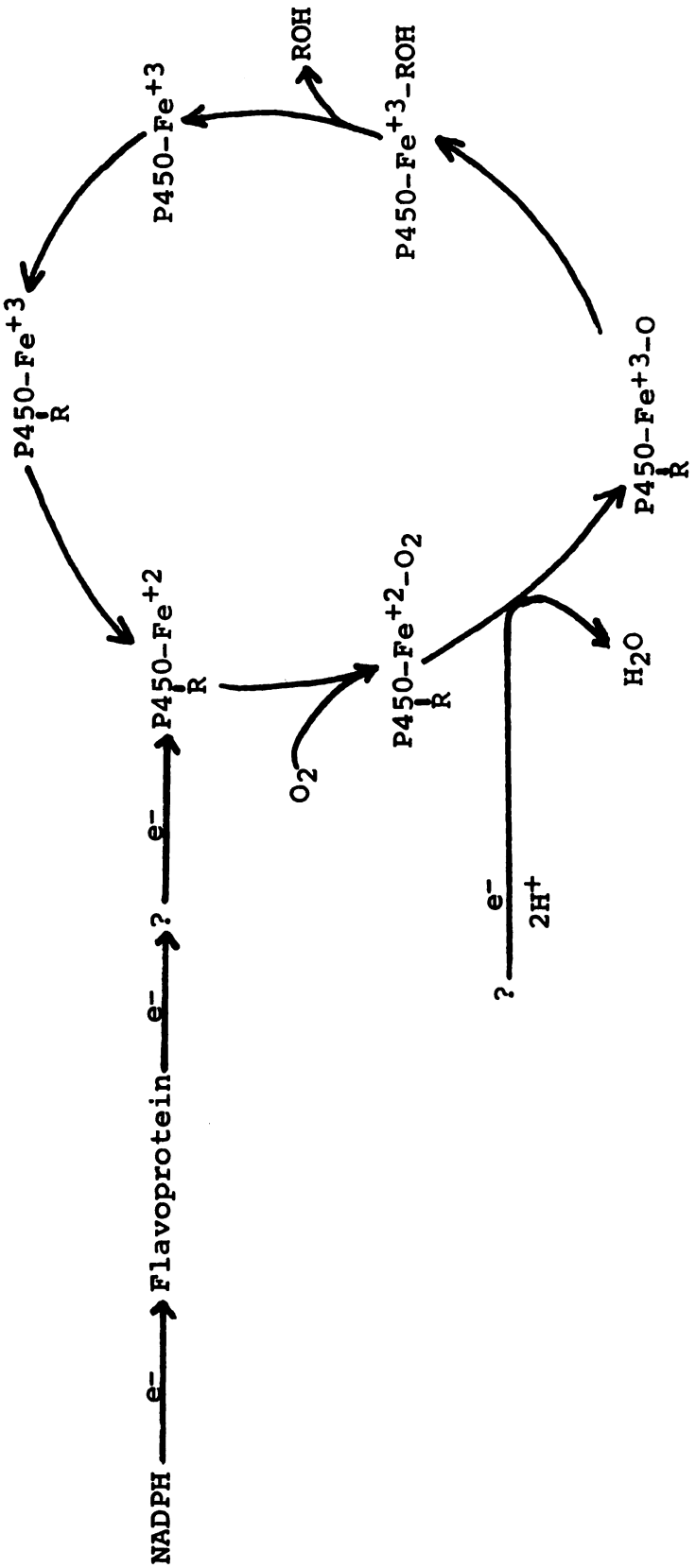
NADPH
e⁻
Flavoprotein
e⁻
?
e⁻
P450-Fe+2
R
O2
P450-Fe+2-O2
R
?
e⁻
2H+
H2O
P450-Fe+3-O
R
P450-Fe+3-ROH
ROH
P450-Fe+3
P450-Fe+3
R

Figure 1.

present in the mixed-function oxidase system. Studies by various investigators have produced lines of evidence for the existence of this multiplicity.

The first evidence for multiplicity was differences observed in spectral forms for cytochromes induced by pretreatment of rats with PB or 3-MC(1,2). The maximum absorbance of cytochrome P450 from microsomes in PB-pretreated rats was 450 nm while that from microsomes in 3-MC-pretreated rats was 448 nm (cytochrome P448). Kinetic studies by Alvares, et.al.(13) indicated that 3-MC-induced microsomal proteins lowered the K_m for hydroxylation of 3,4-benzpyrene compared to PB-induced microsomal proteins. These experiments implied that the inducing properties of PB and 3-MC were not due entirely to the amount of enzyme stimulated but the type of enzyme synthesized. Further kinetic studies of rat liver microsomal aminopyrine demethylase activity supported the evidence of more than one system that can demethylate aminopyrine(14,15). Recently a reconstituted mixed-function oxidase system composed of a carbon monoxide binding pigment (cytochrome P450 or P448), NADPH-dependent reductase, and lipid, was used to show that the specificity for hydroxylation resided primarily in the cytochrome fraction rather than the reductase or lipid fractions(16,17). In addition their results

indicated that cytochrome P450 and cytochrome P448 had different substrate specificities and were, therefore, different hemoproteins. It thus appears that specificity is imposed upon the system by the existence of multiple forms of cytochrome P450.

Work by Dr. A.F. Welton in our laboratory provided more evidence that multiple forms of P450 do exist(18). By staining 0.1% SDS gels with benzidine and hydrogen peroxide, three hemoproteins were identified. Pretreatment of rats with PB induced the hemoprotein with molecular weight 44,000 while pretreatment with 3-MC induced the hemoprotein of molecular weight 53,000. The predominant hemoprotein from control rats had a molecular weight of 50,000.

The work cited above does provide evidence for multiplicity but is not direct proof. Proof will exist only after the components have been purified to homogeneity and used in reconstituted systems to determine the specificity of reactions. In the meantime, techniques to be used in the present stage of development and in the future can be developed. One approach is to make antibodies to the particular components and observe their interactions in the system.

Antibodies have been raised against NADPH-cytochrome c reductase and cytochrome P450 from various organisms. Wada, et.al.(19) made an antibody to NADPH-cytochrome c reductase to show the participation of the microsomal electron transport in ω-oxidation of fatty acids. An anti-reductase made by Masters(20) inhibited NADPH-cytochrome c reductase and ethylmorphine-N-demethylase activities in pig liver microsomes. More importantly, the antibody acted as a specific inhibitor, blocking only the mixed-function oxidase system found in pig liver microsomes, not that found in bovine adrenocortical mitochondria. In 1971, experiments by Glazer(21) provided immunological evidence that substrate specificity was determined at the level of cytochrome P450, not the reductase. An antibody to rat liver reductase inhibited NADPH-cytochrome c reductase (not NADH-cytochrome c reductase) and drug oxidation of aminopyrine-N-demethylation and aniline hydroxylation in microsomes from PB-pretreated rats and benzpyrene hydroxylation in microsomes from 3-MC-pretreated rats. Since the reductase was inhibited to the same degree in both systems, it was concluded that induction with PB or 3-MC altered the expression of a particular cytochrome P450 and not different types of reductase.

In addition to using an antibody to reductase to study the types of reactions occurring in the mixed-function oxidase system, the antibody has been used to characterize the molecular weight of the native protein(22). By immunoprecipitating reductase from detergent solubilized rat liver microsomes labelled with ^{125}I and analyzing the precipitate by SDS gel electrophoresis and gamma counting, the molecular weight of the native reductase was estimated and compared to bromelain solubilized reductase.

Antibodies to cytochrome P450 have also been prepared. Mitani, et.al.(23) raised antibodies against solubilized cytochrome P450 from rat liver microsomes and chick embryo livers. No cross-reactivity was observed between anti-rat P450 and chick P450 and anti-chick P450 and rat P450. In the bacterial system Pseudomonas putida, an electron transport system similar to that found in rat liver microsomes has been studied(24,25). In both systems, the terminal hydroxylase is a cytochrome P450; the terminal hydroxylase is referred to as cytochrome $P450_{cam}$ because of its function in the camphor methylene hydroxylase system of P. putida. It was found that bacterial and rabbit liver microsomal P450 have similar immunological properties. An antibody to cytochrome $P450_{cam}$ cross-reacted 60%-70% with

rabbit liver cytochrome P450 and inhibited benzphetamine hydroxylation in the rabbit liver microsomal system(26). Thomas(27) has made an antibody to partially purified cytochrome P448 and has shown immunological differences between cytochrome P448 and cytochrome P450. The antibody appeared to be specific for the hemoprotein induced by 3-MC and not PB.

In all cases described above, antibodies have been made to partially purified membrane proteins and used to help elucidate structural properties and mechanisms. Although these membrane proteins have not been purified to homogeneity, they can still be used to elicit antibody responses. By using various immunological techniques it is possible to show: 1) the similarity of various antigens isolated from different sources of material; 2) the specificity of the antibody; and 3) the location of antigenic sites and their interaction with enzymatic sites. If the membrane proteins described above can be purified to homogeneity, then the types of experiments that could be performed are exemplified below.

An antibody against purified (Na^+-K^+)-ATPase from the outer medulla of pig kidney did not inhibit the enzyme when the antibody was exposed to the outer surface of the medulla(28). When the inner surface of the medulla was

exposed to the antibody, no enzymatic reaction was observed; therefore, the active site of the enzyme was present on the inner surface of the medulla. The antibody also inhibited ATPase isolated from rabbit kidney and ox brain, implying that the antigenic determinants were the same regardless of the origin of the Na^+ pump.

The effects on several reactions mediated by the rat brain (Na^+-K^+)-ATPase were studied using an antibody prepared against the ATPase complex(29). The antibody completely inhibited the (Na^+-K^+)-ATPase activity but had no effect on the K^+-dependent p-nitrophenylphosphatase activity. On the basis of these studies, the authors have hypothesized that ATPase may be a complex composed of several components, each of which are specific for given reactions. For example, a component for Na^+-dependent formation of phosphoprotein is different from a component of a K^+-dependent reaction. Thus, the different functional units have various antigenic determinants which influence partial reactions within the system.

Kyte has made an antibody to native (Na^+-K^+)-ATPase from canine renal medulla(30,31). His studies with the antigen-antibody complexes proved several points. 1) The antibody was specific for (Na^+-K^+)-ATPase; similar complement fixation curves were obtained with preparations of

enzymes at different stages of purification. 2) The antigen-antibody complexes were located on the inner surface of the plasma membrane of the intact cell. 3) The antibodies did not inhibit (Na^+-K^+)-ATPase when bound to the enzyme at saturating concentrations, implying that a diffusional carrier mechanism for active Na^+ and K^+ transport was impossible.

Since various components of the mixed-function oxidase system have not been purified to homogeneity, it is impossible to prove the specificity of an antibody or the effects of interaction between antibody and antigen. It is possible to present evidence that implies some structural relationships and mechanisms. In our laboratory, for example, an antibody to cytochrome P450 partially purified from microsomes isolated from PB-pretreated rats has been made. It has been shown that this cytochrome is one of three hemoproteins detected on 0.1% SDS gels(18); it corresponds to the 44,000 dalton protein found in microsomes from PB-pretreated rats. Dr. Welton has provided evidence that the antibody immunoprecipitated only hemoprotein 3 from cytochrome P450 partially purified from liver microsomes of PB-pretreated rats(32,33).

By using this antibody in conjunction with various immunological techniques, this thesis will present evidence

to support further the specificity of the antibody for hemoprotein 3. Techniques used include inhibition of aminopyrine-N-demethylation by anti-hemoprotein 3, agglutination of anti-hemoprotein 3 to various types of rat liver microsomes and complement fixation of various cytochrome P450s to anti-hemoprotein 3.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats weighing between 200-250 g and 75-100 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan. Male New Zealand rabbits (6-8 lbs.) were obtained from the Center for Laboratory Animals Resources, Michigan State University. Sheep red blood cells were obtained through Mr. George Good from the Department of Animal Husbandry, Michigan State University.

Butylated hydroxytoluene, 3-methylcholanthrene, mercuripapain, Trizma base, NADP-isocitrate dehydrogenase, isocitric acid, lactoperoxidase, bovine serum albumin, sodium dodecyl sulfate, Brilliant Blue R (Coomassie blue), nicotinamide, sodium azide, and dithiothreitol were products of Sigma Chemical Co., St. Louis, Missouri.

Glucose and phenobarbital were purchased from Merck & Company, Inc., Rahway, New Jersey. Diethylaminoethyl cellulose and carboxymethyl cellulose were obtained from Bio-Rad Laboratories, 32nd and Griffin, Richmond, California. Sucrose and sodium deoxycholate were products of Schwartz-Mann Division of Becton and Co., Orangeburg,

New York. Aminopyrine was obtained through Canalco, Inc., Rockville, Maryland. Trichloroacetic acid and citric acid were products of Mallinckrodt, St. Louis, Missouri.

Freund's complete adjuvant and agar (Nobel Grade) were purchased from Difco Laboratories, Detroit, Michigan. Hemolysin and guinea pig complement were obtained from Cappel Laboratories, Downington, Pennsylvania. Carrier-free Na-^{125}I was obtained from New England Nuclear, Boston, Massachusetts.

Cytochrome $P450_{cam}$ isolated from Pseudomonas putida and the antibody against cytochrome $P450_{cam}$ were gifts of Drs. Karl Dus and I.C. Gunsalus of the University of Illinois, Urbana, Illinois. Cytochrome P450 partially purified from rabbit liver microsomes was a gift of Dr. M.J. Coon of the University of Michigan, Ann Arbor, Michigan. Samples of cytochrome P450 from rat lung microsomes were provided by Mr. John Buege.

PB and 3-MC Pretreatment of Rats

Rats were pretreated with either phenobarbital or 3-methylcholanthrene. Rats weighing between 200-250 g were pretreated by including 0.1% PB in their drinking water 10 days prior to sacrifice. Rats weighing between 75-100 g were injected i.p. with 3-MC (20 mg/kg in corn

oil) 36 and 24 hours prior to sacrifice. Liver microsomes prepared from each will be referred to as PB or 3-MC microsomes.

Preparation of Microsomal Membranes

Rats were fasted 18 hours before killing by decapitation. The liver was extracted and the total microsomal fraction was isolated by differential centrifugation as previously described(34). The membranes were stored by suspension in 0.05 M Tris-HCl, pH 7.5, containing 50% glycerol to a protein concentration of approximately 50 mg/ml and frozen at -15°C under nitrogen in the presence of 0.01% BHT. All isolation and washing procedures were carried out at 0°-4°C.

Partial Purification of Cytochrome P450 and P448

The isolation procedure described by Levin, et.al.(35) and modified by Welton(32) was followed. The final solutions, containing 3-8 mg/ml of microsomal protein, were frozen at -15°C under nitrogen.

Immunological Techniques

Preparation of Antibody

Antibody was raised against cytochrome P450 (hemoprotein 3) in rabbits by Dr. A.F. Welton(32). Adult male

rabbits were immunized with three injections of hemoprotein 3 (3 mg protein/injection), administered in 2 ml of 50% Freund's complete adjuvant at one week intervals. Multiple intradermal injections of 0.1 ml were made into the abdomen. Ten days after the last injection, blood was collected from the marginal ear vein and serum separated from whole blood by allowing the blood to clot at room temperature for 3-4 hours. The clotted blood was placed at 0°-4°C overnight to constrict the clot; the blood was then centrifuged at 10,000 x g for 20 minutes at room temperature. The whole sera was removed by decantation and the IgG fraction isolated.

The IgG fraction from both immune and pre-immune serum was prepared by ammonium sulfate fractionation and DEAE chromatography(36). Serum was centrifuged in a Sorval RC-2B centrifuge at 4°C for 20 minutes at 34,800 x g. The supernatant was decanted and the pH adjusted to 7.8. Ten ml of saturated ammonium sulfate (76.6 g in 100 ml) was added to 20 ml of supernatant at room temperature, stirred for 20 minutes, and centrifuged in an International Centrifuge at 3000 x g for 20 minutes. The pellet was resuspended in 20 ml of 0.85% sodium chloride and the pH adjusted to 7.8. The ammonium sulfate fractionation was performed

again. The final pellet was dissolved in 15 ml of 0.015 M sodium phosphate buffer, pH 7.8 and dialyzed overnight against the same buffer. This partially purified IgG was run over a DEAE column (1 g DEAE per 50 mg protein) pre-equilibrated with 0.015 M sodium phosphate buffer, pH 7.8. The IgG fraction came through in the void (Figure 2). Fractions were pooled and stored at 4°C. Purity was determined by SDS gel electrophoresis.

Fab fragments were prepared from purified IgG(37,38). Forty-five mg of lyophilized IgG was dissolved in 3.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.01 M cysteine and 0.002 M EDTA. Twenty µl of mercuripapain (25 mg/ml) was added and the mixture incubated for 16 hours at 37°C in a Dubanoff metabolic shaker. The solution was dialyzed against water and then against 0.01 M acetate buffer, pH 5.5, overnight at 0°-4°C. After centrifuging the solution in an International Centrifuge at 3000 x g for 10 minutes, the supernatant was applied to a carboxymethyl cellulose column and eluted with a 0.01 M to 0.9 M acetate buffer, pH 5.5, gradient. The fractions were analyzed by measuring their optical density at 280 nm (Figure 3). The Fab fragments were eluted in two major protein peaks.

Figure 2. ELUTION PROFILE OF IMMUNE IGG FROM DEAE CELLULOSE

Partially purified IgG from serum containing anti-hemoprotein 3 was applied to a DEAE column and eluted with 0.015 M sodium phosphate, pH 7.8. The IgG fraction eluted in the void volume, leaving albumin and other impurities on the column. Each fraction contained 3.1 ml.

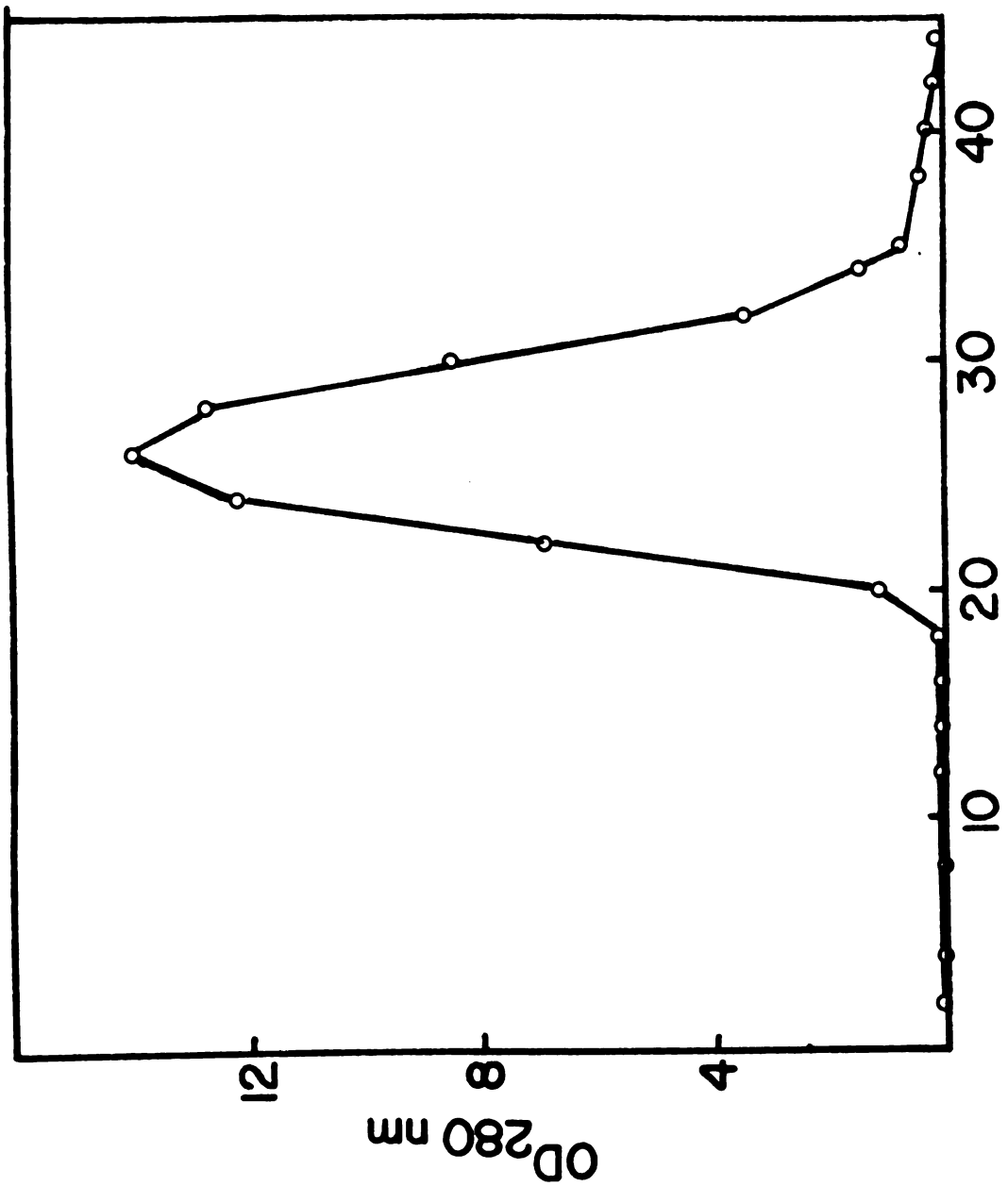

Figure 2.

Figure 3. ELUTION PROFILE OF FAB FRAGMENTS FROM CARBOXYMETHYL CELLULOSE

Fab fragments were prepared from purified IgG as described under Materials and Methods. The degraded IgG solution containing Fab and Fc fragments was applied to a carboxymethyl cellulose column and eluted with a 0.01 M to 0.9 M acetate buffer, pH 5.5, linear gradient. Four ml fractions were collected. The first peak contains Fab fragments and the second peak contains Fab and Fc fragments.

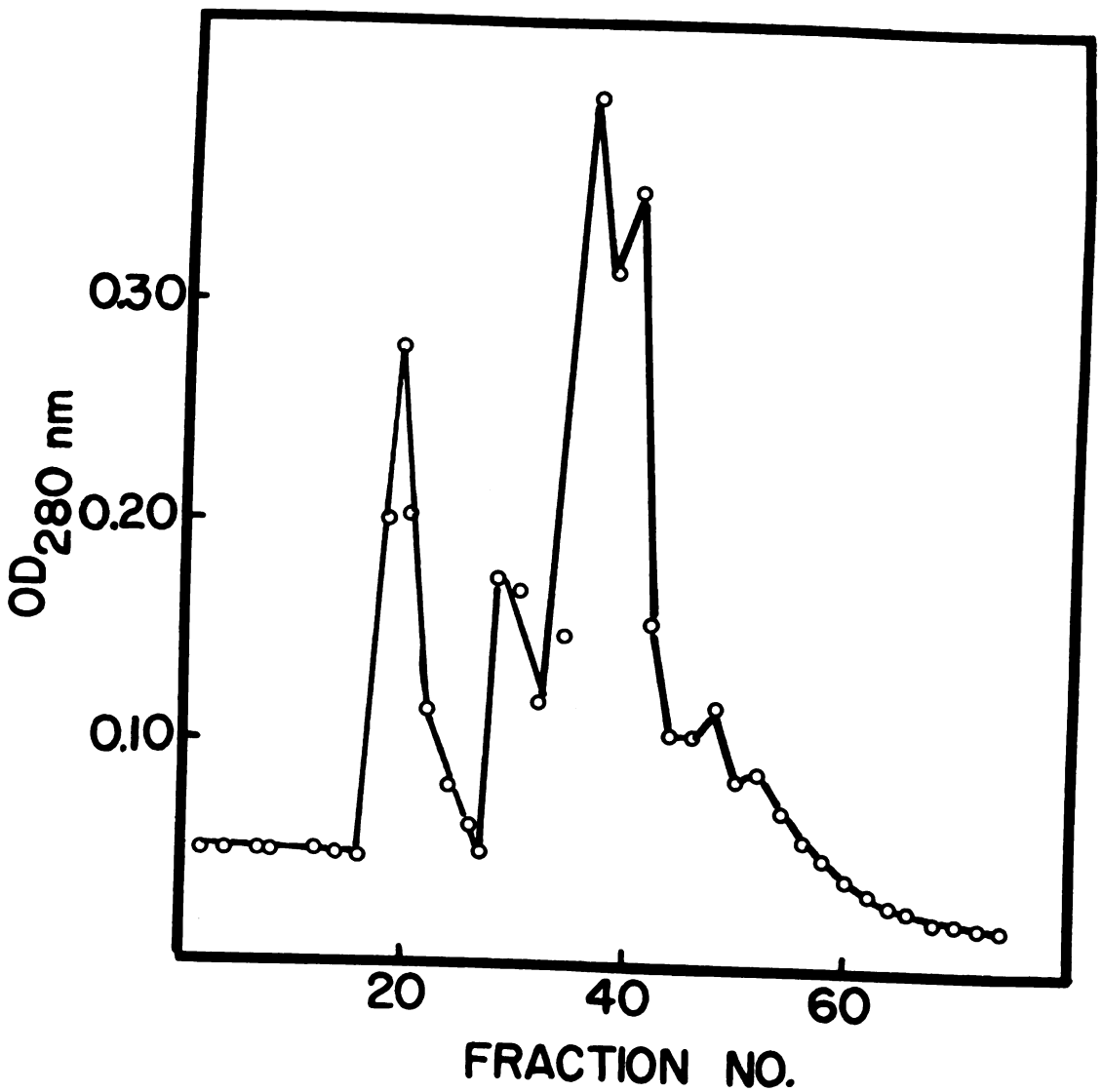

Figure 3.

Ouchterlony Double Diffusion Analysis

Ouchterlony double diffusion analyses were performed as described by Welton(32). Thirteen ml of 1% agar dissolved in 0.015 M sodium phosphate, pH 7.8, 0.1% sodium deoxycholate, and 0.02% sodium azide were added to a disposable plastic petri dish (100 x 15 mm). The well pattern was cut, and antigen and antibody were added to appropriate wells. Plates were developed at room temperature for 24 hours.

Inhibition of Aminopyrine Demethylation Activity with Anti-Hemoprotein 3

N-demethylase activity was assayed by determining formaldehyde production(39,40). Reaction mixtures were incubated at 37°C under air in a Dubanoff metabolic shaker and contained microsomes (PB microsomes, 0.75 mg/ml or 3-MC microsomes, 1.25 mg/ml); 20 mM aminopyrine; 0.05 M Tris-HCl buffer, pH 7.5, to a final volume of 5 ml; NADP-isocitrate dehydrogenase (0.10 units/ml); 0.4 ml of Solution K (433 mg $MgCl_2 \cdot 6H_2O$; 155 mg isocitric acid, trisodium salt; 25 mg NADP in 0.05 M Tris-HCl buffer, pH 7.5) and appropriate concentrations of antibody, either as IgG or Fab fragments. All but Solution K were pre-incubated on ice 3 minutes. After the addition of Solution K, the

mixtures were incubated at 37°C. At 3 minute intervals, 0.5 or 1.0 ml aliquots were removed and mixed with 0.5 or 1.0 ml of 10% TCA to precipitate protein. At the end of 10 minutes, 1.0 or 2.0 ml of NASH reagent (2 M ammonium acetate, 0.05 M acetic acid and 0.02 M acetylacetone; diluted to 1 liter with water) were added and samples incubated at 60°C for 1 hour. Samples were centrifuged and the optical density at 412 nm was measured on a Perkin-Elmer model 124 spectrophotometer.

Agglutination Assays

The agglutination studies were conducted by an adaptation of the methods described by Kwapinski(41). Each assay (1.5 ml total volume) contained 0.14 mg of microsomal protein, varying amounts of IgG and 0.85% sodium chloride. This mixture was incubated at 37°C for 5 hours and then centrifuged at 3000 x g for 10 minutes. The resulting pellets were washed once in 0.5 ml of 0.85% sodium chloride and then resuspended in 0.85% sodium chloride for protein assays. As a control, microsomes incubated without antibody were also carried through this procedure, and the amount of protein found in the resulting pellet was subtracted from the amount pelleted in the presence of antibody. Incubation with pre-immune IgG gave the same results as controls.

The agglutination assays were also performed with iodinated microsomal protein. Iodination of microsomes was performed as described by Welton(32,42); pellets were diluted with cold microsomes and used in the assays.

Complement Fixation Assays

Complement fixation assays were used to test various antigen-antibody systems. The procedures outlined by Levine(43) and Kabat and Mayer(44) were followed.

Reagents: Stock diluent - To 800 ml of distilled water were added 81.6 g of NaCl, 12.1 g of Tris base, 6.6 ml of concentrated HCl, 33 ml of 0.15 M $MgSO_4$, and 15 ml of 0.1 M $CaCl_2$. The solution was mixed, the volume adjusted to 1 liter, and the pH adjusted to 7.4. This stock buffer was diluted 1 in 10 just before use and 0.1% bovine serum albumin was added.

Alsever's Solution - To 1200 ml of distilled water were added 24.6 g of glucose, 9.6 g of sodium citrate (dihydrate), and 5.04 g of NaCl. The pH was adjusted to 6.1 with citric acid, and the solution was sterilized by passing it through an ultrafine sintered glass filter.

Sheep Red Blood Cells - Sheep red blood cells (50 ml) were drawn aseptically into 70 ml of Alsever's solution and stored at 2^{o}-5^{o}C for one week prior to use. Four ml SRBC

were centrifuged at 4300 x g for 10 minutes; the cells were then washed 3 times by suspending the cells in 1.0 ml of diluent and centrifuging at 4300 x g for 10 minutes. After the last wash, cells were resuspended in 18.0 ml of diluent and filtered through glass wool. To determine cell concentration, 1.0 ml of suspended SRBC was lysed with 14.0 ml of 0.1% Na_2CO_3 and the optical density at 541 nm measured. Adjustments were made until an OD_{541} of 0.680 was obtained. This corresponds to a RBC concentration of 1×10^9 cells per ml.

Hemolysin - Hemolysin was reconstituted, then diluted 1 in 50 with diluent and stored at -20°C. For use, 10 ml of hemolysin, diluted 1 in 1000, was added slowly and with constant swirling to 10 ml of suspended SRBC, incubated at 37°C for 15 minutes and diluted with 180 ml of diluent. The sensitized cells were prepared daily and stored at 4°C.

Complement - Complement was reconstituted and stored as 2 ml aliquots in screw cap tubes at -20°C. Once restored, the complement was good for 2-4 weeks.

Titration of Complement: To determine the optimum concentration of complement to be used in assays, complement was titered. One ml of complement, diluted in the range of 1/100 to 1/250, was mixed with 5.5 ml of diluent in a 40 ml Sorval centrifuge tube and incubated at 0°-4°C

for 16-18 hours. The samples were then incubated with 1.0 ml of sensitized SRBC for 60 minutes at 37°C in a Dubanoff metabolic shaker. At the end of the incubation period, samples were cooled on ice to stop the reaction and centrifuged at 4300 x g for 10 minutes. The supernatants were measured at 413 nm. The percent hemolysis for each sample was calculated and plotted against complement dilution. The highest dilution giving 90% hemolysis was used in future assays (Figure 4).

Titration of Hemolysin: Hemolysin (antibody to sheep red blood cells) was serially diluted from 1/400 to 1/6400. Five ml of hemolysin was added to 5.0 ml of washed SRBC with constant swirling; thus, for each hemolysin diluted, cells were sensitized. Each assay (total volume, 6.5 ml) contained diluent, 1.0 ml of complement appropriately diluted, and 1.0 ml of sensitized SRBC. The samples were incubated at 37°C for 60 minutes, cooled on ice and centrifuged at 4300 x g for 10 minutes; optical densities at 541 nm of the supernatant were measured. Percent hemolysis was calculated and plotted against hemolysin dilution (Figure 5). The largest dilution giving 50% hemolysis was used.

Titration of Antibody: To determine the concentration of antibody to be used and the range of antibody-antigen

Figure 4. TITRATION OF GUINEA PIG COMPLEMENT

Guinea pig complement was titrated in the complement fixation assay as described under Materials and Methods. The largest dilution of guinea pig complement giving 90% hemolysis was used in future complement fixation assays. A dilution of 1/200 was determined from this titration.

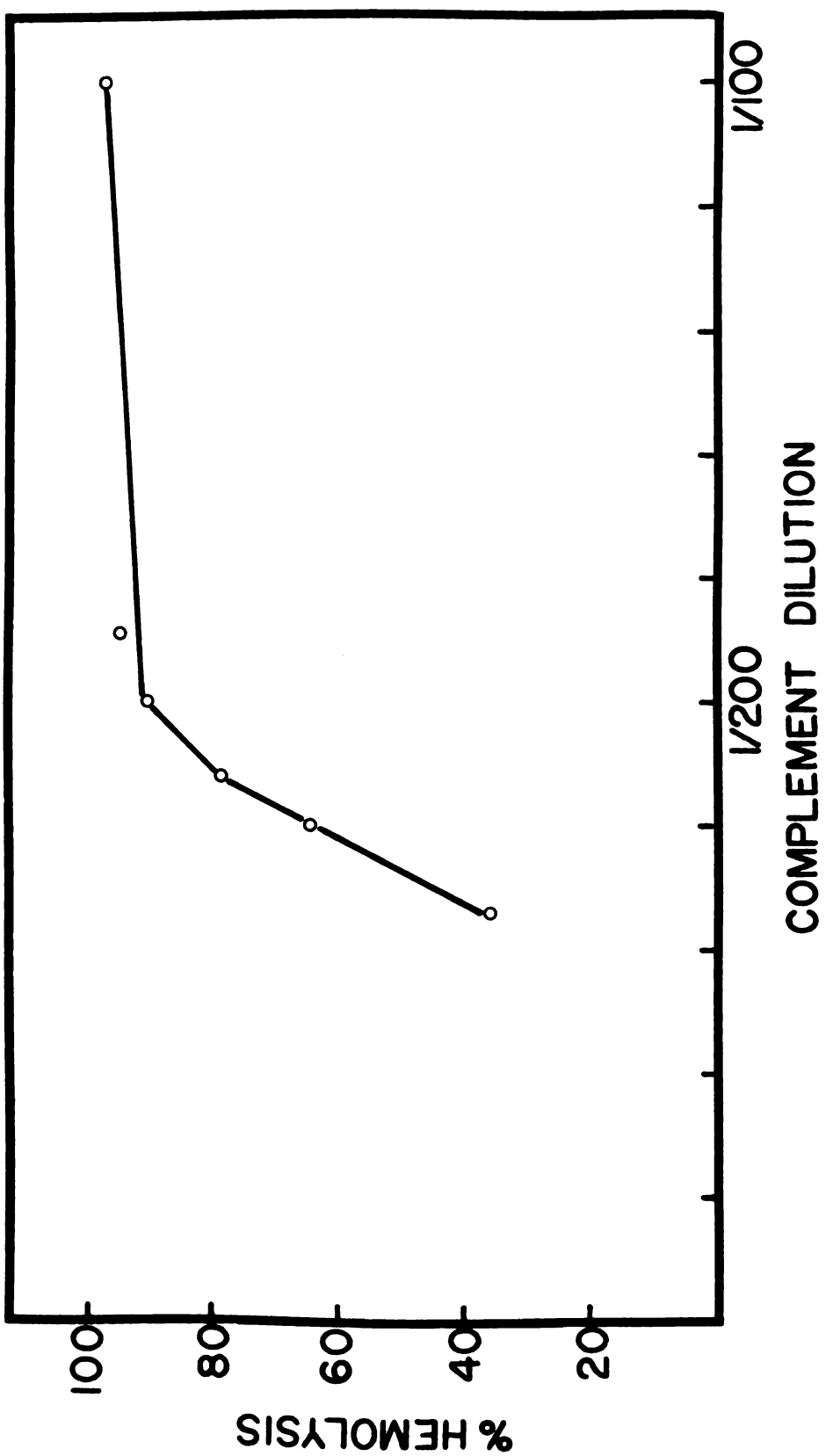

Figure 4.

Figure 5. TITRATION OF HEMOLYSIN

Hemolysin was titered as described under Materials and Methods. The largest dilution giving 50% hemolysis was used in subsequent complement fixation assays. This corresponded to a dilution of 1/1000.

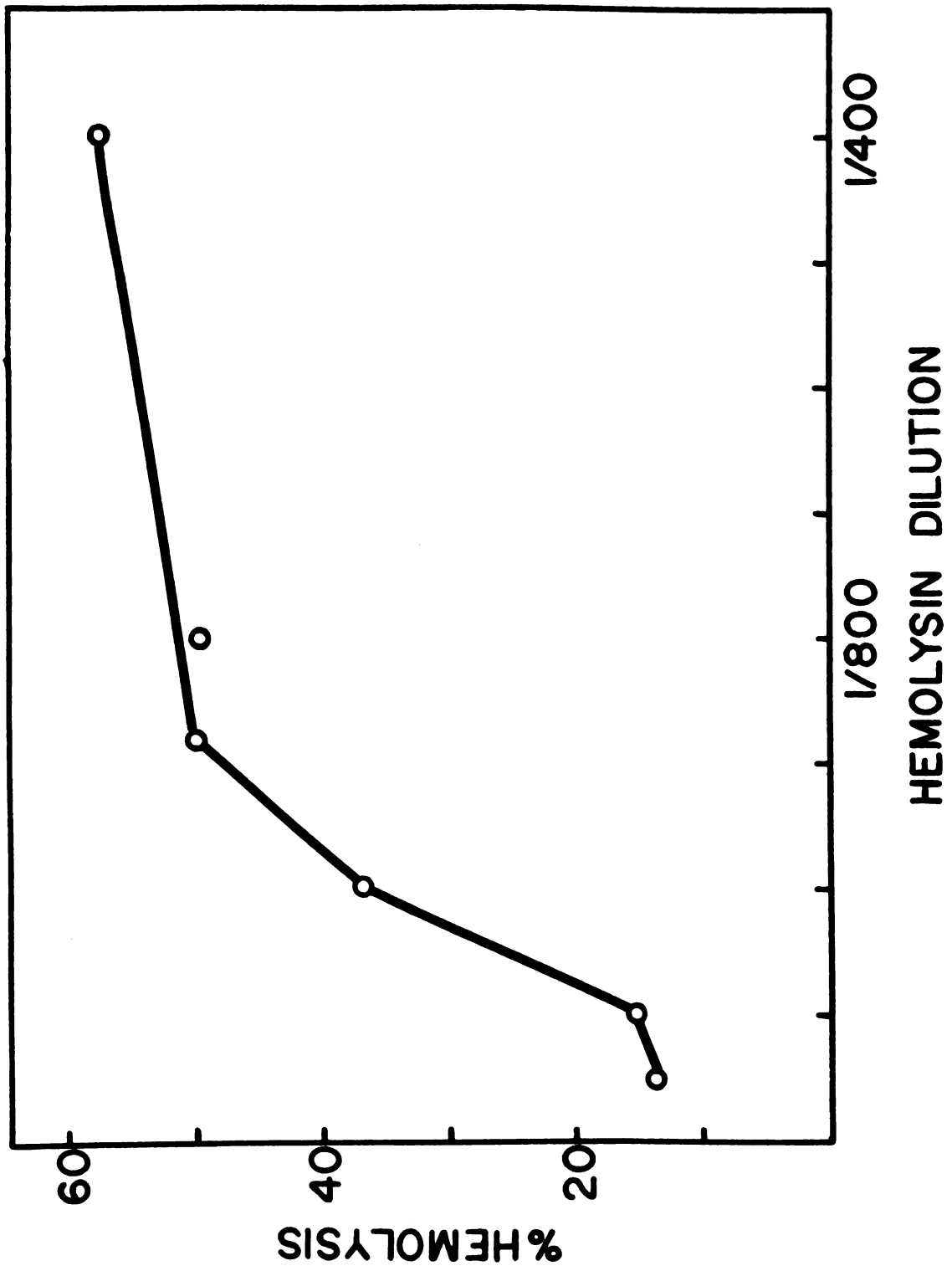

Figure 5.

interaction, antibody was serially diluted from 1/200 to 1/6400 and antigen from 0.01 to 10 μg of protein. To each 40 ml Sorval centrifuge tube (total volume, 6.5 ml) was added: 1.0 ml of appropriately diluted antibody, diluent, 1.0 ml of complement, and 1.0 ml of antigen. The tubes were vortexed and incubated at 0°-4°C for 16-18 hours. One ml of sensitized SRBC was added and samples incubated at 37°C for 60 minutes. At the end of the incubation, samples were cooled on ice and centrifuged at 4300 x g for 10 minutes. Supernatants were measured at 413 nm and the percent of complement fixed was determined. In all assays four controls were included: 1) diluent and antibody; 2) diluent and antigen; 3) diluent and complement; and 4) diluent only. The highest antibody dilution giving a maximum fixation of 70% was used (Figure 6).

Complement Fixation Assay: The complement assay was performed by adding, in order, to a 40 ml Sorval centrifuge tube: 1.0 ml of antibody, diluent, 1.0 ml of complement, and 1.0 ml of antigen. The four controls described under "Titration of Antibody" were included. After samples had incubated at 0°-4°C for 16-18 hours, 1.0 ml of sensitized SRBC was added. The tubes were then incubated at 37°C for 60 minutes, cooled on ice, and centrifuged at 4300 x g for 10 minutes. Optical densities at 413 nm of the supernatants

Figure 6. TITRATION OF ANTI-HEMOPROTEIN 3

The titration of anti-hemoprotein 3 was described under Materials and Methods. The largest dilution giving a maximum of 70% complement fixed was used in subsequent complement fixation assays. This corresponded to a 1/300 dilution for anti-hemoprotein 3. Antibody dilutions: 1/200 (O—O); 1/250 (■--■); 1/300 (Δ—Δ); 1/350 (●--●).

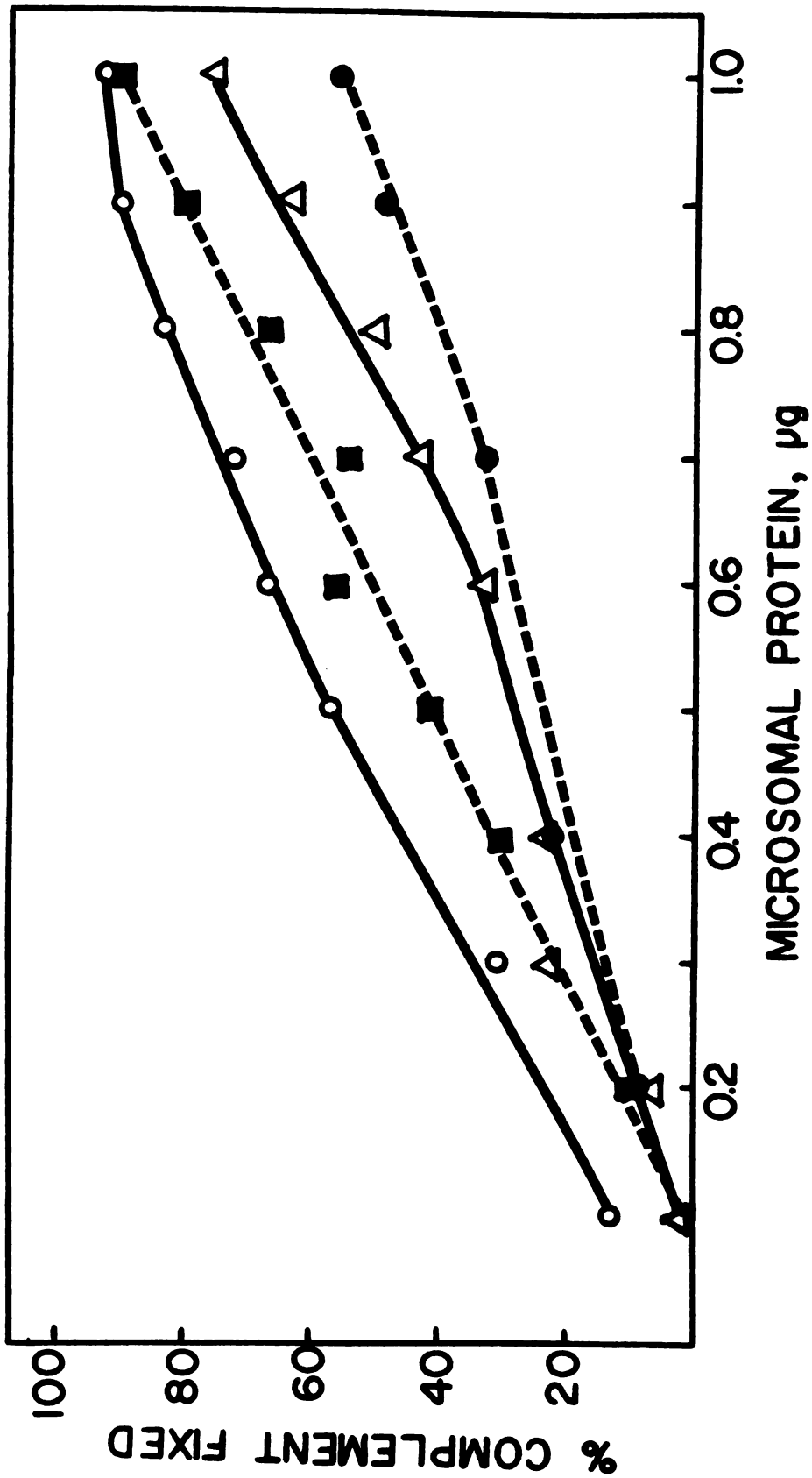
% COMPLEMENT FIXED
100
80
60
40
20
0.2
0.4
0.6
0.8
1.0
MICROSOMAL PROTEIN, µg

Figure 6.

were measured and used to calculate the percent of complement fixed. A typical protocol is given in Table I.

SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Fairbanks(45) and modified by Welton(32). Gels were scanned at 550 nm in a Gilford spectrophotometer equipped with a gel scanning attachment.

Other Assays

Protein concentrations were determined by the method of Lowry(46). Cytochromes P450 and P420 were assayed as described by Imai and Sato(47). A computer program was used to calculate the concentrations; extinction coefficients of 11 and -11 $cm^{-1}mM^{-1}$ between 450 nm and 490 nm for P450 and P420, respectively, and -41 and 110 $cm^{-1}mM^{-1}$ between 420 nm and 490 nm for P450 and P420, respectively, were assumed.

Table I. PROTOCOL FOR COMPLEMENT FIXATION ASSAYS

A typical protocol for complement fixation assays are given below. Components are added in the order listed and each series of assays include four controls. The change in optical density is calculated by subtracting the optical density of the samples from the control. The percent complement fixed is determined as follows:

$$\frac{\Delta OD_{413}}{OD_{control}} \times 100\%$$

Table I

Tube No.	Ab, ml	Diluent, ml	C', ml	Antigen, ml	EA, ml	OD_{413}	OD_{413}	% C'Fixed
1	1.0	3.0	1.0	1.0	1.0	0.008	0.530	101
2	1.0	3.0	1.0	1.0	1.0	0.005	0.533	101
3	1.0	3.0	1.0	1.0	1.0	0.103	0.435	83
4	1.0	3.0	1.0	1.0	1.0	0.139	0.399	76
5	1.0	3.0	1.0	1.0	1.0	0.165	0.373	71
6	1.0	3.0	1.0	1.0	1.0	0.267	0.271	52
7	1.0	3.0	1.0	1.0	1.0	0.340	0.198	38
8	1.0	3.0	1.0	1.0	1.0	0.393	0.145	28
9	1.0	3.0	1.0	1.0	1.0	0.477	0.068	13
10	1.0	3.0	1.0	1.0	1.0	0.515	0.023	4
11	---	4.0	1.0	1.0	1.0	0.545		
12	1.0	4.0	1.0	---	1.0	0.525		
13	---	5.0	1.0	---	1.0	0.545		
14	---	6.0	---	---	1.0	0.013		

Ab - antibody diluted 1/300
C' - complement
EA - sensitized sheep red blood cells

RESULTS

An antibody directed against hemoprotein 3, the 44,000 dalton microsomal protein which is induced in rats by pretreatment with PB, was prepared and used in these studies. Since the antibody appeared to have a low titre (1/300 dilution = 4.7 μg IgG) as assayed by complement fixation, two other cytochrome P450 antigens were injected into rabbits in attempts to elicit better antibody responses. The first antigen was the degradative form of hemoprotein (cytochrome P420) in which the solubilizing SDS reagent had been removed. The second antigen was composed of hemoprotein 3 crosslinked to rabbit serum albumin(48,49). Neither antigen elicited an antibody response as tested in Ouchterlony double diffusion with solubilized whole microsomes. In either case the structure of the antigen may have been drastically altered so that the antibody no longer recognized the hemoprotein found in microsomes or the antibody titre was extremely low. Thus, the original antibody to hemoprotein 3 was used and will be referred to as anti-hemoprotein 3.

The two major components present in all systems were microsomes from various sources containing cytochrome P450 and the antibody to hemoprotein 3. SDS gel electrophoresis was performed on all proteins used. The gel scans, shown in Figures 7 and 8, refer to whole microsomes from PB, 3-MC, and control rats; partially purified cytochrome P450 from microsomes of PB, 3-MC, and control rats; rat lung microsomes; cytochrome $P450_{cam}$ from P. putida; partially purified cytochrome P450 from rabbit liver microsomes; hemoprotein 3 (antigen); and IgG containing anti-hemoprotein 3.

Ouchterlony double diffusion analyses were performed to determine the degree of cross-reactivity of the antibody with various sources of cytochrome P450. In Figure 9, a precipitin line was observed only with microsomes from PB-pretreated rats; no cross-reaction occurred with $P450_{cam}$, rabbit liver P450, or rat lung microsomes. In Figures 10 and 11 precipitin lines were observed with PB, 3-MC, and control microsomes and with partially purified cytochrome P450 from PB, 3-MC, and control microsomes. No spurs were observed, indicating common antigenic sites. There was no observable reaction of pre-immune IgG with any of the microsomes.

Figure 7. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS AND THE CYTOCHROME P450S PARTIALLY PURIFIED FROM MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS

The upper scans are of microsomes isolated from control and PB- and 3-MC-pretreated rats. The lower scans represent cytochrome P450 partially purified from microsomes isolated from control and PB- and 3-MC-pretreated rats.

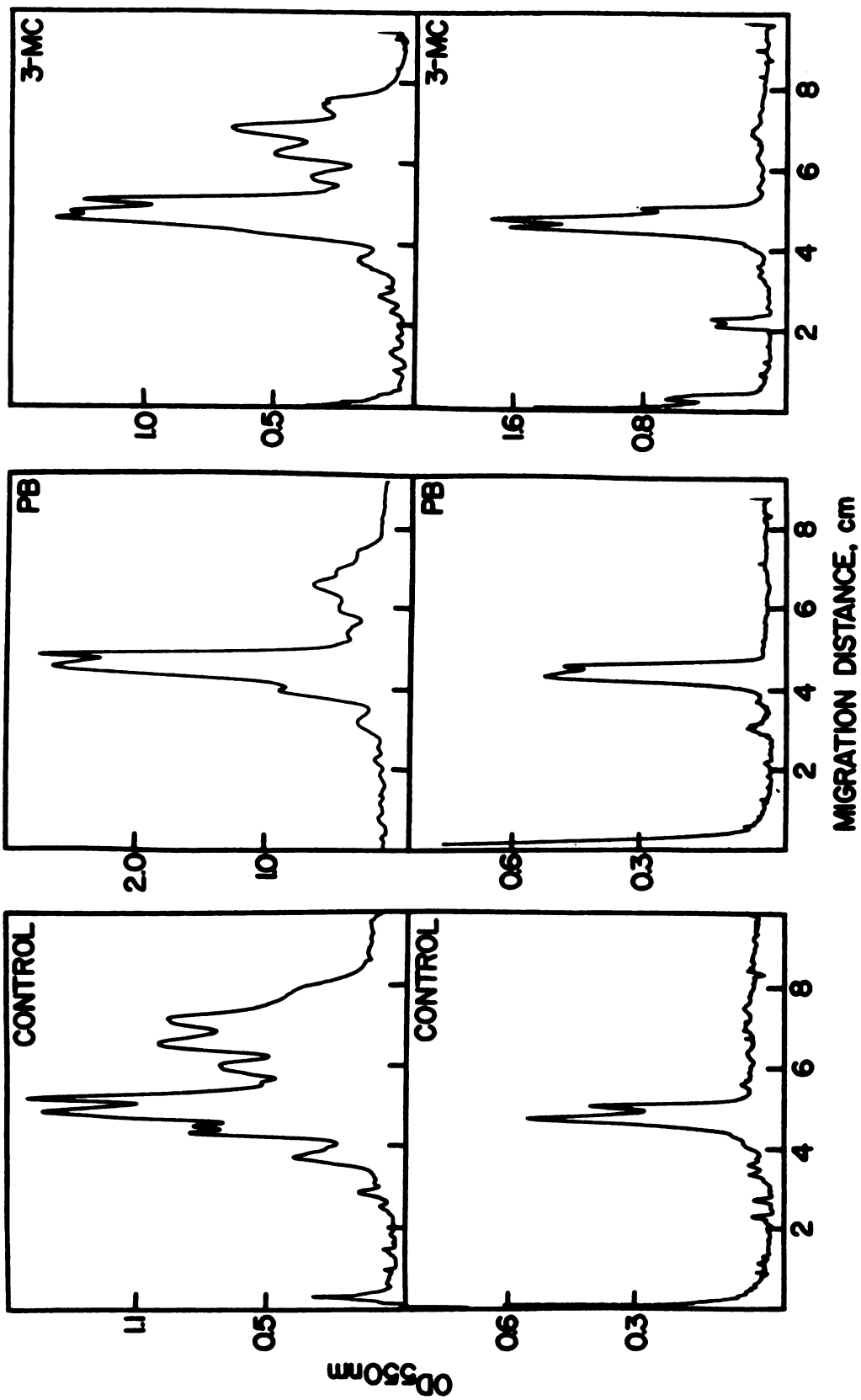
CONTROL
PB
3-MC
CONTROL
PB
3-MC
1.1
0.5
2.0
1.0
0.6
0.3
1.0
0.5
1.6
0.8
0.6
0.3
2
4
6
8
OD550nm
MIGRATION DISTANCE, cm

Figure 7.

Figure 8. 1% SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF CYTOCHROME P450 FROM RAT LUNG MICROSOMES, PSEUDOMONAS PUTIDA, AND RABBIT LIVER MICROSOMES AND HEMOPROTEIN 3 AND ANTI-HEMOPROTEIN 3

The upper scans are of cytochrome P450 from (left to right): rat lung microsomes, P. putida, and rabbit liver microsomes. The lower scans are of hemoprotein 3 (antigen) and anti-hemoprotein 3 (antibody).

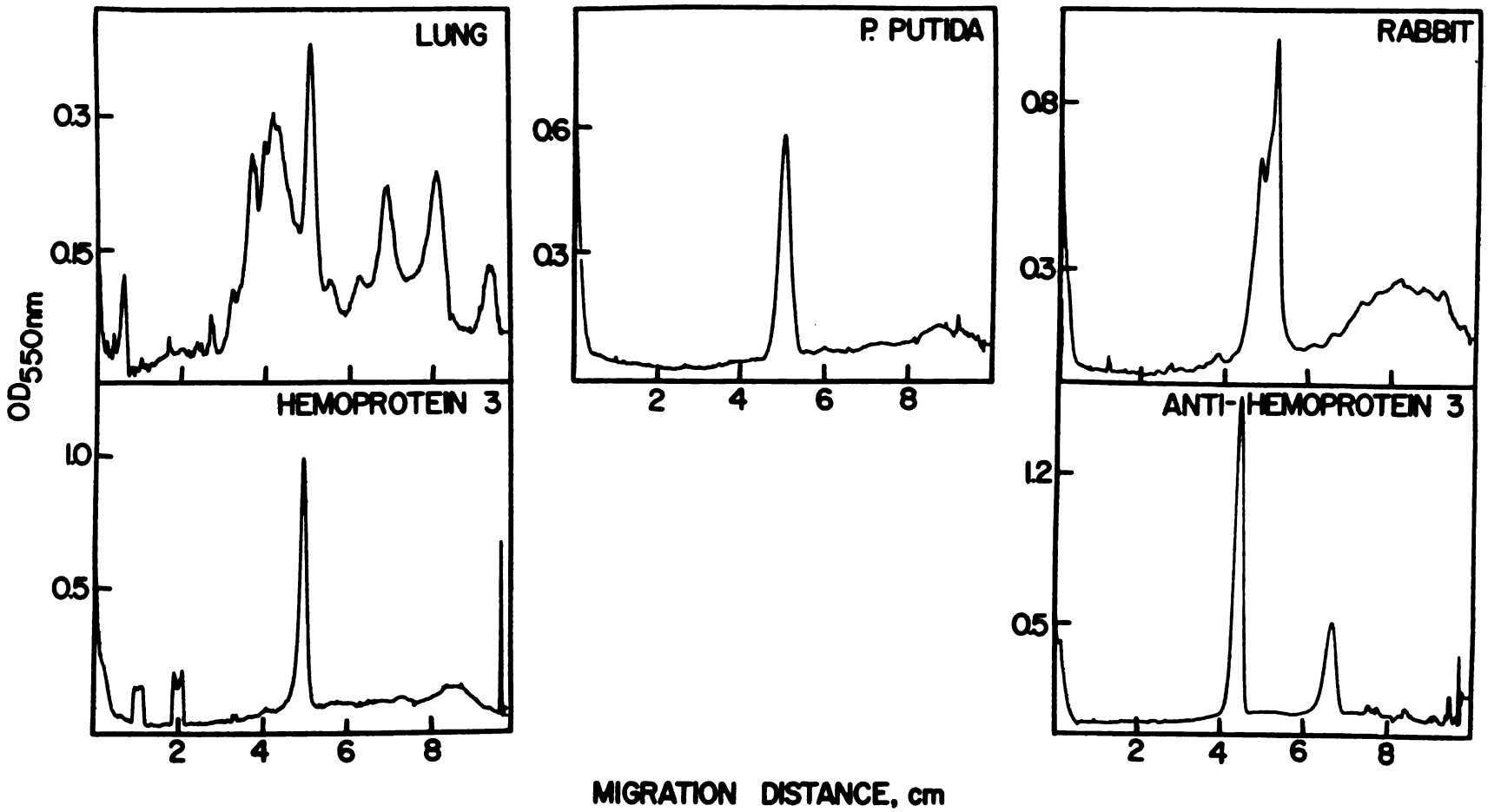
LUNG
P. PUTIDA
RABBIT
HEMOPROTEIN 3
ANTI-HEMOPROTEIN 3
OD550nm
MIGRATION DISTANCE, cm

Figure 8.

Figure 9. OUCHTERLONY DOUBLE DIFFUSION ANALYSES OF THE PRECIPITIN REACTIONS BETWEEN ANTIBODY TO HEMOPROTEIN 3 AND CYTOCHROME P450 ISOLATED FROM VARIOUS MICROSOMAL SOURCES

Ouchterlony double diffusion analyses were performed as described under Materials and Methods. The center well contains anti-hemoprotein 3 (260 μg of protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Well 1 contains 0.015 M sodium phosphate, pH 7.8. Well 2 contains cytochrome $P450_{cam}$ (26 μg). Well 3 contains cytochrome P450 from microsomes isolated from PB-pretreated rats (49 μg). Well 4 contains cytochrome P450 from rabbit liver microsomes (20 μg). Wells 5 and 6 contain lung microsomes from rats (23 μg and 27 μg, respectively).

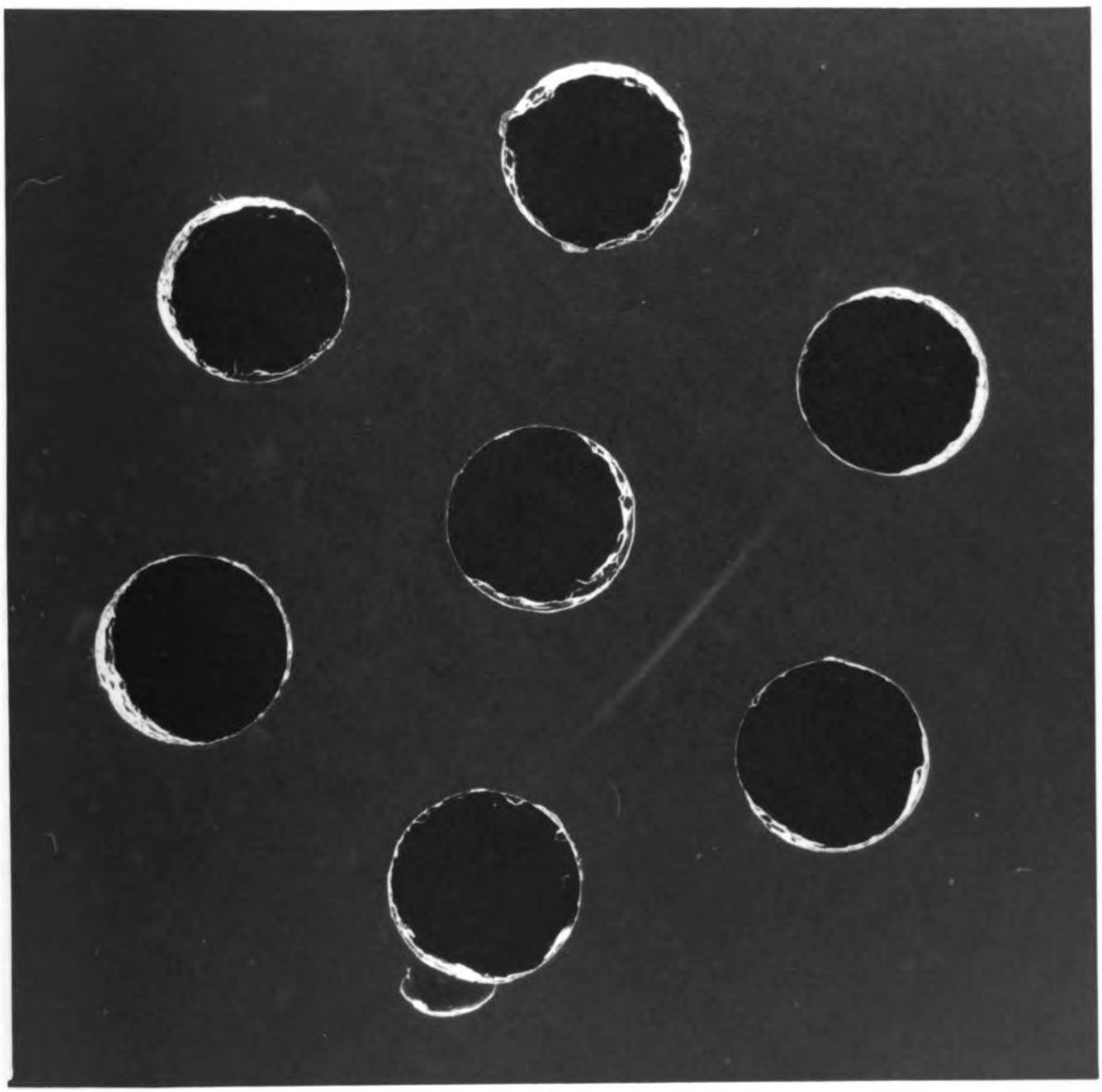

Figure 9.

Figure 10. OUCHTERLONY DOUBLE DIFFUSION ANALYSES OF THE PRECIPITIN REACTIONS BETWEEN ANTIBODY TO HEMOPROTEIN 3 AND MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS

Ouchterlony double diffusion analyses were performed as described under Materials and Methods. The center well contains anti-hemoprotein 3 (260 μg of protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Well 1 contains 0.015 M sodium phosphate, pH 7.8. Wells 2 and 5 contain microsomes from PB-pretreated rats (49 μg each). Well 3 contains microsomes from 3-MC-pretreated rats (40 μg). Wells 4 and 6 contain microsomes from control rats (34 μg).

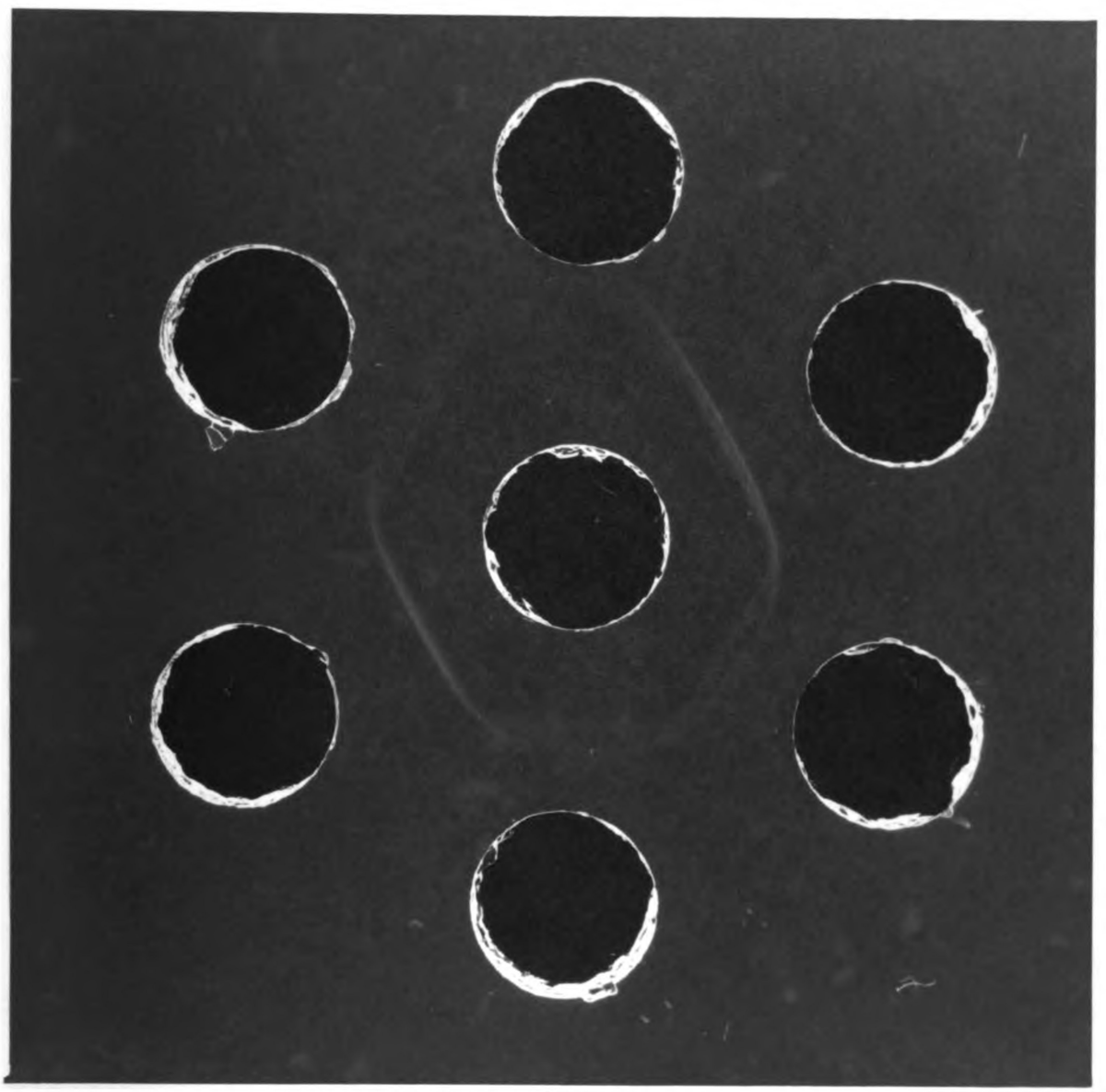

Figure 10.

Figure 11. OUCHTERLONY DOUBLE DIFFUSION ANALYSES OF THE PRECIPITIN REACTIONS BETWEEN ANTI-HEMOPROTEIN 3 AND CYTOCHROME P450 PARTIALLY PURIFIED FROM MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS

Ouchterlony double diffusion analyses were performed as described under Materials and Methods. The center well contains anti-hemoprotein 3 (260 μg of protein). The outer wells contain various antigens and are numbered clockwise from the top (No. 1). Well 1 contains 0.015 M sodium phosphate, pH 7.8. Wells 2 and 5 contain cytochrome P450 partially purified from microsomes isolated from PB-pretreated rats (28 μg). Well 3 contains cytochrome P448 partially purified from microsomes isolated from 3-MC-pretreated rats (23 μg). Wells 4 and 6 contain cytochrome P450 partially purified from control microsomes (29 μg).

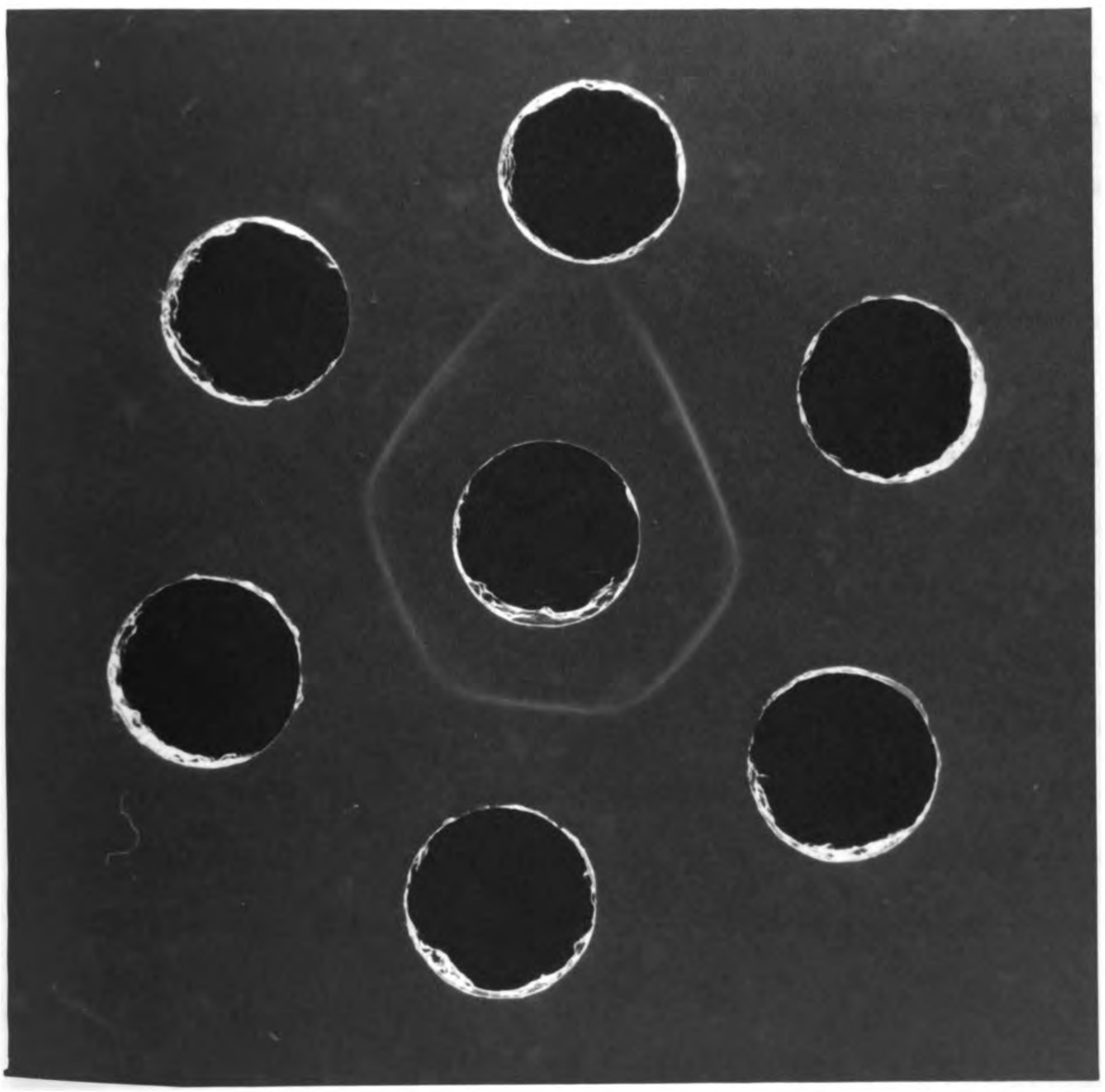

Figure 11.

The inhibition of aminopyrine-N-demethylation in microsomes isolated from PB and 3-MC-pretreated rats with purified IgG to hemoprotein 3 is shown in Figure 12. The reaction was not inhibited, even at IgG to microsomal protein ratios of 4.5:1 and 3.1:1 for microsomes isolated from PB- and 3-MC-pretreated rats, respectively. Microsomes also agglutinated at the higher antibody to protein ratios, indicating that the antigenic sites were located on the exterior surface of the microsomal membranes and were not interfering with the enzymatic sites.

To avoid the problem of agglutination, Fab fragments instead of IgG were used in the assay. The results, given in Figure 13, show only slight inhibition of aminopyrine-N-demethylation in microsomes isolated from PB-pretreated rats at a Fab to protein ratio of 11.6:1. Thus, the inhibition of aminopyrine-N-demethylation in microsomes from PB- or 3-MC-pretreated rats with immune IgG or Fab fragments was unsuccessful. The enzymatic sites may be, therefore, buried within the microsomal membrane or are on the exterior surface of the membrane and not affected by the binding of antibody.

Agglutination studies were performed to determine if anti-hemoprotein 3 would preferentially precipitate microsomes from PB-pretreated rats. Differences were observed

Figure 12. INHIBITION OF AMINOPYRINE-N-DEMETHYLATION IN MICROSOMES ISOLATED FROM PB- AND 3-MC-PRETREATED RATS WITH IMMUNE IGG

Inhibition of aminopyrine-N-demethylation with anti-hemoprotein 3 was assayed by determining formaldehyde production. The reaction mixtures, described under Materials and Methods, for A included 3.75 mg of microsomal protein from PB-pretreated rats without immune IgG (O—O) and with 1.1 mg of IgG (■--■), 3.5 mg of IgG (Δ—Δ), or 16.9 mg of IgG (●--●). The reaction mixtures in B included 6.0 mg of microsomal protein from 3-MC-pretreated rats without antibody (O—O) and with 1.1 mg of IgG (■--■), 3.9 mg of IgG (Δ—Δ), or 18.6 mg of IgG (●--●).

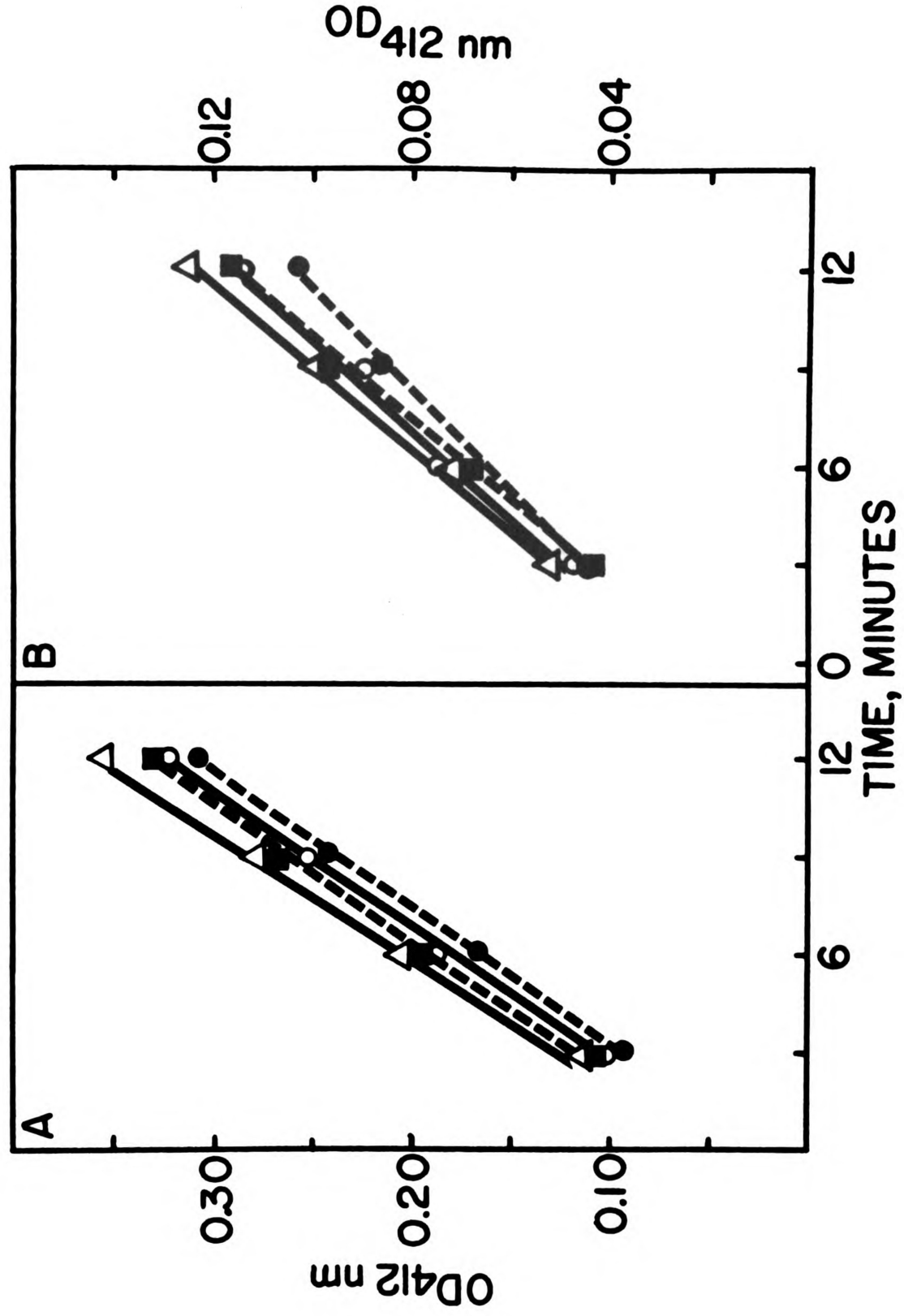

Figure 12.

Figure 13. INHIBITION OF AMINOPYRINE-N-DEMETHYLATION IN MICROSOMES ISOLATED FROM PB-PRETREATED RATS WITH PRE-IMMUNE AND IMMUNE FAB FRAGMENTS

Inhibition of aminopyrine-N-demethylation with anti-hemoprotein 3 Fab fragments was assayed by formaldehyde production. The reaction mixtures, described under Materials and Methods, included 1.55 mg of microsomal protein from PB-pretreated rats without antibody (O—O) and with 10 mg of pre-immune Fab fragments (Δ—Δ) or 18 mg of immune Fab fragments (■—■).

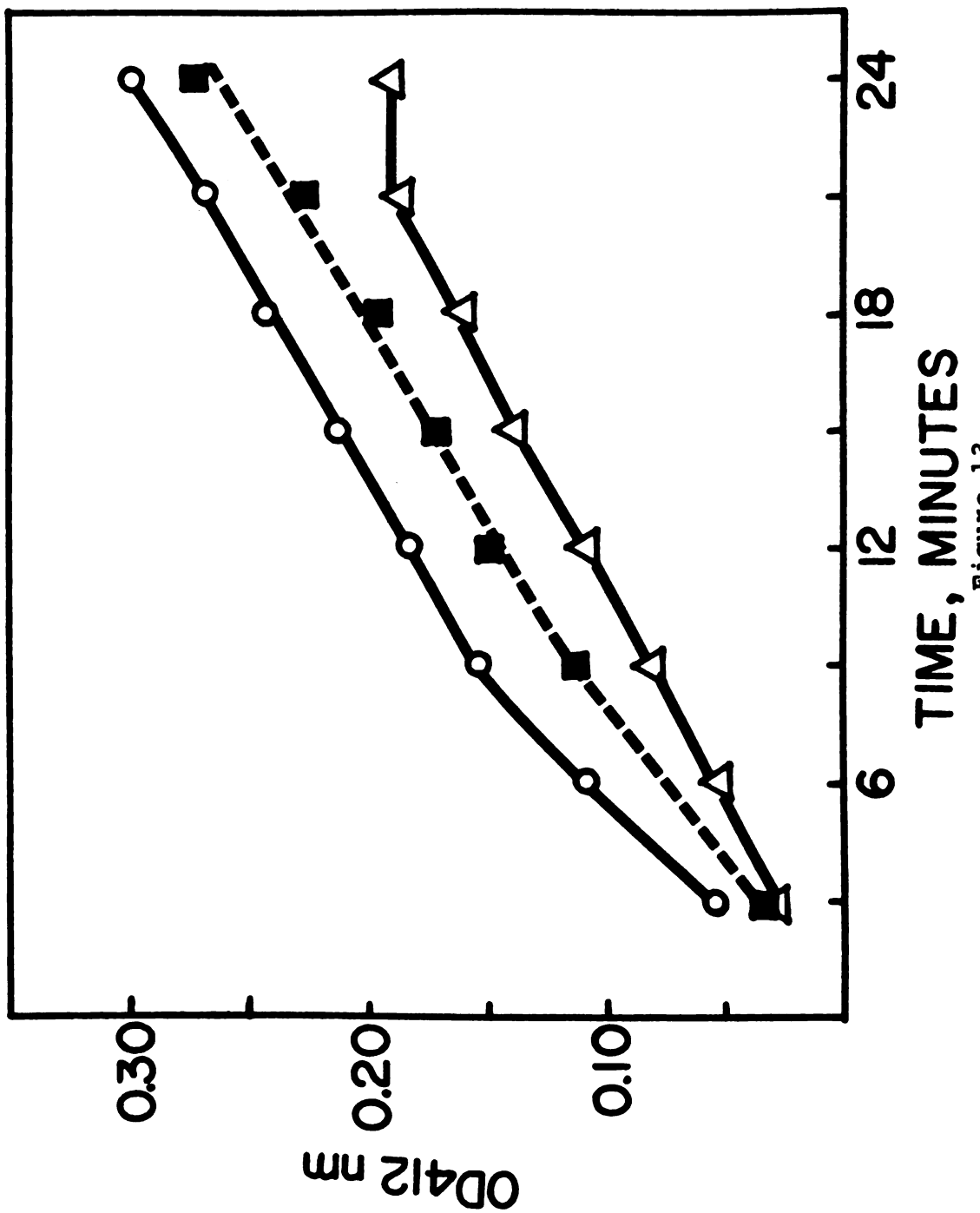

Figure 13.

in agglutination of microsomes from control and PB- and 3-MC-pretreated rats (Figure 14). If the 44,000 dalton cytochrome P450 is induced in microsomes from PB-pretreated rats and is present in higher levels than in microsomes from control or 3-MC-pretreated rats, then one would expect microsomes from PB-pretreated rats to agglutinate more readily than the other two. Such a relationship was observed. Microsomes from PB-pretreated rats agglutinated three times as much as microsomes from control rats; microsomes from 3-MC-pretreated rats precipitated twice as much as microsomes from control rats.

Attempts to assay the degree of agglutination with iodinated microsomes were unsuccessful. The differences that were observed in Figure 14 are missing in Figure 15. If hemoprotein 3 is induced in PB-pretreated rats and is, therefore, present in greater quantities than in control microsomes, then the amount of labelled hemoprotein 3 in PB microsomes should be greater than the amount of labelled hemoprotein 3 in control microsomes. As a result more labelled microsomal protein from PB-pretreated rats should be agglutinated than labelled protein from control rats. The observation that equal amounts of labelled microsomal protein from either control or PB-pretreated rats implies that iodinated microsomal protein is preferentially

Figure 14. **AGGLUTINATION OF MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS WITH ANTI-HEMOPROTEIN 3**

Agglutination of microsomes isolated from control and PB- and 3-MC-pretreated rats was assayed as described under Materials and Methods. The agglutination of control microsomes (Δ—Δ) was compared to that of liver microsomes isolated from 3-MC-pretreated (■--■) and PB-pretreated rats (O—O).

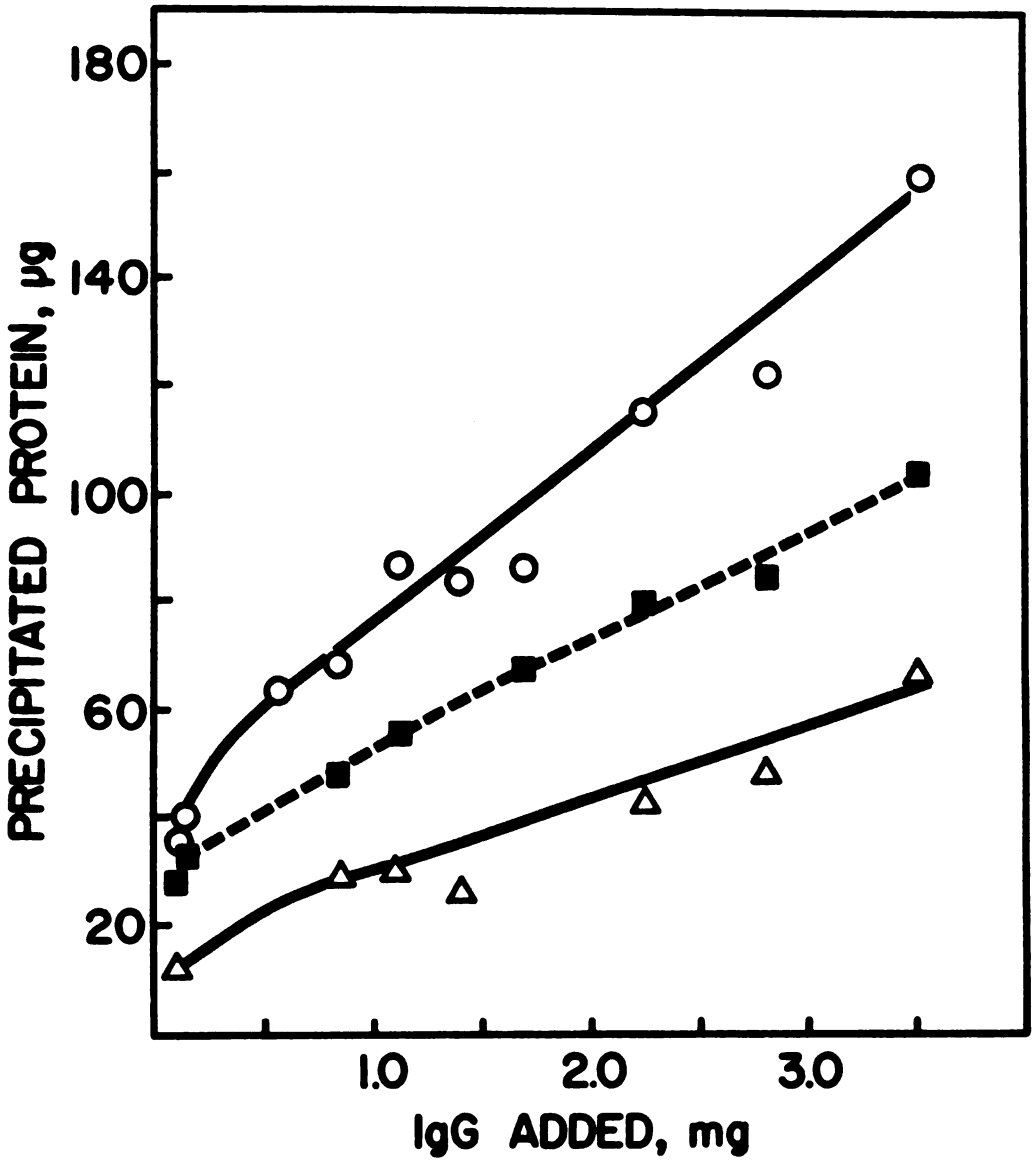
180
140
100
60
20
PRECIPITATED PROTEIN, µg
1.0
2.0
3.0
IgG ADDED, mg

Figure 14.

Figure 15. AGGLUTINATION OF ^{125}I-MICROSOMES ISOLATED FROM CONTROL AND PB- AND 3-MC-PRETREATED RATS WITH ANTI-HEMOPROTEIN 3

Agglutination of microsomes isolated from control and PB- and 3-MC-pretreated rats and iodinated with ^{125}I were assayed as described under Materials and Methods. The agglutination of control ^{125}I-labelled microsomes (Δ—Δ) was compared to that of microsomes isolated from PB-pretreated rats (O—O).

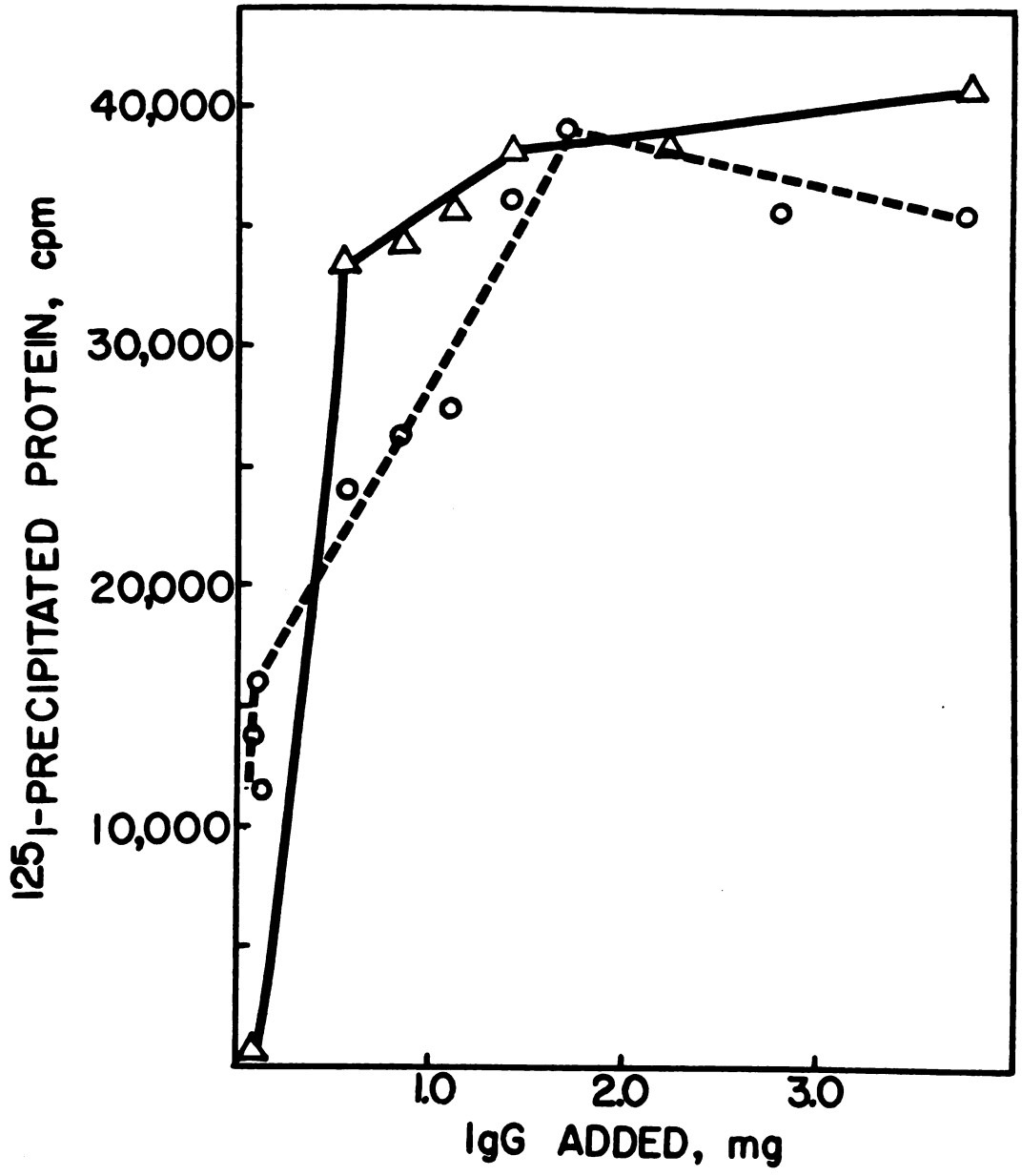
40,000
30,000
20,000
10,000
125I-PRECIPITATED PROTEIN, cpm
1.0
2.0
3.0
IgG ADDED, mg

Figure 15.

agglutinated. On the basis of the original protein assay which quantitatively determined the amount of agglutinated protein, differential agglutination by microsomes from control and PB- and 3-MC-pretreated rats was observed.

Complement fixation assays were performed with various microsomal sources to demonstrate the specificity of anti-hemoprotein to hemoprotein 3. In experiments using whole microsomes from control or PB- and 3-MC-pretreated rats, differences were observed in the amount of complement fixed (Figure 16), thus implying differences in the amount of antibody bound by each. If more antibody bound to liver microsomes from PB-pretreated rats than to control or 3-MC-pretreated rats, then the antibody would appear to be specific for the hemoprotein induced by pretreatment with PB. This effect was observed. At low concentrations of microsomal protein (0.8 μg), it is possible to quantitate the relative amount of antibody bound to the three types of microsomes. Assuming that the amount of complement fixed is directly proportional to the amount of antibody bound, it appears that microsomes from PB-pretreated rats bind approximately three times the amount of antibody as do control microsomes while microsomes from 3-MC-pretreated rats bind twice as much as do control microsomes.

Figure 16. COMPLEMENT FIXATION PROFILES OF ANTI-HEMOPROTEIN 3 WITH WHOLE MICROSOMES

Complement fixation assays were performed as described under Materials and Methods. The fixation of complement with microsomes isolated from PB-pretreated rats (O—O) was compared to the amount of fixation with microsomes isolated from control (Δ—Δ) and 3-MC-pretreated rats (■--■). Each assay contained 4.7 μg of immune IgG.

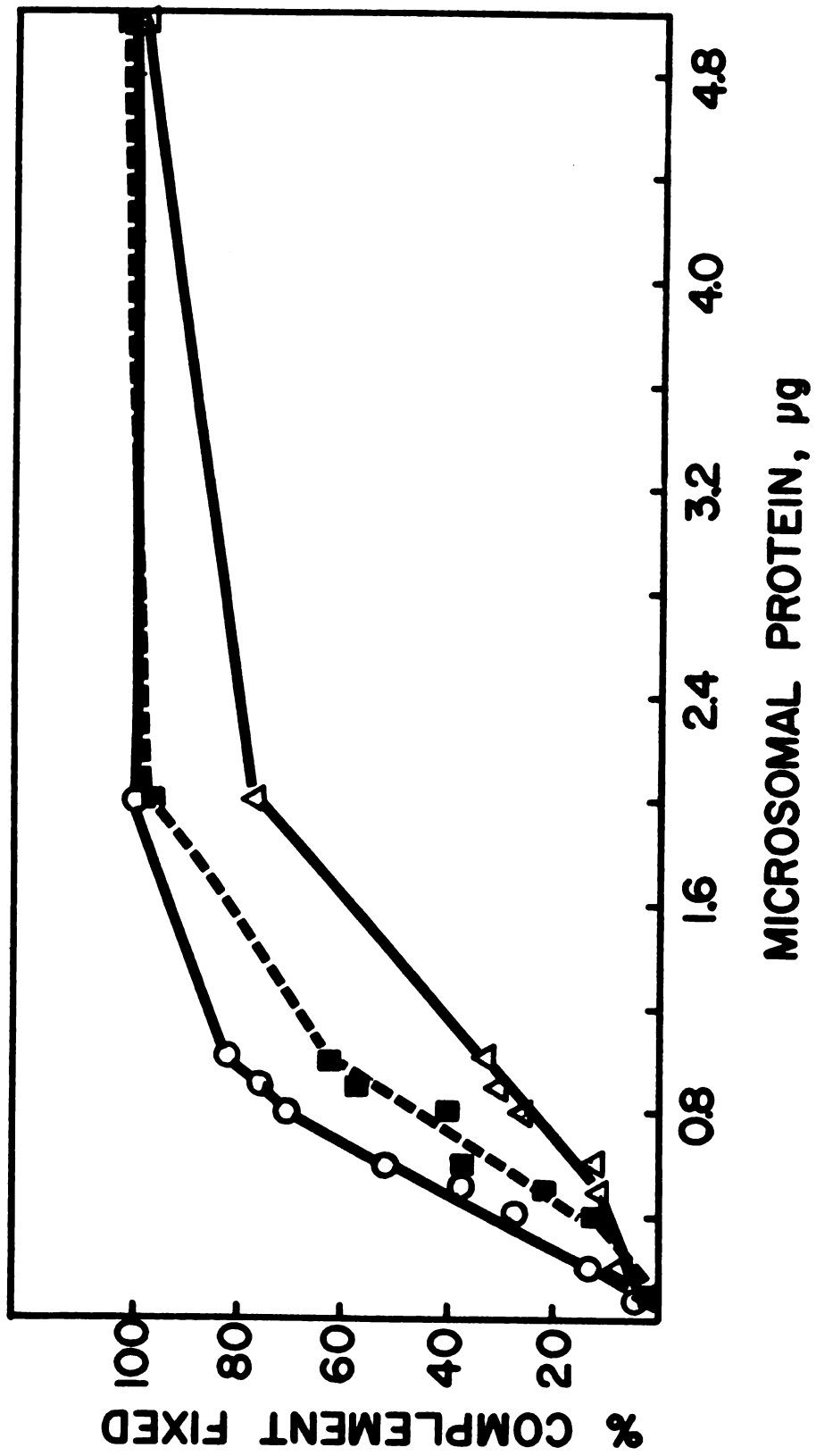
% COMPLEMENT FIXED
100
80
60
40
20
0.8
1.6
2.4
3.2
4.0
4.8
MICROSOMAL PROTEIN, µg

Figure 16.

In experiments using partially purified microsomes from control or PB- or 3-MC-pretreated rats, the relative amount of antibody bound to each type of microsome differed from that observed above (Figure 17). More complement was fixed and hence, more antibody was bound to liver microsomes from PB-pretreated rats. Approximately equal amounts of antibody bound to microsomes from control and 3-MC-pretreated rats. If comparisons are made at 0.4 μg of microsomal protein, microsomes from PB-pretreated rats bound 2.5 times the amount of antibody as do microsomes from control or 3-MC-pretreated rats.

To determine if other microsomal sources of cytochrome P450 would cross-react with anti-hemoprotein 3, complement fixation assays were performed. Lung microsomes from rats fixed complement in the presence of ten to thirty times the amount of microsomal protein used in assays with liver microsomes from rats (Figure 18). If the cytochrome P450 content of lung microsomes is significantly less than the cytochrome P450 content of liver microsomes, then one expects more microsomal protein to be needed to obtain an equivalent amount of cytochrome P450. An observation made by Hook(50) that the concentration of cytochrome P450 in rabbit lung microsomes was approximately 1/8 the amount found in rabbit liver microsomes is probably applicable to

Figure 17. COMPLEMENT FIXATION PROFILES OF ANTI-HEMOPROTEIN 3 WITH CYTOCHROME P450 PARTIALLY PURIFIED FROM WHOLE MICROSOMES

Complement fixation assays were performed as described under Materials and Methods. Each assay contained 4.7 μg of immune IgG. The fixation of complement with cytochrome P450 partially purified from microsomes isolated from PB-pretreated rats (O—O) was compared to the amount of fixation with that from control (Δ—Δ) and 3-MC-pretreated rats (■--■).

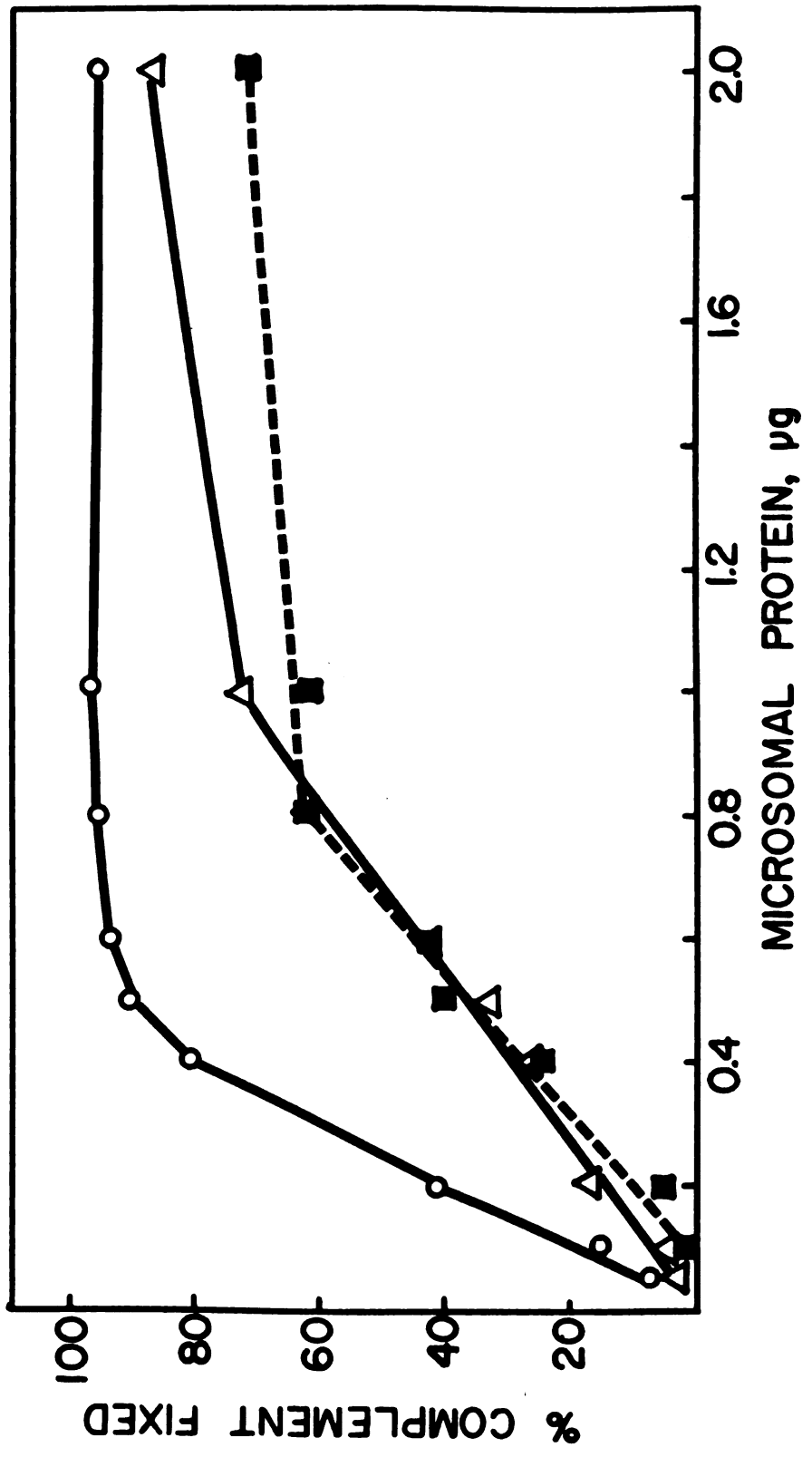
% COMPLEMENT FIXED
100
80
60
40
20
0.4
0.8
1.2
1.6
2.0
MICROSOMAL PROTEIN, µg

Figure 17.

Figure 18. COMPLEMENT FIXATION PROFILE OF RAT LUNG MICROSOMES WITH ANTI-HEMOPROTEIN 3

Complement fixation assays were performed as described under Materials and Methods. Each assay contained 4.7 μg of immune IgG and varying amounts of rat lung microsomal protein.

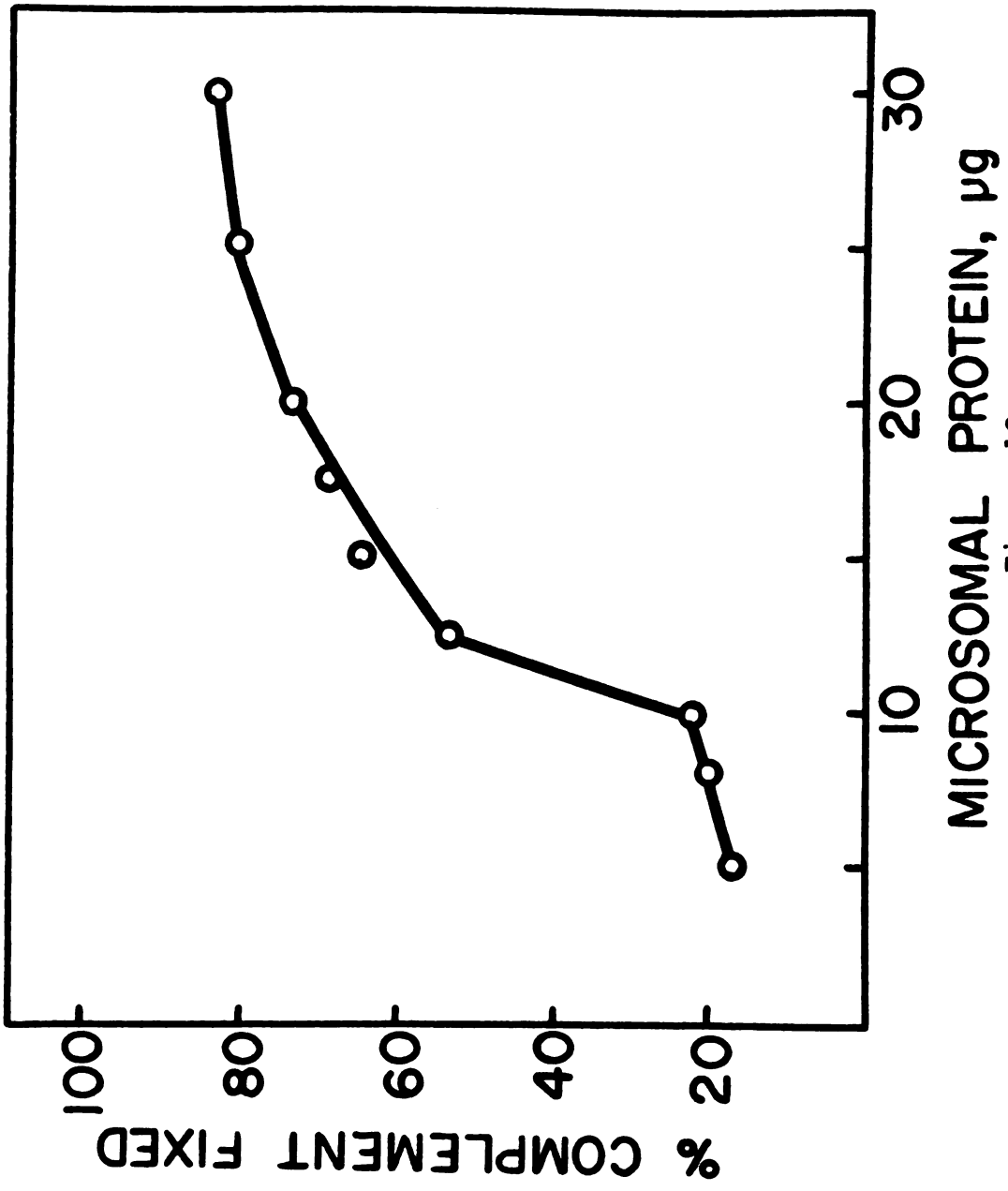
% COMPLEMENT FIXED
100
80
60
40
20
10
20
30
MICROSOMAL PROTEIN, µg

Figure 18.

those organs in rats, i.e., lung microsomes in rats contain much less cytochrome P450 than liver microsomes. Since lung microsomes from rats did bind antibody, the antigenic sites must be similar to those found in liver microsomes from rats.

Cytochrome P450 partially purified from rabbit liver microsomes and cytochrome $P450_{cam}$ purified from P. putida were compared with cytochrome P450 in whole microsomes isolated from PB-pretreated rats. One can infer from Figure 19 that no antibody is bound to cytochrome P450 from rabbit liver microsomes or cytochrome $P450_{cam}$ within the protein range examined (up to ten μg of protein). The antibody is specific for cytochrome P450 from rat liver microsomes. These results imply that few or no antigenic determinants for hemoprotein 3 are found on cytochrome P450 from rabbit liver microsomes or cytochrome $P450_{cam}$.

An antibody to cytochrome $P450_{cam}$ was tested in the complement fixation system with cytochrome $P450_{cam}$, cytochrome P450 from rabbit liver microsomes and cytochrome P450 in whole microsomes from PB-pretreated rats. As shown in Figure 20, complement was fixed by cytochrome $P450_{cam}$ but not by cytochrome P450 from rabbit or rat liver microsomes. The antibody appears to be specific for cytochrome $P450_{cam}$ implying that no common antigenic determinants

Figure 19. COMPLEMENT FIXATION PROFILES OF ANTI-HEMOPROTEIN 3 WITH CYTOCHROME P450 ISOLATED FROM RAT LIVER AND RABBIT LIVER MICROSOMES AND P. PUTIDA

Complement fixation assays were performed as described under Materials and Methods. Each assay contained 4.7 μg of immune IgG and varying concentrations of proteins: microsomal cytochrome P450 isolated from PB-pretreated rats (O—O); partially purified cytochrome P450 from rabbit liver microsomes (Δ—Δ); cytochrome $P450_{cam}$ from P. putida (■—■).

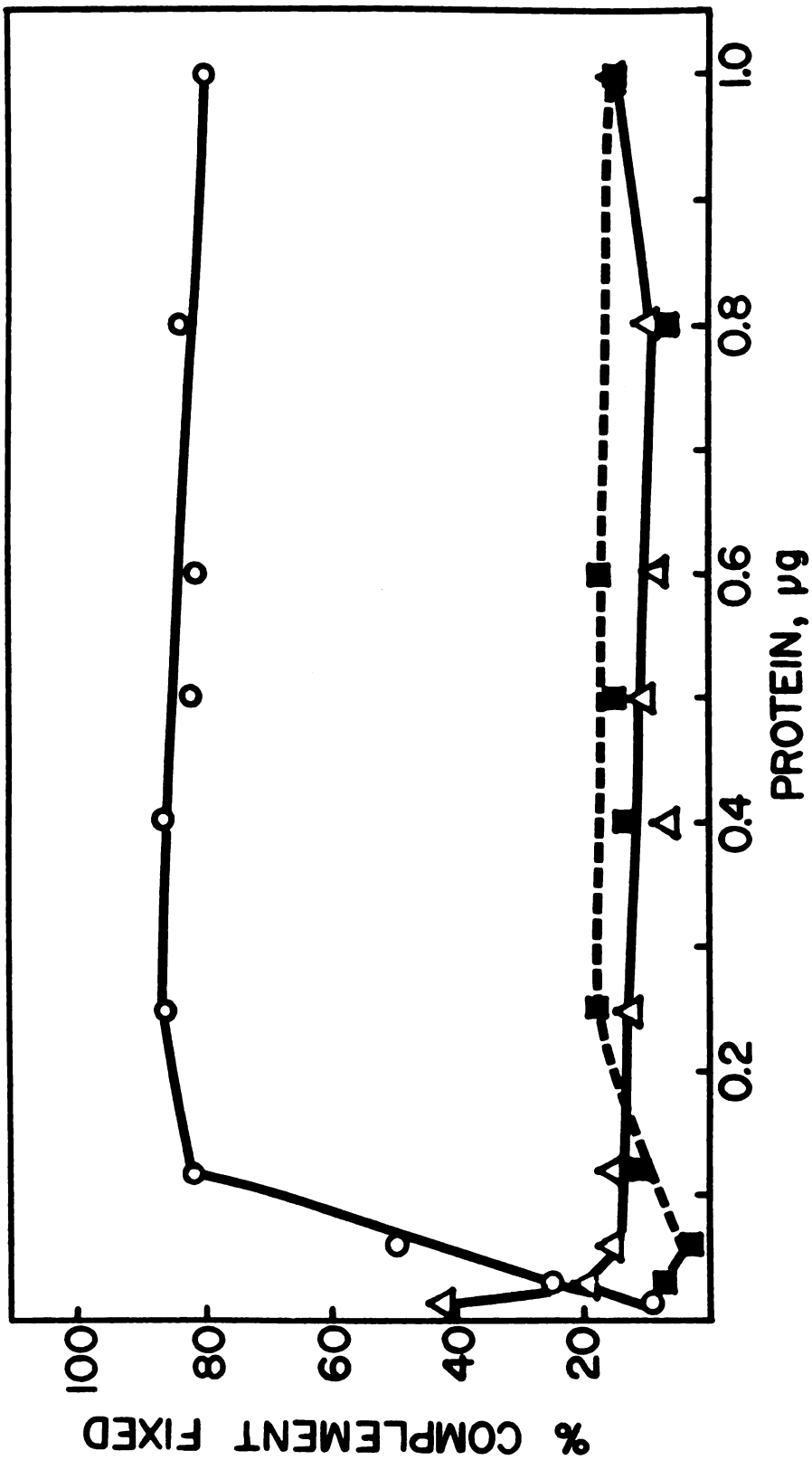

Figure 19.

Figure 20. COMPLEMENT FIXATION PROFILES OF ANTI-CYTOCHROME $P450_{CAM}$ WITH CYTOCHROME P450 FROM RAT LIVER AND RABBIT LIVER MICROSOMES AND P. PUTIDA

Complement fixation assays were performed as described under Materials and Methods. Each assay contained anti-$P450_{cam}$ diluted 1/600 and varying concentrations of proteins. Cytochrome $P450_{cam}$ (O—O); cytochrome P450 partially purified from rabbit liver microsomes (■--■); microsomes isolated from PB-pretreated rats (Δ—Δ).

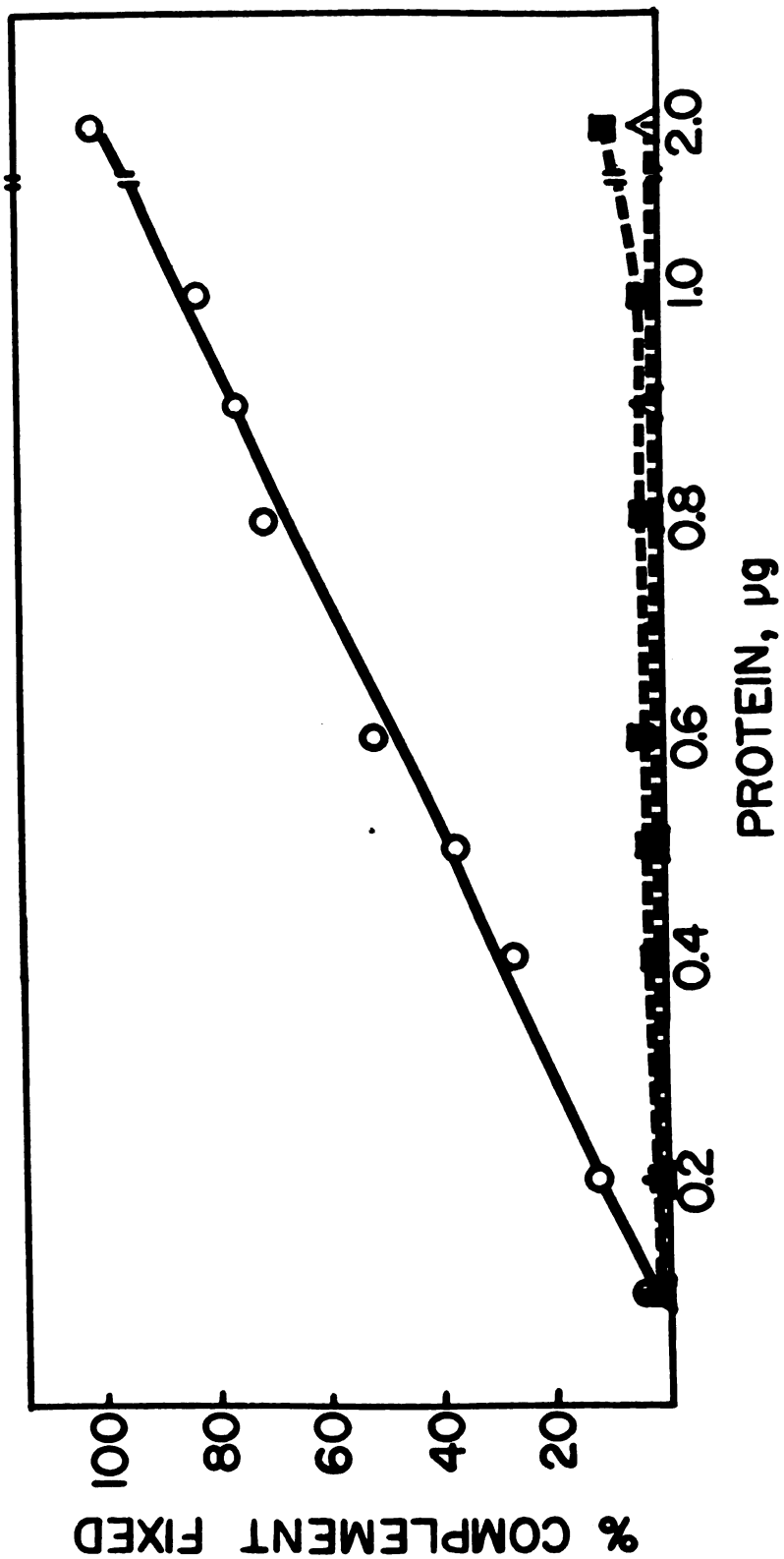

Figure 20.

exist among cytochrome $P450_{cam}$, cytochrome P450 from rabbit liver microsomes and cytochrome P450 from rat liver microsomes.

DISCUSSION

The results presented in this thesis are further evidence that anti-hemoprotein 3 is specific for hemoprotein 3 and that multiplicity of cytochrome P450 does exist. By using four different immunological techniques (Ouchterlony double diffusion, antibody inhibition of enzymatic assays, agglutination, and complement fixation) the experiments conducted have provided consistent results which support three ideas: 1) the antibody is specific for a particular antigen; 2) the same protein (hemoprotein 3) may be found in other organs of the same species; 3) the active site of cytochrome P450 appears to be buried within the microsomal membrane.

The first line of evidence for specificity was provided by Ouchterlony double diffusion analyses. If the antibody to the 44,000 dalton rat liver microsomal cytochrome P450 were specific, then it should bind only to hemoprotein 3. This was observed. The antibody reacted with those microsomes that contained hemoprotein 3 (microsomes from control and PB- and 3-MC-pretreated rats) and failed to cross-react

with cytochrome $P450_{cam}$, rabbit liver cytochrome P450 and rat lung microsomes.

The second and third lines of evidence were observed in the agglutination and complement fixation studies of whole microsomes from control and PB- and 3-MC-pretreated rats. Since hemoprotein 3 is induced in PB-pretreated rats and the antibody has been raised against this protein, one would expect more antibody to bind to microsomes from PB-pretreated rats. As more antibody was bound, more agglutination would occur and more complement would be fixed. By using results in each assay to quantitate the relative amount of antibody bound to the three types of microsomes, one observes that liver microsomes from PB-pretreated rats bound three times as much antibody as did microsomes from control rats and microsomes from 3-MC-pretreated rats bound twice as much antibody as did microsomes from control rats (Table II).

It is difficult to quantitate the concentration of the 44,000 dalton hemoprotein found in each type of microsome to make relative comparisons, but a rough estimate can be obtained by comparing 1% SDS-polyacrylamide gel electrophoresis protein profiles. Integration of peak areas can be made to quantitate the amount of hemoprotein 3. In terms of relative proportions, liver microsomes from

Table II. SUMMARY OF RESULTS FROM AGGLUTINATION AND COMPLEMENT FIXATION STUDIES WITH RESPECT TO THE AMOUNT OF HEMOPROTEIN 3 PRESENT ON 1%-SDS GELS AND THE SPECIFIC ACTIVITY OF CYTOCHROME P450

Table II summarizes the results of various studies with respect to the amount of hemoprotein 3 present. The first column denotes the source of cytochrome P450. Column 2 gives the amount of protein precipitated at 2.5 mg of IgG from agglutination studies. Column 3 gives the amount of complement fixed at 0.8 and 0.4 μg of microsomal protein from whole microsomes and partially purified cytochrome P450, respectively. Column 4 gives the % of Band 6 (hemoprotein 3) found from 1% SDS gel protein profiles. Column 5 gives the amount of complement fixed at 0.8×10^{-3} and 2.0×10^{-3} nmoles of cytochrome P450 from whole microsomes and partially purified cytochrome P450, respectively. Column 6 summarizes the specific activity of cytochrome P450. In the parentheses following each number is the proportion of that number with respect to the number given for control samples in that particular series.

Table II

Sample	Agglutination Precipitated Protein	% C'F Microsomal Protein	% Band 6 From SDS Gel Scan	% C'F nmoles P450	Specific Activity nmoles P450 per mg Protein
Whole Microsomes					
PB	124 (2.4)	71 (2.9)	15.3 (2.9)	20 (0.5)	2.6 (3.7)
3-MC	83 (1.6)	47 (1.9)	9.0 (1.7)	28 (0.7)	1.4 (2.0)
Control	51 (1.0)	24 (1.0)	5.3 (1.0)	40 (1.0)	0.7 (1.0)
Partially Purified Cytochrome P450					
PB	-------	82 (2.6)	22.8 (1.2)	62 (1.24)	6.3 (2.3)
3-MC	-------	27 (0.9)	15.3 (0.8)	30 (0.6)	4.5 (1.7)
Control	-------	31 (1.0)	19.7 (1.0)	50 (1.0)	2.8 (1.0)

% C'F - % complement fixed

PB-pretreated rats contained three times as much hemoprotein 3 as did microsomes from control rats and microsomes from 3-MC-pretreated rats contained twice as much protein as did microsomes from control rats. The correspondence between the proportions obtained with agglutination and complement fixation assays and with integration of peak areas on 1% SDS-polyacrylamide gel protein profiles argues for the specificity of the antibody.

The fourth line of evidence supporting antibody specificity was observed in complement fixation assays involving cytochrome P450 partially purified from three types of whole microsomes. The proportion of hemoprotein 3 found in each type of microsome can be roughly estimated by integration of peak areas from 1% SDS-polyacrylamide gel electrophoresis protein profiles. The ratios calculated for cytochrome P450 from microsomes isolated from PB- and 3-MC-pretreated rats as compared to that from control rats were 1.2 and 0.8, respectively. A similar relationship was observed with respect to the amount of antibody bound in complement fixation assays except that cytochrome P450 from PB-induced rats bound 2.5 times as much antibody as did cytochrome P450 from control rats. The discrepancy observed with respect to the relative amount of antibody bound by cytochrome P450 from PB-induced rats compared to

that from control rats, i.e., 1.2 and 2.5, may be an idiosyncrasy of the purification process of cytochrome P450. More antigenic sites per molecule of cytochrome P450 from PB-induced rats may be exposed during purification.

If the mixed-function oxidase system is found in other organs of the same species and contains the same protein components, then one may hypothesize that the systems also have a similar hemoprotein 3. Rat lung microsomes contain cytochrome P450, but in much smaller quantities than is found in rat liver microsomes. Given that the antibody is specific for hemoprotein 3, the antibody can be used in the complement fixation assay with rat lung microsomes to ascertain if hemoprotein 3 is present in rat lung microsomes. It was found that rat lung microsomes fixed complement when at least ten times the amount of rat liver microsomal protein was present. These results imply that similar antigenic sites for anti-hemoprotein 3 exist in the cytochrome P450s isolated from various organs within the same species.

Additional evidence that anti-hemoprotein 3 is specific for hemoprotein 3 from rat microsomes was suggested from results of complement fixation with cytochrome P450 and cytochrome $P450_{cam}$ isolated from rabbit liver microsomes and *P. putida*, respectively. No complement was fixed by either cytochrome in the presence of anti-hemoprotein 3.

These results indicate the lack of similar antigenic determinants among cytochrome $P450_{cam}$, rabbit liver cytochrome P450 and rat liver cytochrome P450. Anti-hemoprotein 3 is specific for rat microsomal hemoprotein 3, which apparently is not present in the other two systems.

The antibody to cytochrome $P450_{cam}$ was also tested by complement fixation with cytochrome $P450_{cam}$, rabbit liver cytochrome P450, and rat liver cytochrome P450. Only the system containing cytochrome $P450_{cam}$-anti-cytochrome $P450_{cam}$ fixed complement; therefore, there appear to be no common antigenic determinants among cytochrome $P450_{cam}$, rabbit liver cytochrome P450, and rat liver cytochrome P450.

The results with the anti-cytochrome $P450_{cam}$ and anti-hemoprotein 3 were quite surprising since recent experiments performed in Coon's laboratory(26) were contrary to what we expected. In their reconstituted system, the hydroxylation of benzphetamine in the presence of purified cytochrome P450 isolated from rabbit liver microsomes was inhibited by antibodies to rabbit liver cytochrome P450 and to cytochrome $P450_{cam}$. Both antibodies must have similar antigenic determinants in order to cross-react with rabbit liver cytochrome P450. This discrepancy indicates the necessity to test their antibodies and antigens in our complement fixation assay. If complement is fixed only for specific

antigen-antibody reactions, then the inhibition of benzphetamine hydroxylation by both antibodies is rather dubious. If, on the other hand, cross-reactions do occur, then there are distinct differences between our antibody to rat liver hemoprotein 3 and their antibodies. It is inconceivable, though, that the same antibody to cytochrome $P450_{cam}$ would not fix complement with rabbit liver cytochrome P450 but would inhibit an enzymatic reaction.

Returning to the initial complement fixation studies discussed above, one can observe an interesting problem when the amount of complement fixed is plotted against the concentration of cytochrome P450 (Figures 21 and 22). In Figure 21, the relationships among cytochrome P450 isolated from control and PB- and 3-MC-induced rats are no longer 3:2:1 for cytochrome P450 isolated from PB, 3-MC, and control microsomes, respectively. At 0.8×10^{-3} nmoles of cytochrome P450, it appears that cytochrome P450 from control microsomes binds twice as much antibody as does cytochrome P450 isolated from PB microsomes. If, in terms of microsomal protein, three times as much antibody was bound by microsomes from PB-induced rats than from control rats, then it is not logical that expressing the amount of antibody bound in terms of nmoles of cytochrome P450 would alter the relationship between the amount of

Figure 21. COMPLEMENT FIXATION PROFILES OF CYTOCHROME P450 IN WHOLE MICROSOMES WITH ANTI-HEMOPROTEIN 3

Complement fixation assays were performed as described under Materials and Methods. The concentration of cytochrome P450 was extrapolated from the specific activities of cytochrome P450 in whole microsomes and microsomal protein used in each assay. Cytochrome P450 in whole microsomes from PB-pretreated rats (O—O); cytochrome P448 in whole microsomes from 3-MC-pretreated rats (■--■); cytochrome P450 in whole microsomes from control rats (Δ—Δ).

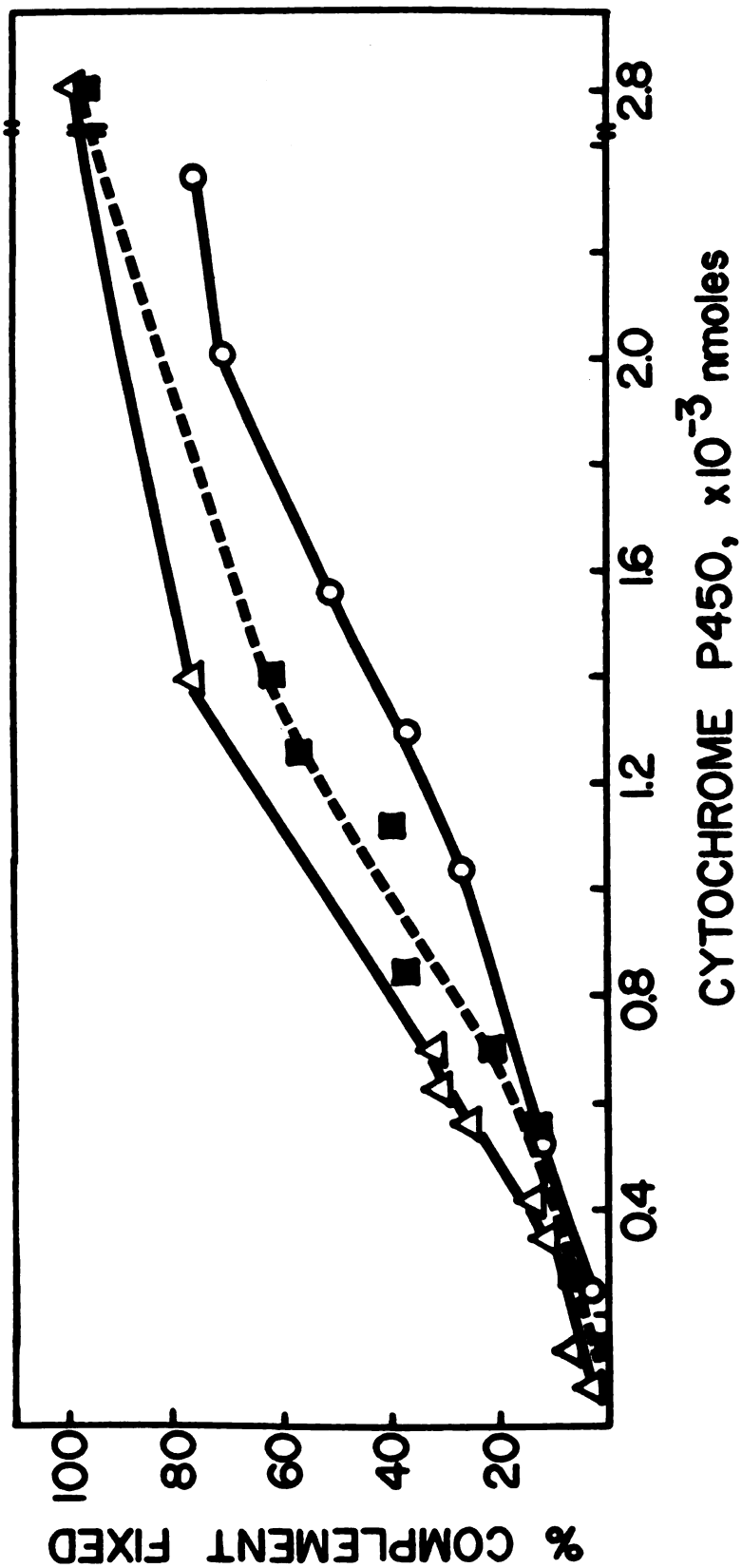
% COMPLEMENT FIXED
100
80
60
40
20
0.4
0.8
1.2
1.6
2.0
2.8
CYTOCHROME P450, x10^{-3} nmoles

Figure 21.

Figure 22. **COMPLEMENT FIXATION PROFILES OF PARTIALLY PURIFIED CYTOCHROME P450 WITH ANTI-HEMOPROTEIN 3**

Complement fixation assays were performed as described under Materials and Methods. The concentration of cytochrome P450 was determined from specific activities of partially purified cytochrome P450 and microsomal protein used in each assay. Cytochrome P450 partially purified from PB-microsomes (O—O); cytochrome P448 partially purified from 3-MC microsomes (■—■); cytochrome P450 partially purified from control microsomes (Δ—Δ).

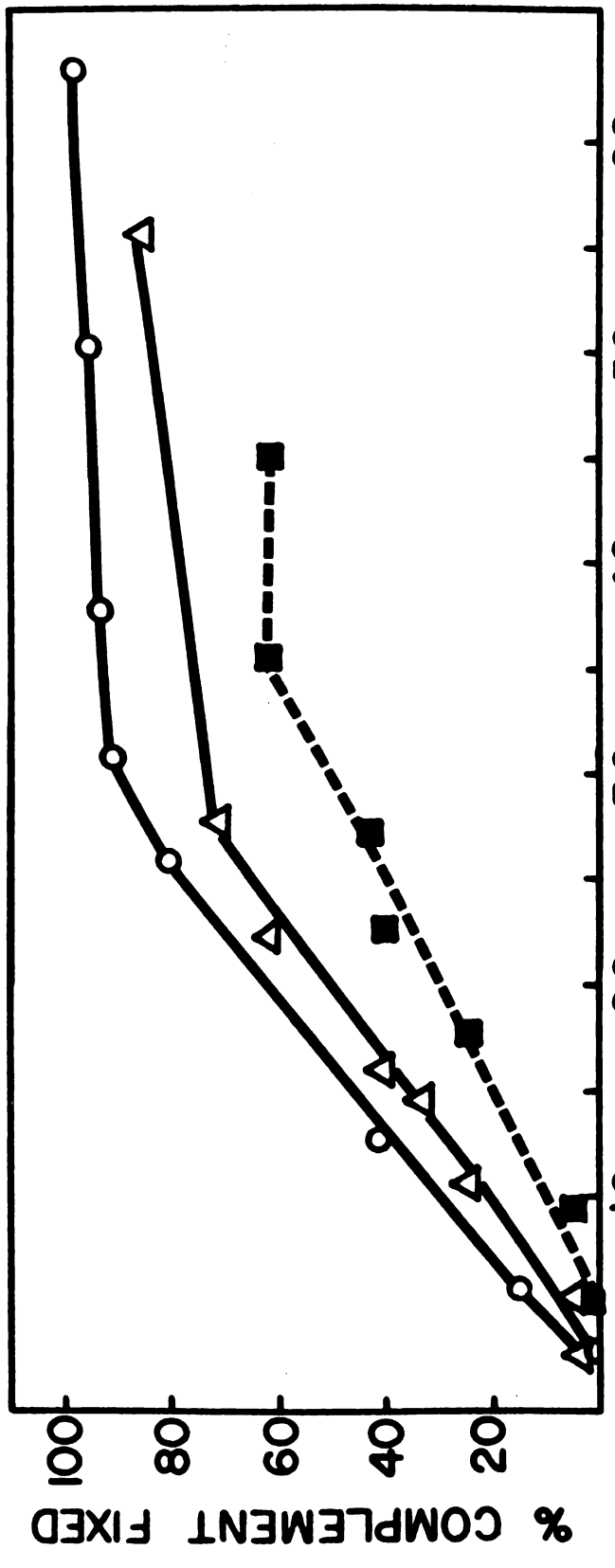
% COMPLEMENT FIXED
100
80
60
40
20
1.0
2.0
3.0
4.0
5.0
6.0
CYTOCHROME P450, x10-3 nmoles

Figure 22.

antibody bound by cytochrome P450 from PB microsomes and that by cytochrome P450 from control microsomes. This might be explained if the spectrophotometric assay of cytochrome P450 is not valid. It has recently been proposed that apoprotein exists which cannot be detected by the spectrophotometric assay(51). If antibody binds to apoprotein which is not detected as cytochrome P450, then the amount of cytochrome P450 is underestimated and will give the results shown in Figure 21. It seems reasonable, therefore, that the spectrophotometric assay of cytochrome P450 should be scrutinized.

If the percent of complement fixed is plotted against the concentration of cytochrome P450 partially purified from microsomes isolated from control and PB- and 3-MC-pretreated rats, the relationships observed in Figure 17 on the basis of microsomal protein are also observed in Figure 22. Unfortunately the ratios of specific activity for cytochrome P450 from partially purified PB, 3-MC, and control microsomes do not correspond to the ratios observed on the basis of amount of bound antibody (Table II). Again, the amount of cytochrome P450 can be underestimated if the assay is dependent upon heme bound to apoprotein.

Evidence for the location of the enzymatic site with respect to antigenic sites has also been presented. The

results of agglutination and enzyme inhibition assays indicate that some antigenic sites for cytochrome P450 are exposed on the exterior surface of the membrane, i.e., on the cytoplasmic side. The agglutination process relies upon externally exposed antigenic sites. The failure to inhibit aminopyrine-N-demethylation suggests that the enzymatic site is buried within the membrane and is exposed upon solubilization. Preliminary studies by Frederick O'Neal in Dr. Aust's laboratory with benzphetamine and benzpyrene hydroxylations in a reconstituted system show specific inhibition in the presence of anti-hemoprotein 3. Benzphetamine hydroxylation, specific for cytochrome P450 in PB-induced systems, was inhibited by the antibody but benzpyrene hydroxylation (specific for cytochrome P448) was not. The results imply that the antibody is specific for the 44,000 dalton cytochrome P450 and that antigenic sites exposed in a soluble, reconstituted system do inhibit enzymatic reactions.

In summary, the results presented in this thesis provide further evidence that: 1) anti-hemoprotein 3 is specific for the 44,000 dalton hemoprotein from microsomes induced in PB-pretreated rats and 2) the active site is not freely exposed to the exterior but probably buried within the membrane. If specific antibodies for each type

of cytochrome or hemoprotein can be made, it will be possible to conduct studies specific for one component in the presence of other similar proteins. With the use of antibodies, one will be able to investigate the relative rate of turnover and enzyme specificity and, perhaps, develop more reliable assays for individual hemoproteins.

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APPENDIX

APPENDIX

In Review:

Ann F. Welton, Fredrick O. O'Neal, Linda C. Chaney and Steven D. Aust, "Multiplicity of Cytochrome P450 Hemoproteins in Rat Liver Microsomes: Preparation and specificity of an antibody to the hemoprotein induced by phenobarbital," J. Biol. Chem.

Abstracts:

A.F. Welton, L.C. Mandorf, and S.D. Aust, "Studies of Multiple Cytochrome P450 Hemoproteins in Rat Liver Microsomes," Fed. Proc., 33, 1437 (1974).

R.W. Moore, F.O. O'Neal, L.C. Chaney, and S.D. Aust, "Specificity of Antibody to the Cytochrome P450 Hemoprotein Induced by Phenobarbital," Fed. Proc. (in press).