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LIVER MICROSOMAL NADPH-CYTOCHROME P-450 REDUCTASE: PURIFICATION AND CHARACTERIZATION

by

Janice Lynn Vermilion

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1976

Doctoral Committee:

Professor Minor J. Coon, Chairman
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Assistant Professor John T. Groves
Professor Vincent Massey
Professor Raymond W. Ruddon

To My Parents

ACKNOWLEDGMENTS

I would like to express my gratitude to the numerous people who have aided me during these studies. I wish to thank my thesis advisor, Dr. Minor J. Coon, for encouragement throughout the course of this work, and especially for his expert advice and comments in regard to the preparation of the manuscript. To my coworkers, in Dr. Coon's laboratory, particularly Dr. Fred P. Guengerich and Dr. David A. Haugen, I wish to express my appreciation for invaluable aid contributed in the form of critical discussion and technical advice. I am especially indebted to Susan B. Krezoski for performing the SDS-polyacrylamide gel electrophoresis experiments. In addition, I would like to thank Dr. Vincent Massey and the members of his laboratory for numerous helpful suggestions and discussions, as well as for their generosity in regard to the use of equipment and reagents. I also extend my deepest gratitude to Dr. Eglis T. Lode, who has been a source of invaluable information concerning the study of electron transfer proteins, both for his interest in this work and many thoughtful suggestions.

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ABBREVIATIONS

Abbreviation or Trivial Name	Systematic Name
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADH	Reduced nicotinamide adenine dinucleotide
FAD	Flavin adenine dinucleotide
FMN	Riboflavin-5'-phosphate
EDTA	Ethylenediaminetetraacetate
SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin
Tris	Tris(hydroxymethyl)aminomethane
DEAE-cellulose	Diethylaminoethyl-cellulose
DCPIP	2,6-Dichlorophenolindophenol
Menadione	2-Methyl-1,4-napthoquinone
3-AcPyADP	3-Acetyl pyridine adenine dinucleotide phosphate
Dilauroyl-GPC	Dilauroylglycerol-3-phosphoryl choline
Reductase, P-450 reductase, or NADPH-cytochrome P-450 reductase	NADPH:ferricytochrome oxidoreductase (EC 1.6.2.4), detergent-solubilized; NADPH:ferricytochrome P-450 oxidoreductase

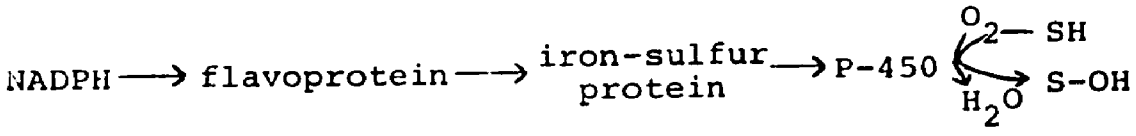
Abbreviation or Trivial Name	Systematic Name
Cytochrome c reductase or NADPH-cytochrome c reductase	NADPH:ferricytochrome oxidoreductase (EC 1.6.2.4), refers to preparations solubilized by protease or lipase treatment
P-450	Liver microsomal cytochrome P-450, unless otherwise noted
$P\text{-}450LM_2$	Phenobarbital-induced form of rabbit liver microsomal P-450, designated (2) by SDS-polyacrylamide gel electrophoretic mobility relative to that of other forms of rabbit liver microsomal P-450
$P\text{-}450LM_4$	β-Napthoflavone-induced form of rabbit liver microsomal cytochrome P-450, designated (4) by SDS-polyacrylamide gel electrophoretic mobility relative to that of other forms of rabbit liver microsomal P-450

INTRODUCTION

The enzyme system responsible for the NADPH-dependent hydroxylation of a wide variety of compounds by liver microsomes has received attention from biochemists, pharmacologists, and toxicologists because of the diversity of the substrates oxidized and because of its important role in detoxification (1). As shown by Omura *et al.* (2) and Estabrook and associates (3,4), cytochrome P-450 is the component of the enzyme system which participates in the reaction of substrate with oxygen, in what is believed to be a typical mixed function oxidation reaction, as defined by Mason (5). The reducing equivalents required for the P-450-catalyzed hydroxylation reactions are supplied indirectly from NADPH in a series of electron transfer steps. The research described in this thesis is concerned with the identification and characterization of the intermediate electron transfer protein, NADPH-cytochrome P-450 reductase.

Due to difficulty in solubilizing the membrane-bound system of liver microsomes, the identification of the essential components of similar P-450-containing enzyme systems from adrenocortical mitochondria and from the bacterium, *Pseudomonas putida*, preceded the resolution of the individual components of the liver microsomal system. In studies

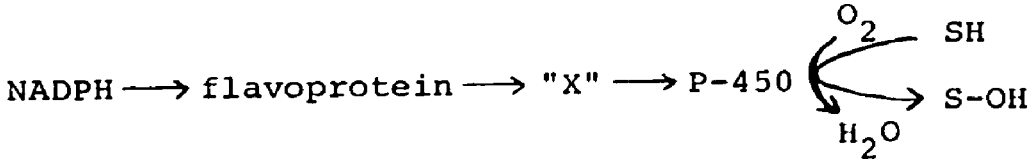
summarized by Gunsalas et al. (6), the soluble bacterial enzyme system which is responsible for the hydroxylation of camphor was shown to require a flavoprotein and an iron-sulfur protein as intermediate electron carriers in the overall transfer of reducing equivalents from NADH to cytochrome P-450. Similarly, the studies of Omura and coworkers (2,7) and of Schleyer et al. (8), also showed the involvement of both a flavoprotein and an iron-sulfur protein in electron transfer from NADPH to cytochrome P-450 in the 11β-hydroxylation of steroids by adrenocortical mitochondria. The sequence of electron flow in adrenocortical mitochondria is as follows (2):



where S represents substrate.

By analogy to these two systems, electron flow in liver microsomes was assumed to occur in a similar sequence of electron transfer steps. At this time, there was already good evidence for the participation of the flavoprotein, NADPH-cytochrome c reductase, in the microsomal electron transport system; the role of this enzyme will be discussed shortly. The nature of the electron carrier, if any, between the flavoprotein and cytochrome P-450 was somewhat

uncertain. Immunochemical (9) and EPR studies (10,11) failed to provide evidence for the presence of an iron-sulfur protein in microsomes analogous to the adrenodoxin of the mitochondrial system, or the putidaredoxin of the bacterial system. Therefore, the postulated sequence of electron transfer in liver microsomes was as follows (12, 13):



where the identity of "X" was unknown.

In 1968, Lu and Coon (14) reported the solubilization of the NADPH-dependent hydroxylating system from liver microsomes by the use of detergent in the presence of protective agents. This system was separated by chromatographic procedures into three fractions which contained P-450, an NADPH-specific reductase, and a heat-stable factor (15). The heat-stable factor was subsequently identified as phosphatidylcholine (16). When the two enzyme fractions and lipid were combined, this reconstituted system catalyzed the hydroxylation of fatty acids and a variety of drugs and hydrocarbons (15,17). Subsequently, similar techniques were used to study the hydroxylation reactions catalyzed by liver microsomal P-450 from several species, including rat (18), rabbit (19), mouse (20), and human (21), and by P-450 from a yeast (22), and in each case

a requirement for phospholipid and a reductase fraction was demonstrated.

More recently, the phenobarbital-induced P-450 from rabbit liver microsomes has been obtained in apparently homogeneous form (23-25). The enzyme was characterized by Coon and coworkers (23,26-30) in a series of studies, and is a single polypeptide chain of molecular weight 49,000, containing one molecule of heme. The enzyme has the spectral properties of a low-spin hemeprotein and exhibits maximal absorbance as the reduced-CO complex at 452 nm. It is catalytically most active in drug demethylation and hydrocarbon hydroxylation. The midpoint oxidation-reduction potential at pH 7.0, which is not altered by substrate binding, is -0.330 V and the enzyme contains, in addition to the iron atom of the heme, an as yet unidentified electron acceptor with a similar potential (31,32).

Previous evidence that suggested the presence of different forms of P-450 in liver microsomes (1,33-39) has been substantiated by the separation and purification of nonidentical forms of the enzyme from both rabbit (28) and rat (40,41) liver. The phenobarbital-induced form of P-450, described above, may be distinguished from other forms of P-450 by differences in molecular weight, pattern of induction, spectral and catalytic properties, and on the basis of immunochemical cross reactivity (28,30,42,43). The

existence of multiple forms of P-450 may, in part, account for the broad substrate specificity of the microsomal system.

The reductase fraction described by Lu *et al.* (15), which was shown to catalyze the reduction of P-450, also exhibited cytochrome *c* reductase activity. The involvement of the microsomal flavoprotein, NADPH-cytochrome *c* reductase, in microsomal electron transport had been suggested earlier (1,44). This enzyme, first isolated from mammalian liver by Horecker in 1950 (45), was shown to be localized in the microsomal membrane by Williams and Kamin (46) and by Phillips and Langdon (47). Since cytochrome *c* is not a constituent of microsomes, the physiological electron acceptor for this reductase was unknown; however, several lines of evidence implicated this enzyme as a component of the electron transfer system leading to P-450-dependent hydroxylation reactions. It was demonstrated that induction of P-450 and associated drug oxidation activities was also accompanied by an increase in the amount of microsomal cytochrome *c* reductase (4,48,49). Compounds which served as artificial electron acceptors for the enzyme were known to inhibit drug hydroxylation in microsomes (1,50) and, in addition, immunochemical studies established that antibodies prepared against the purified reductase were effective inhibitors of microsomal oxidation reactions (9,51,52).

Although these experiments provided strong support for a role for NADPH-cytochrome *c* reductase in electron transfer

to P-450, the purified lipase-solubilized enzyme of Williams and Kamin (46) could not substitute for the detergent-solubilized reductase fraction of the three-component system of Lu et al. (15). While several laboratories have reported the isolation of purified cytochrome c reductase (46,47,53-56), none of these preparations have been shown to function as a P-450 reductase. In each case, the enzyme was isolated by procedures which relied on protease or lipase treatment of the microsomes to solubilize the enzyme; therefore, as suggested by Lu et al. (15), it was possible that these methods of solubilization somehow altered the enzyme so that it no longer functioned in P-450 reduction. Alternatively, it was possible that yet a fourth essential component of the system remained unresolved by the fractionation procedure of Lu et al. (15).

Support for the hypothesis that proteolytically solubilized enzymes represent altered forms of the native enzymes was provided by studies on microsomal cytochrome b_5 and NADH-cytochrome b_5 reductase by Sato and coworkers (57,58). It was subsequently established that proteolytic treatment of these proteins led to cleavage of the membrane-bound enzyme into two fragments (59,60). The larger of the two fragments is a soluble protein and contains the bound cofactor, but it has catalytic properties significantly different from those of the detergent-solubilized enzyme (58,60). The smaller fragment, lost in subsequent purification, contains a large

proportion of hydrophobic residues and is believed to be involved in binding of the native enzyme to the membrane (61,62). The possibility that solubilization of NADPH-cytochrome c reductase by proteolytic treatment of the microsomes also resulted from cleavage of a portion of the polypeptide chain received support from the work of Welton et al. (63). In experiments utilizing antibodies prepared against the bromelain-solubilized reductase (55), it was found that the polypeptide chain precipitated from detergent-solubilized microsomes by treatment with the antibody had a molecular weight of 79,000, significantly higher than that of 71,000 for the purified enzyme. These experiments left little doubt that the protease-solubilized reductase was a physically modified form of the native enzyme, and therefore might have altered catalytic properties as well. Clearly, a better understanding of the role of this enzyme in electron transfer to P-450 required isolation of the purified enzyme without the use of proteolytic agents.

While the early studies on the cytochrome c reductase indicated that the enzyme contained FAD (45,46) in the amount of two mol of flavin per mol of enzyme (64), Iyanagi and Mason (56) recently reported that the enzyme contained equimolar quantities of FAD and FMN. Partial purification of the reductase by detergent procedures has been accomplished in several laboratories from liver (26,56,65-67) and kidney microsomes (68), and the presence of both

flavins in these preparations has also been reported (26, 56); however, the relationship between the P-450 reductase and cytochrome c reductase activities still remained somewhat uncertain. A satisfactory demonstration that these two activities are common to a single protein has only recently been made possible by the extensive purification of the detergent-solubilized enzyme (69-71). The studies presented in this thesis with the purified detergent-solubilized flavoprotein and purified P-450 establish the direct involvement of this enzyme in electron transfer to P-450 in the absence of the participation of other electron carriers. It is hoped that the purification and characterization of P-450 reductase will assist in a better understanding of the role of this important flavoprotein in microsomal electron transport.

EXPERIMENTAL PROCEDURES

Materials

Reagents and Chemicals: The following materials were obtained from Sigma: DEAE-cellulose (0.91 meq per gm), Tris base (reagent grade), di-sodium EDTA, dithiothreitol, cytochrome c (horse heart, Type III and Type VI), NADPH, NADH, NADP, 3-AcPyADP, menadione, DCPIP, FAD, riboflavin, Naja naja venom, bovine serum albumin, glycine, N,N,N',N'-tetramethylethylenediamine, acetylacetone, and phenylmethane sulfonyl fluoride. Potassium ferricyanide, nitro blue tetrazolium, formaldehyde solution, and methanol were obtained from the J. T. Baker Chemical Co. Ammonium persulfate was purchased from Mallinckrodt and phenobarbital from Merck and Co. Acrylamide and N,N'-bis-methylene acrylamide (electrophoresis purity), and agarose (A-1.5m, 100-200 mesh) were from Biorad Laboratories. Sodium deoxycholate, guanidinium chloride and ammonium sulfate (ultra pure) were obtained from Schwarz-Mann. Benzphetamine hydrochloride was purchased from Upjohn Co., cyclohexane (spectral grade) and 2-mercaptoethanol from Eastman Organic Chemicals, potassium bromide from Matheson, Coleman, and Bell, and SDS from BDH Chemicals, Ltd. Perchloric acid was

a product of the G. Frederick Smith Chemical Co. and Coomassie Blue R-250 of Canalco, Inc. Ethylmorphine hydrochloride was obtained from The University of Michigan Hospital stores, and aniline-C^{14}(U)-hydrochloride (9.55 mc per mmol) from New England Nuclear. MN-polygram Cel 300 and MN-polygram Sil S-HR precoated thin layer plates were purchased from Brinkmann Instruments. Renex 690 was obtained from ICI, America. Chromatographically pure FAD and a sample of FMN prepared from flavodoxin were kindly furnished by Dr. Vincent Massey. Dilauroyl-GPC was obtained as a solution in chloroform from Serdary Research Laboratories. Prior to use, the solvent was removed under a stream of nitrogen, and a 1 mg per ml aqueous suspension was prepared by sonication.

DEAE-cellulose: DEAE-cellulose was thoroughly washed before use by the following procedure: Six hundred grams of the cellulose were suspended in 16 liters of water and defined several times. Sodium hydroxide was added to a final concentration of 0.5 N, the suspension was stirred for 10 min, and was then filtered on a large Buchner funnel. The wet cake was resuspended in water and then stirred and filtered as described above. This process was repeated with 16 liters each of 0.1 N HCl, water, 0.5 N NaOH, and water. The material was defined several more times before use.

Calcium Phosphate Gel: The preparation of calcium phosphate gel was carried out as described by Swingle and Tiselius (72). The concentration of the final suspension

was determined by the dry weight of evaporated samples and adjusted to 30 mg per ml. Freshly prepared gel was no less effective in the enzyme purification than material aged for over a year.

Enzymes: P-450LM$_2$, purified by the method of van der Hoeven et al. (23,26) was provided by Ms. Sylvia B. Dahl. P-450 concentrations were determined using an extinction coefficient of 91 m$\underline{M}^{-1}$ for $A_{450-490}$ for the CO-difference spectrum of the reduced hemeprotein (73). The preparations used for the majority of this work were homogeneous as judged by their electrophoretic properties when submitted to SDS-polyacrylamide gel electrophoresis and contained 14.5 to 16.9 nmol of P-450 per mg of protein. P-450LM$_4$, purified from β-napthoflavone-induced rabbit liver microsomes (28), was provided by Dr. D. A. Haugen and contained 14.8 nmol per mg of protein.

Partially purified detergent-solubilized rabbit liver cytochrome $\underline{b}_5$ was generously supplied by Dr. F. P. Guengerich. The concentration of cytochrome $\underline{b}_5$ was determined using an extinction coefficient of 185 m$\underline{M}^{-1}$ cm^{-1} for $A_{424-409}$ for the reduced difference spectrum (73). Bromelain-solubilized NADPH-cytochrome $\underline{c}$ reductase, purified by the method of Pederson et al. (55), was also furnished by Dr. Guengerich. The enzyme preparation catalyzed the reduction of 54 μmol cytochrome $\underline{c}$ per min per mg of protein and contained 49% of the total flavin as FMN.

Methods

Preparation of Microsomes: Phenobarbital was administered to 150-g male rats (Sprague Dawley strain) as a 0.1% solution in their drinking water for 3 1/2 days. Animals received no food during the 16-hr period prior to sacrifice and were killed by decapitation. Excised livers were immediately chilled and rinsed with 0.05 M Tris, pH 7.4, containing 1.15% KCl and 1.0 mM EDTA. Tris-chloride buffers were used throughout the entire procedure and subsequent operations were performed at 4°. The livers were minced with scissors and homogenized in a Waring blendor with 4 volumes of the above buffer for two 40-sec intervals. The homogenate was centrifuged at 10,000 x g for 30 min, and the resulting supernatant solution was then centrifuged in a Beckman Type 35 preparative rotor for 70 min at 35,000 rpm to pellet the microsomal fraction. The microsomes were carefully removed, leaving behind the small glycogen-containing portion of the pellet, and resuspended in 0.05 M Tris, pH 7.7, containing 1.0 mM EDTA, by use of a Potter-Elvehjem glass homogenizer with a teflon pestle. The total volume was adjusted to that of the 10,000 x g supernatant solution and centrifugation was repeated. The microsomes were then resuspended as described above in 0.05 M Tris, pH 7.7, containing 1.0 mM EDTA and 20% (v/v)

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glycerol at a protein concentration of 25 to 35 mg per ml, and stored at -20° for periods not exceeding 2 weeks.

Protein Determination: Protein was estimated by the method of Lowry et al. (74) using bovine serum albumin as a standard, and absorbance readings were taken at 750 nm. The concentration of albumin was determined independently using $E^{1\%}_{279}$ of 6.67 (75). Each determination included a series of samples and standards, all containing an equivalent amount of sample buffer.

Assay for Cytochrome P-450 Reductase Activity: NADPH oxidation in the presence of a saturating amount of P-450LM$_2$ and phospholipid, with benzphetamine as the substrate, was routinely used as a measure of the ability of the reductase to transfer electrons to the cytochrome. The reaction mixture contained, in a final volume of 1.0 ml, the following components in the order of addition indicated: 30 µg of dilauroyl-GPC, 0.5 nmol of P-450LM$_2$, varying amounts of reductase, 100 µmol of potassium phosphate buffer, pH 7.4, and 1.0 µmol of benzphetamine. The mixture was preincubated at 30° for 3 min, and the reaction was initiated by the addition of 0.15 µmol of NADPH. The oxidation of NADPH was followed at 340 nm by use of a Beckman DU spectrophotometer equipped with a Gilford Model 2000 multiple sample absorbance recorder or by use of an Aminco DW-2 spectrophotometer. Rates were determined using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Preparations of

purified P-450LM$_2$ were free of detectable amounts of reductase activity; therefore, the control rates were determined in the absence of the cytochrome. This rate reflected the NADPH-oxidase activity of the flavoprotein and was generally undetectable under the assay conditions described above. The specific activity of the reductase was calculated on the basis of this spectral assay and expressed as μmol of NADPH oxidized per min per mg of protein.

The exact amount of P-450LM$_2$ required for maximal rates varied somewhat with the preparation of hemeprotein used. Fig. 1 shows a typical saturation curve obtained when the amount of reductase and phospholipid were held constant and P-450LM$_2$ was increased to a nearly saturating level. At a concentration of about 0.5 μM of cytochrome, the rate of NADPH oxidation showed a linear dependence on added reductase up to 0.04 nmol, as shown in Fig. 2. It has been estimated that phenobarbital-induced liver microsomes contain approximately 0.1 nmol of reductase and 3 nmol of total P-450 per mg of protein (13,56), giving a molar ratio of cytochrome to flavoprotein of about 30. It should be noted that the ratio of the two enzymes under the conditions of this assay is very similar.

When it was necessary to quantitate hydroxylated product formed in the above reaction, formaldehyde liberation resulting from the N-demethylation of benzphetamine was determined by a colorimetric method. The reaction volume

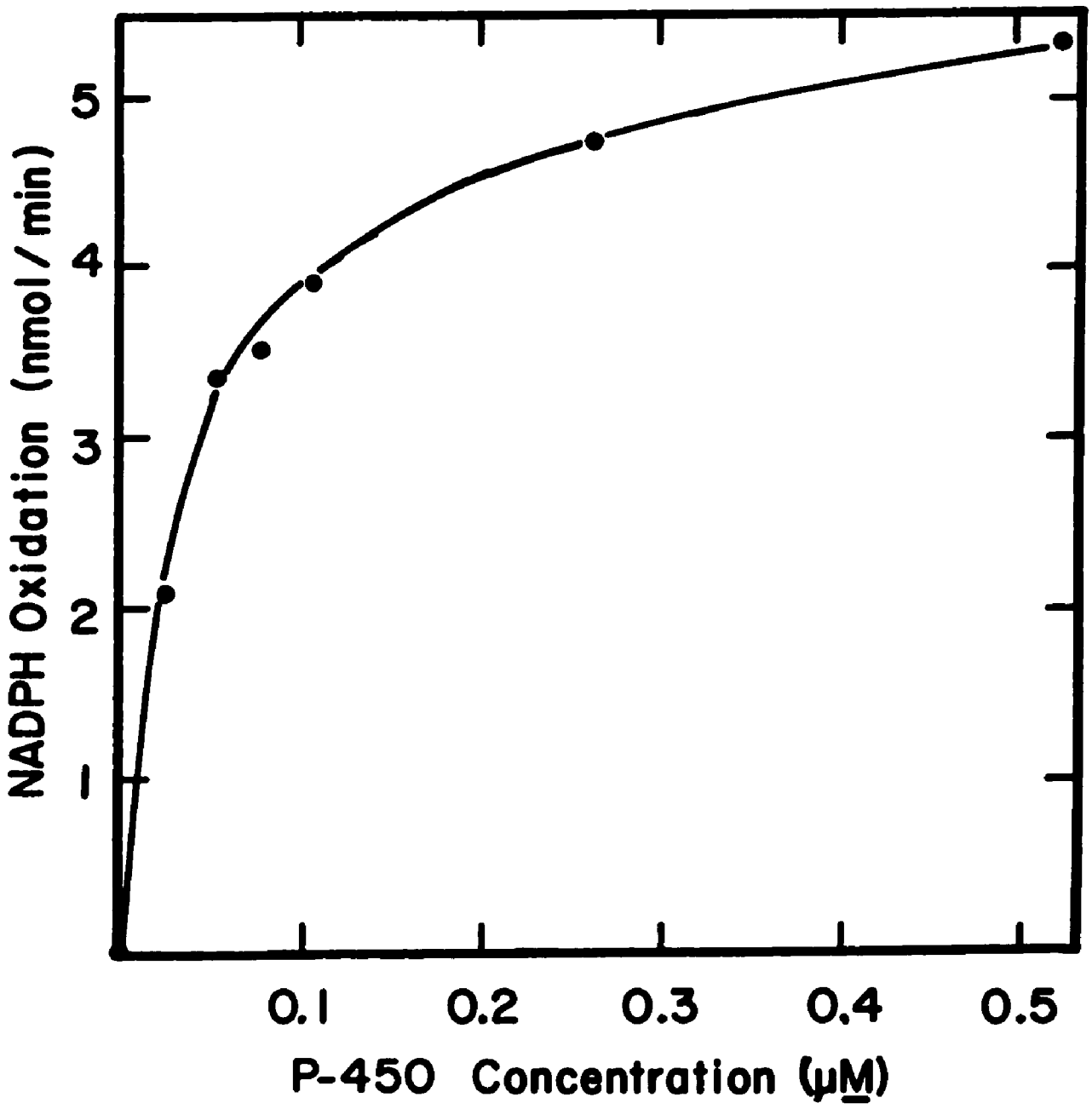


Fig. 1. Rate of NADPH oxidation as a function of P-450LM$_2$ concentration. The amount of dilauroyl-GPC (30 μg)[2] and reductase (0.028 nmol of FMN) was held constant and the concentration of P-450LM$_2$ was varied. Benzphetamine was used as the substrate.

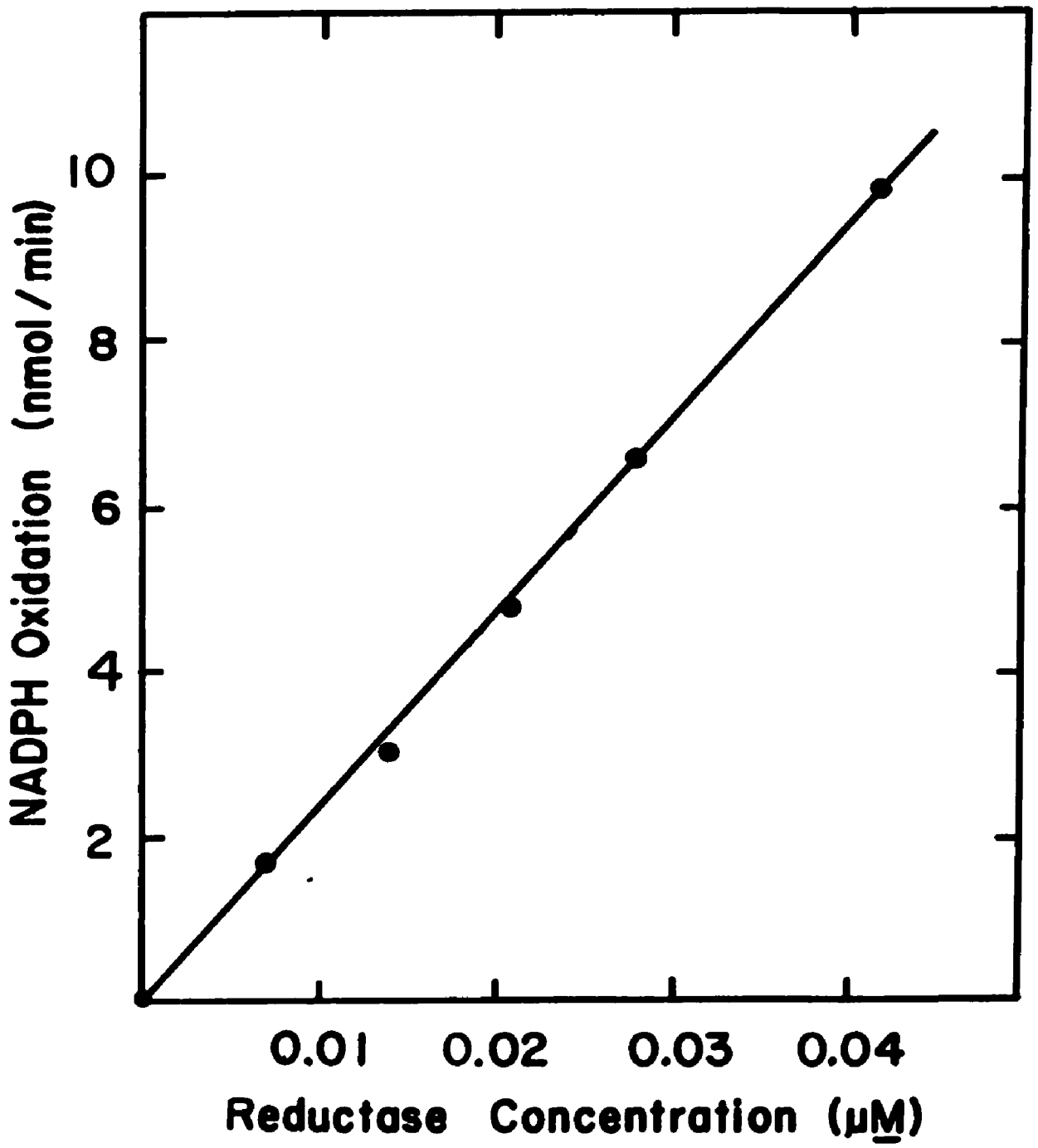


Fig. 2. Rate of NADPH oxidation as a function of reductase concentration. The amount of dilauroyl-GPC (30 μg) and P-450LM$_2$ (0.53 nmol, 16.9 nmol per mg of protein) was held constant and the concentration of reductase was varied. Benzphetamine was used as the substrate. Reductase concentration is expressed as enzyme-bound FMN.

was increased to 1.5 ml, and the concentrations of the added components were the same as described in the spectral assay except that 0.5 μmol of NADPH was added. After a 3-min preincubation the reaction was initiated with NADPH and allowed to proceed for 10 min, at which time 0.5 ml of 17.5 % perchloric acid was added. Formaldehyde was determined by the method of Nash (76) as modified by Cochin and Axelrod (77). The experimentally determined extinction coefficient of 3.25 $m\underline{M}^{-1}$ cm^{-1} for the resulting chromophore at 412 nm was used in calculating the amount of formaldehyde formed. The control reaction in this case contained no reductase.

Assay for Cytochrome c Reductase Activity: Reduction of cytochrome c was used as a convenient measure of reductase activity during purification of the enzyme and in mechanistic studies. Reaction rates were routinely measured in a high ionic strength buffer, as described by Phillips and Langdon (47). The 1.0-ml assay mixture contained the following components at the indicated concentrations: potassium phosphate buffer, pH 7.7, 0.3 M; cytochrome c, 40 μM; EDTA, 0.1 mM; and NADPH, 0.1 mM. The mixture was preincubated for 3 min at 30° and the reaction was initiated by the addition of enzyme. The rate of cytochrome c reduction was calculated from the absorbance change at 550 nm using an extinction coefficient of 21 $m\underline{M}^{-1}$ cm^{-1} (78) for the conversion of oxidized cytochrome to the reduced

form. A linear dependence of the initial reduction rate on added reductase was observed, as shown in Fig. 3. Potassium cyanide at a concentration of 1.0 mM was found to have no significant effect on the rate of cytochrome c reduction by microsomes and was not included during measurement of microsomal reductase activity.

Although the specific activity of the enzyme, expressed as μmol of cytochrome c reduced per min per mg of protein, was always determined in 0.3 M phosphate, the use of lower ionic strength buffers in kinetic and some other studies will be indicated where appropriate. Both Sigma Type III and Type VI cytochrome c were used in routine measurements at saturating levels of acceptor. Type VI was used in kinetic studies since it contains a smaller amount of high molecular weight polymers and a negligible amount of the reduced form.

Cytochrome c reduction is practically insensitive to the presence of detergents in the reaction mixture and has served as the most reliable method for location and quantitation of the reductase in fractions from column chromatography during purification of the enzyme. Concentrations of Renex 690 up to 0.1% in the final reaction mixture did not inhibit reduction, and deoxycholate at the same level produced only about 15% inhibition.

The pH profile for cytochrome c reduction in 0.1 M phosphate buffer is shown in Figure 4. Maximum activity

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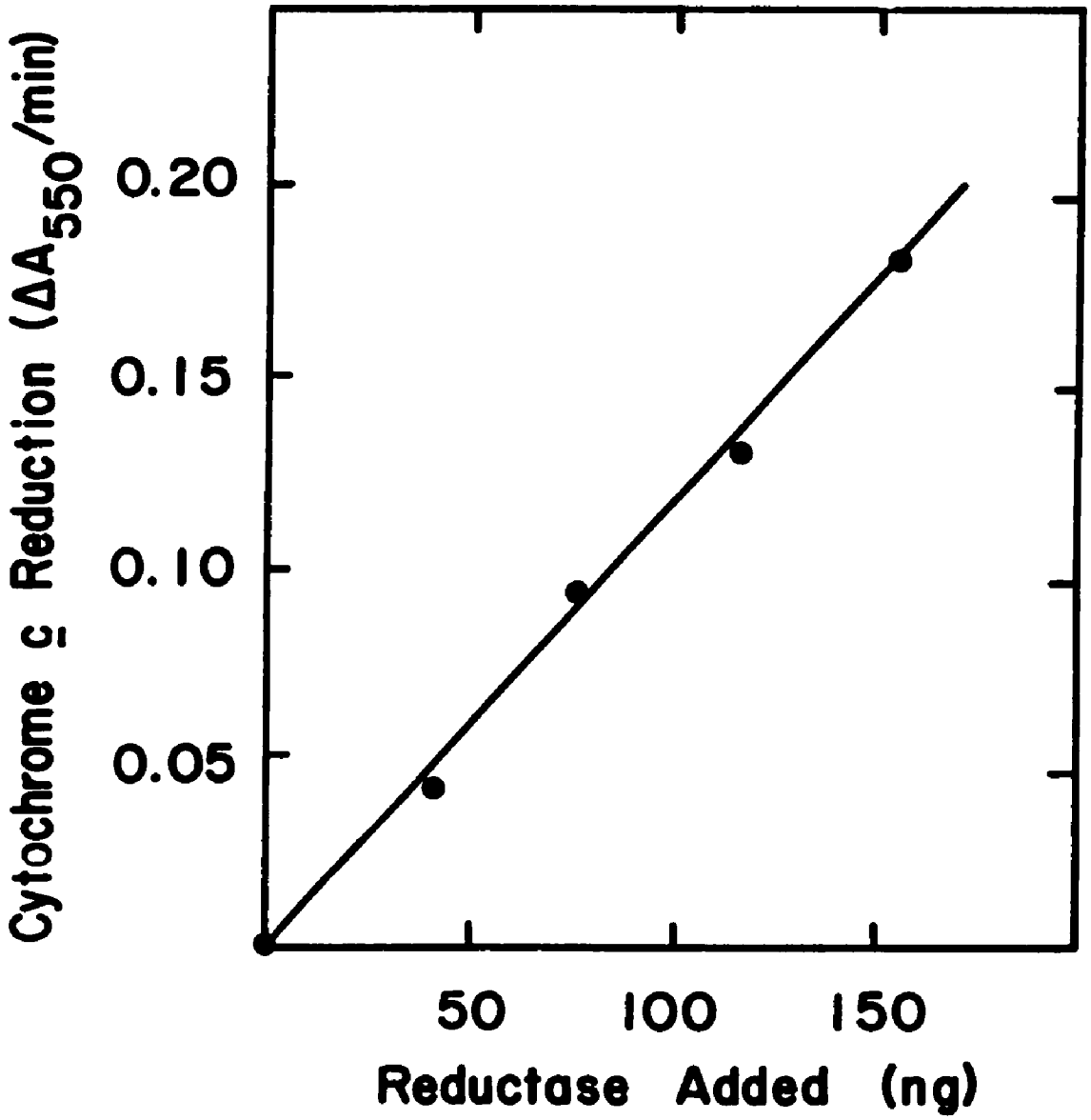


Fig. 3. Rate of cytochrome c reduction as a function of reductase added.

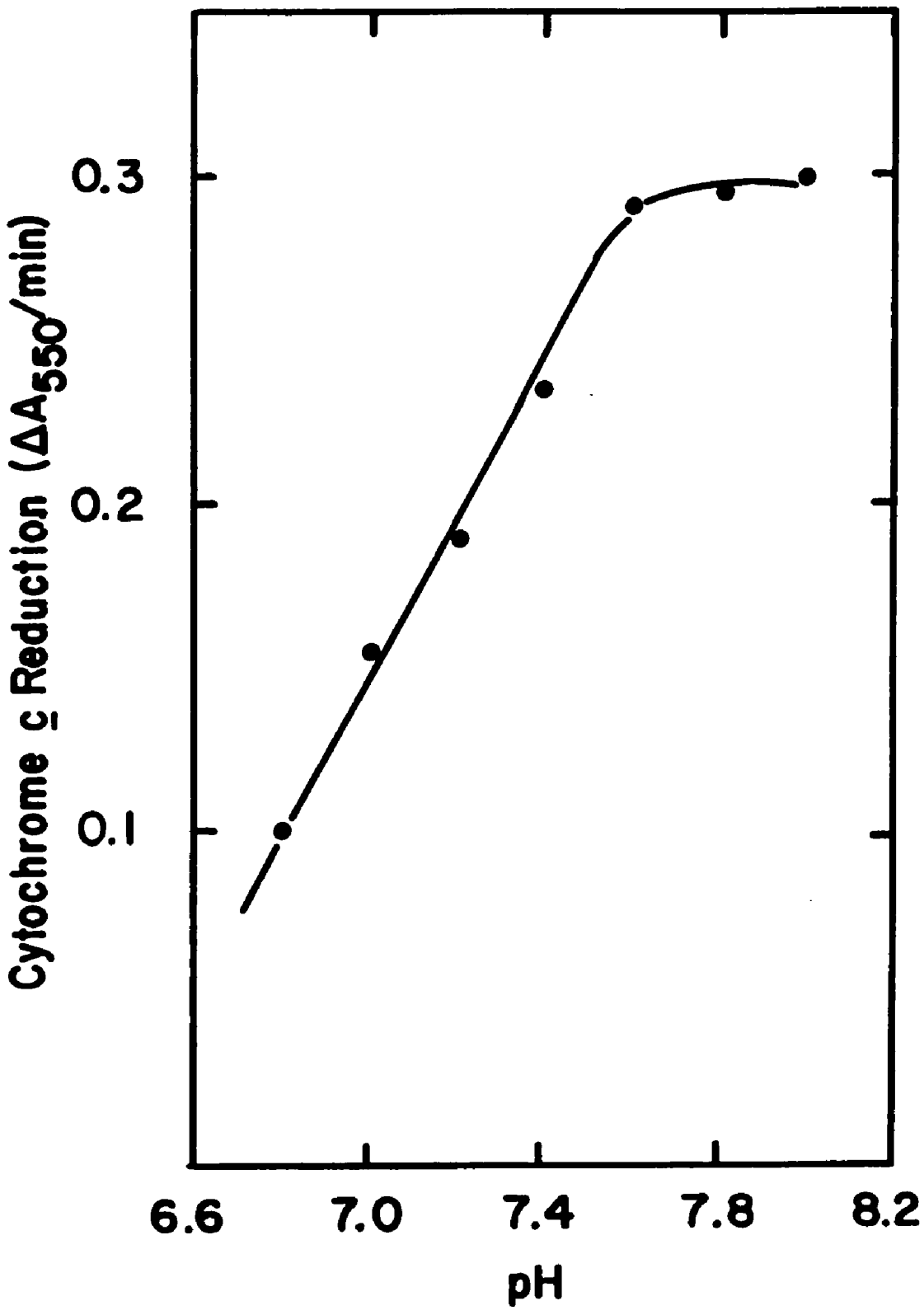


Fig. 4. Rate of cytochrome c reduction in 0.1 M phosphate buffer as a function of pH. All buffers contained 0.1 mM EDTA.

was observed above pH 7.6; the dependence of the rate of reduction on pH is similar to that observed by others for NADPH-cytochrome c reductase (46,47). The rate of reduction at pH 6.8 was not enhanced when enzyme and FMN were included during the preincubation and the reaction was initiated with NADPH.

Assays for Reduction of Other Electron Acceptors: Reduction of various other electron acceptors was measured at pH 7.7 at 30° in a manner similar to that described for assay of cytochrome c reduction at the following final concentrations: DCPIP, 50 μM; ferricyanide, 0.5 mM; menadione, 50 μM; nitro blue tetrazolium, 20 μM; and 3-AcPyADP, 0.2 mM. Menadione was added as 0.05 ml of a 1.0 mM solution in 20% ethanol. For assay of ferricyanide and 3-AcPyADP reduction, the concentration of NADPH was increased to 0.2 mM, and for menadione reduction, to 0.15 mM. A 0.3 M potassium phosphate buffer was used unless stated otherwise. Nitro blue tetrazolium assay mixtures contained 250 μg of dilauroyl-GPC and 0.1 M phosphate buffer. The rate of DCPIP reduction was determined by the decrease in absorbance at 600 nm using an extinction coefficient of 21 mM^{-1} cm^{-1} (79) and the rate of ferricyanide reduction by the decrease at 420 nm using an extinction coefficient of 1.02 mM^{-1} cm^{-1} (80), with appropriate correction in both cases for the nonenzymatic rates. Nitro blue tetrazolium and 3-AcPyADP reduction were determined by the

increase in absorbance at 550 and 363 nm, respectively, using extinction coefficients of 28.6 mM^{-1} (81) and 5.6 mM^{-1} cm^{-1} (82). The transhydrogenase-type activity was measured in a Cary 14 recording spectrophotometer with an identical reaction mixture containing no enzyme in the reference cell. Menadione reduction was monitored indirectly following NADPH oxidation at 340 nm. No NADPH oxidaoxidation in 1% ethanol in the absence of menadione was observed. The linearity of reaction rates with added reductase was verified in each case.

Purification of Flavins: FAD was purified by chromatography on DEAE-cellulose as described by Massey and Swoboda (83). FMN was purified in a similar manner by the method of Mayhew (84) after enzymatic hydrolysis of FAD. Individual fractions were examined for purity by thin layer chromatography on silica gel with n-butanol-acetic acid-water (4:3:3), as developing solvent (85) and compared to authentic samples of FMN and FAD. Flavins were visualized by their fluorescence under long wavelength ultraviolet light. Peak fractions from column chromatography were reserved for use as standards in the fluorometric determinations. Flavin samples were stored at -20° and care was taken in handling the solutions to exclude light.

Flavin Determination: Fluorescence measurements were made on a recording ratio spectrofluorimeter assembled by Dr. David P. Ballou, which was available for use through the

courtesy of Dr. Vincent Massey. Determinations were made at 20° using exciting light of wavelength 450 nm, and emitted light was measured at 535 nm. Slit widths were 2.0 and 2.5 mm for excitation and emission, respectively.

The amount of FAD and FMN present in mixtures of the two flavins was quantitated from fluorescence measurements made before and after hydrolysis with snake venom phosphodiesterase (Naja naja), and from the relative fluorescence at pH 7.7 versus pH 2.6, employing a slight modification of the method of Faeder and Siegel (86). Flavin was released from purified enzyme (200 µg in 0.2 ml) by boiling in the dark for 3 min, and the samples were immediately cooled in an ice-water bath. Under these conditions, the protein remained soluble, and determinations on these samples without further treatment to precipitate protein were not always reproducible. For more precise measurements, protein was precipitated by the addition of 4 volumes of cold methanol and removed by centrifugation for 10 min at 10,000 x g. An aliquot of the supernatant solution was diluted with an equal volume of 0.1 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA. Fluorimetric measurements on 0.1 to 0.4 ml aliquots in a total volume of 2.0 ml in the above buffer were made at pH 7.7, and again, after the addition of 0.2 ml of 0.1 N HCl, at pH 2.6. Alternatively, readings were made at pH 7.7 in the above buffer before and after addition of a few crystals of Naja naja venom.

The concentration of the flavin solutions used as standards was determined using extinction coefficients for A_{450} of 11.3 $m\underline{M}^{-1}cm^{-1}$ (87) and of 12.2 $m\underline{M}^{-1}cm^{-1}$ (88) for FAD and FMN, respectively. The relative fluorescence constants obtained by the method of Faeder and Siegel (86) for the flavin standards used in this study are presented in Table I (methanol absent) and are in good agreement with the values for FMN at pH 7.7 of 1.000; for FMN at pH 2.6 of 0.690; for FAD at pH 7.7 of 0.099; and for FAD at pH 2.6 of 0.456; calculated from the data presented by the above authors. In addition, the fluorescence constants of the FMN standard were compared to those of an FMN sample prepared from flavodoxin and were found to be within 5% of the values obtained with this authentic flavin.

To correct for changes in the fluorescence properties of flavin in the presence of a small amount of methanol, solutions of standards to be used in routine determinations were prepared in buffer containing 40% methanol at appropriate concentrations so that measurements could be made under conditions identical to those used for the reductase samples. The presence of methanol at 2 to 8% during the measurement had little effect on the fluorescence of FMN at pH 7.7 and, as shown in Table I, little effect on the fluorescence of either flavin at pH 2.6. The fluorescence of FAD at pH 7.7 was enhanced about 25%; therefore, these relative values were used in

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TABLE I

FLUORESCENCE CONSTANTS OF FLAVIN STANDARDS IN THE PRESENCE AND ABSENCE OF METHANOL

The fluorescence of each flavin was measured at pH 7.7 and pH 2.6 at 20° as described by Faeder and Siegel (86) and the fluorescence constants were calculated on the basis of the flavin concentration before the addition of HCl. The data are presented as relative values, assigning the fluorescence constant for FMN at pH 7.7 as 1.000. When methanol was present, the final concentration during the measurement ranged from 2 to 8%. The values shown represent an average from at least 3 separate experiments.

Flavin	pH	Fluorescence Constants	
		Methanol Absent	Methanol Present
FMN	7.7	1.000	1.000
FMN	2.6	0.678	0.670
FAD	7.7	0.103	0.125
FAD	2.6	0.456	0.490

calculating the flavin concentration of methanol-treated reductase samples. In other experiments not shown, glycerol and deoxycholate at the concentrations present in enzyme samples had no significant effect on flavin fluorescence.

With the modification of the method of Faeder and Siegel (86) in sample preparation described above, agreement between the values obtained by this procedure and by enzymatic hydrolysis was quite satisfactory. Table II summarizes an analysis on a single enzyme sample by both methods.

SDS-Polyacrylamide Gel Electrophoresis: Slab gel electrophoresis was performed using a slight modification of the discontinuous system of Laemmli (89) in a Hoefer Model SE-500 apparatus. The gels were prepared from a stock solution of 22.2% acrylamide and 0.6% N,N'-bis-methylene acrylamide. The separating gel contained 7.5% acrylamide, 0.375 M Tris-Cl, pH 8.4, and 0.1% SDS, and was polymerized by the addition of 0.033% ammonium persulfate and 0.025% N,N,N',N'-tetramethylethylenediamine (TEMED). Slabs 9 cm in length and 0.75 mm in thickness were prepared one day in advance. The 1 cm stacking gel, prepared the day of the experiment, contained 3% acrylamide, 0.125 M Tris-Cl, pH 6.8, and 0.1% SDS, and was polymerized by the addition of 0.1% ammonium persulfate and 0.05% TEMED. The electrode buffer, pH 8.3, contained 0.05

TABLE II

ANALYSIS OF ENZYME FLAVIN BY TWO METHODS

Method	nmol of FMN/mg	nmol of FAD/mg	nmol of total flavin/mg	FMN/FAD
Enzymatic hydrolysis with venom phosphodiesterase	10.9	12.5	23.4	0.89
Faeder and Siegel (86)	11.1	11.6	22.7	0.95

M Tris, 0.38 M glycine, and 0.1% SDS. Protein samples were diluted in 0.0625 M Tris-Cl, pH 6.8, containing 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue, and were then placed in a 100° water bath for 2 min. Twenty-microliter samples were placed in each well, and electrophoresis carried out using a Heathkit Model IP-17 regulated high voltage power supply with a current of 12 mA per gel during the stacking process, and of 20 mA per gel during the separation. The temperature was maintained at 10° by a circulating water bath and the run was terminated after approximately 3 hrs.

The gel was fixed for 1 hr in a solution of water-isopropanol-acetic acid (65:25:10), and stained for 1 hr with 0.05% Coomassie Blue R-250 in the same solvent. Diffusion destaining was carried out in a Hoefer Model SE-530 apparatus with a solution containing 10% acetic acid and 10% isopropanol. The gels were softened for 15 to 30 min by treatment with 3% glycerol prior to drying, which was performed with a Hoefer Model SE-540 Slab Gel Dryer.

Absorption Spectra: All ultraviolet and visible spectra were obtained in spite of an Aminco DW-2 recording spectrophotometer equipped with a Lauda K-2/R circulating water bath.

Purification Procedure: Solubilization of phenobarbital-induced rat liver microsomes was performed essentially as described by Dignam and Strobel (71) except that protamine sulfate addition was omitted. Tris buffers were Tris-chloride and phosphate buffers were potassium phosphate. Microsomes (4.2 g of protein) were suspended in a final volume of 420 ml of a solution containing 0.1 M Tris, pH 7.7, 1.0 mM EDTA, 0.5 mM dithiothreitol, 20% (v/v) glycerol and 1% (w/v) Renex 690. All components except detergent were stirred for 10 min, and Renex was then added dropwise as a 10% solution. Stirring was continued for an additional 20 min, and the microsomal extract was then centrifuged for one hour at 105,000 x g. The supernatant solution was diluted with an equal volume of water and applied to a column (4.5 x 40 cm) of DEAE-cellulose previously equilibrated with 0.05 M Tris, pH 7.7, containing 0.1 mM EDTA, 10% glycerol, and 0.5% Renex. All buffers used in subsequent steps contained EDTA and glycerol at the above concentrations. The column was washed with one liter of the equilibrating buffer containing 0.1 M KCl, and the reductase was eluted with 2.5 liters of KCl solution in a linear gradient from 0.1 to 0.5 M in the same buffer. Under these conditions most of the microsomal hemeproteins were eluted with the 0.1 M wash and the more tightly bound reductase was not eluted until about midway through the gradient. Preliminary results indicate that

including a protamine sulfate precipitation step after detergent solubilization sharpens the reductase elution profile on DEAE-cellulose, as previously described by Dignam and Strobel (71) for a similar chromatographic step on DEAE-Sephadex A-25. A calcium phosphate suspension (2.0 g in 67 ml) was added to the combined reductase fractions and the mixture was stirred for 10 min. After centrifugation at 8,000 x g for 10 min, the detergent-containing supernatant solution was discarded. The gel, containing adsorbed protein, was suspended in 45 ml of 0.15 M phosphate buffer, pH 7.4, stirred for 20 min, and again centrifuged. Elution was repeated with 25 ml of the same buffer, the mixture was centrifuged, and the supernatant solutions were combined. Recovery of reductase from calcium phosphate under these conditions was 90% or better.

Prior to subsequent chromatography, Tris buffer and deoxycholate were added so that the final solution contained 1 mg of protein per ml, 0.1 M phosphate, 0.1 M Tris, and 0.1% deoxycholate. The pH was adjusted to 7.2 by the addition of dilute HCl. The sample was then applied to a column of DEAE-cellulose (2.2 x 30 cm) previously equilibrated with 0.1 M Tris, pH 7.2, containing 0.1% deoxycholate. The column was washed with 100 ml of equilibrating buffer, also containing 0.1 M phosphate. At this point the reductase could be seen as a bright yellow band migrating ahead of dark colored impurities which were more

tightly bound at the top of the column. The 1 to 2 cm of DEAE-cellulose containing these impurities were removed, and the column was then washed with 250 ml of equilibrating buffer containing 0.15 M KCl. The reductase was eluted with a 500 ml linear gradient of KCl in the same buffer with salt concentration increasing from 0.15 to 0.5 M. Active fractions were pooled, concentrated on calcium phosphate (360 mg), and eluted as described above, in a combined volume of 25 ml.

The sample was diluted 1.5-fold with 10% glycerol and the pH was adjusted to 7.2. Renex and deoxycholate were added to a final concentration of 0.1% of each, and the sample was applied to a small column of DEAE-cellulose (1.5 x 7 cm), previously equilibrated with 0.1 M phosphate buffer, pH 7.2, containing 0.1% deoxycholate. The column was washed with the same buffer until the reductase had migrated at least half way down the column. All column material above the yellow band was removed and the reductase was eluted with the same buffer containing 0.2 M KCl. The enzyme was immediately dialyzed against one liter of 0.02 M Tris, pH 7.4, for at least four hrs, and concentrated by treatment with calcium phosphate (120 mg) as before, to give a final volume of 6 ml.

Stabilization of Reductase: The enzyme was quite stable at 4° in 10% glycerol at pH 7.0 to 8.0 in the presence of either Renex 690 or deoxycholate. A loss

of activity occurred in the absence of glycerol which could not be restored by the addition of flavin, but no loss of activity was observed in the absence of detergent. No requirement for added thiol was observed in this study; however, the enzyme is sensitive to mercurial agents such as p-chloromercuribenzoate, and dithiothreitol is effective in reversing this inhibition. The use of ammonium sulfate in the purification of the reductase has been avoided since treatment of the enzyme with concentrations as low as 30% may cause dissociation of FMN, even if care is taken to exclude light.

Purified enzyme, at a protein concentration of about 1 mg per ml, was stable for several months without significant loss of activity when stored at -20° in phosphate buffer, pH 7.4 to 7.7, containing 0.1 mM EDTA and 10% glycerol. At this concentration the enzyme remained soluble at 4 to 6°, but slowly precipitated out of solution at higher temperatures. The solubility of the enzyme was improved if the concentration of buffer was greater than 0.1 M and was enhanced somewhat by the addition of 0.1% detergent. No solubility problem was observed with more dilute solutions of enzyme (50 to 100 µg per ml). The stability of the dilute enzyme is slightly better in phosphate buffer than in Tris, and preparations diluted in 0.05 M potassium phosphate, pH 7.7, containing EDTA and glycerol, were stable for several hrs at 4°C.

Preparation of FMN-depleted Enzyme: Two procedures were used for the selective removal of FMN from the reductase. Removal by KBr dialysis (90) was carried out as follows: Enzyme at a concentration of 0.6 to 1.2 μM was dialyzed for 36 hours at 4° against at least 3 changes of 0.05 M sodium pyrophosphate buffer, pH 8.5, containing 2 M KBr, 0.1 mM EDTA and 10% glycerol. Potassium bromide was then removed by dialysis against several changes of 0.05 M potassium phosphate buffer, pH 7.6, also containing EDTA and glycerol. FMN-depleted enzyme prepared by this method was stable for several days when stored at -20°.

Selective removal of FMN by irradiation in the presence of ammonium sulfate was carried out using a procedure described in earlier work on NADPH-cytochrome c reductase by Baggot and Langdon (91). Enzyme (0.6 to 1.2 μM) in buffer containing 40% ammonium sulfate, 0.01 mM EDTA, and 10% glycerol, was centered at a distance of 6 inches from a circular array of twelve 15-watt fluorescent lights and irradiated for periods of 6 to 9 hours at a temperature of about 10°. Inactivation by this process was observed in the pH range from 6.8 to 8.5 in 0.05 M phosphate or pyrophosphate buffers and the exact conditions used will be noted where necessary. Assays for cytochrome c reductase or other diaphorase activities were carried out on aliquots of the irradiated solution. Flavin was determined after dialysis against several changes of 0.05 M potassium phosphate buffer,

pH 7.6, containing 0.1 mM EDTA and 10% glycerol, and appropriate correction for volume changes was made. Measurements of the pH of solutions containing high concentrations of salt were made with a Radiometer GK 2302C electrode.

Neither of the above procedures was as effective when higher concentrations of enzyme were used. In the case of the ammonium sulfate procedure, more concentrated samples quickly became very turbid and substantially less activity could be restored by addition of FMN, as described below. Flavin determinations on depleted samples were generally performed using the method of Faeder and Siegel (86) but did not employ methanol treatment of the samples, as described earlier. These values were periodically checked by the venom procedure and no serious discrepancies between the two methods were observed.

Reactivation of FMN-depleted enzyme toward various electron acceptors by the addition of flavin was carried out as follows: The appropriate assay mixture containing acceptor, enzyme, and 4 to 5 μM flavin was preincubated at 30° for 4 min, and the reaction was initiated by the addition of NADPH. Control rates were determined in the presence of flavin without added enzyme. Longer preincubation periods were no more effective, and treatment of the enzyme with flavin at 4 or 25° for periods up to 20 min, followed by assay in the absence of added flavin, gave less reproducible results.

RESULTS

Purification of the Reductase: During the course of this work two different procedures were developed for the purification of the reductase from phenobarbital-induced rat liver microsomes. The first purification procedure, described in detail elsewhere (70), involved deoxycholate solubilization of microsomes essentially as described by Lu and Coon (14), followed by column chromatography on DEAE-cellulose, DEAE-Sephadex A-50, and hydroxylapatite, in the presence of deoxycholate or the nonionic detergent Renex 690. The total recovery of cytochrome c reductase activity was only 10% and the procedure was lengthy. This procedure was abandoned in favor of a second, described in detail in Methods, which is less time consuming and consistently yields enzyme of slightly higher purity. Although both procedures allow isolation of active enzyme purified over 100-fold from the starting microsomes, analysis by SDS-polyacrylamide gel electrophoresis indicated that the final preparations obtained by the two methods differ slightly in apparent molecular weight. It is not a major tenet of this thesis to rigorously establish the existence of multiple forms of rat liver P-450 reductase, but in view of present knowledge regarding the existence of

multiple forms of P-450 (23,40,41,43), the data to be presented on the reductase warrant serious consideration.

There is good evidence that the reductase is the limiting component of the induced microsomal drug hydroxylating enzyme system. In studies with antibodies prepared to the lipase-solubilized enzyme., Masters et al. (9) demonstrated that the inhibition of microsomal cytochrome c reductase activity by the addition of the immune globulin was directly proportional to the inhibition of drug demethylase activity. In similar studies, the sulfhydryl reagent, mersalyl, was also used as a selective inhibitor of reductase activity, and again, the extent of the inhibition of microsomal cytochrome c reductase and drug demethylase activities was similar (92,93). These results suggest that the rate of drug demethylation by induced microsomes may also be used as an estimate of the amount of reductase present. If the flavoprotein responsible for the cytochrome c reductase activity in microsomes is also the only enzyme participating in electron transfer from NADPH to P-450, then it should be possible to demonstrate copurification of cytochrome c and P-450 reductase activities during isolation of the enzyme.

A typical purification of the reductase by the procedure described in Methods is summarized in Table III. The specific activity of the enzyme with P-450 and with cytochrome c as the electron acceptor was determined at each

TABLE III

PURIFICATION OF NADPH-CYTOCHROME P-450 REDUCTASE FROM PHENOBARBITAL-INDUCED RAT LIVER MICROSOMES

Reductase activity was determined by the spectral assays described in Methods. The ratio of specific activities is expressed as cytochrome c/cytochrome P-450. Yields are based on cytochrome c reductase activity. The P-450 used in these experiments contained 16.9 nmol per mg of protein.

Preparation	Protein	Specific Activity with Cyt. c	with P-450	Ratio of Activities	Yield
	mg	units/mg			%
Microsomes	4216	0.424	0.014[a]	30[a]	100
DEAE-cellulose column eluate; calcium phosphate eluate	123	8.58	0.326	26	60
DEAE-cellulose column eluate; calcium phosphate eluate	17	38.6	1.77	22	37
DEAE-cellulose column eluate; calcium phosphate eluate	6	61.4	2.36	26[b]	21

[a] P-450 reductase activity was estimated by benzphetamine demethylation and the specific activity was expressed as μmol of formaldehyde formed per min per mg of protein.

[b] The ratio shown here is higher than that reported previously (70), because deoxycholate, which enhances P-450 reductase activity was included in this assay in the previous experiments, but not in the present work.

stage of the purification. As described earlier, P-450-dependent NADPH oxidation in the presence of saturating amounts of purified P-450LM$_2$ and phospholipid, with benzphetamine as the substrate, was used to determine the P-450 reductase activity of the partially purified and the final preparations of enzyme. However, measurements of P-450-dependent NADPH oxidation in microsomes is difficult, since even in the absence of benzphetamine, the NADPH-oxidase activity of microsomes is high. Therefore, the P-450 reductase activity of the microsomes was estimated by measuring formaldehyde production due to the N-demethylation of benzphetamine. Comparison of the rate of product formation in microsomes to the rate of NADPH oxidation in the reconstituted system is assumed to be valid, since the P-450-dependent reactions of the microsomal system generally exhibit a one-to-one stoichiometry of NADPH oxidized to product formed (94,95).

As shown in Table III, the ratio of the specific activity with cytochrome c to that with P-450 as the electron acceptor did not vary significantly during an extensive purification. Based on P-450 reductase activity, a 170-fold purification was achieved, and based on cytochrome c reductase activity, a 150-fold purification was achieved with an overall recovery of 21%. Copurification of these activities provides additional evidence that the microsomal NADPH-dependent P-450 and cytochrome c reductase

activities are common to a single enzyme. The enzyme obtained by this purification procedure had a specific activity with cytochrome c as the electron acceptor of 59 to 64 units per mg of protein, and with P-450 of 2.2 to 2.4 units per mg of protein.

Absorption Spectrum: The spectrum of the isolated enzyme is shown in Fig. 5, and is that of the fully oxidized enzyme. The spectrum is characterized by absorbance maxima at 276, 384, and 456 nm, with a shoulder at 485 nm. The ratio of absorbance at 276 nm to that at 456 nm is 8.7, and is slightly higher than the value of 6.7 obtained for the bromelain-solubilized NADPH-cytochrome c reductase (55) and of 6.3 for the trypsin-solubilized enzyme (54), both from rat liver.

Estimation of Minimal Molecular Weight and Evidence for Two Forms of the Reductase: When purified enzyme was treated with SDS and mercaptoethanol and submitted to polyacrylamide gel electrophoresis, it did not show a single protein band, but consisted of a major band and a more diffuse minor band of slightly lesser mobility, as shown in Fig. 6. The exact proportions of each of the two components was difficult to assess because resolution of the upper band was sometimes poor, but the lower band was clearly the major constituent in each preparation analyzed, and accounted for approximately 75 to 95% of the total protein present in these two bands. The gels shown

Fig. 5. Absorption spectrum of the reductase. The enzyme was in 0.05 M phosphate buffer, pH 7.7, containing 10% glycerol and 0.1 mM EDTA, and the temperature was 15°. For the ultraviolet spectrum, the enzyme was diluted 10-fold in the same buffer.

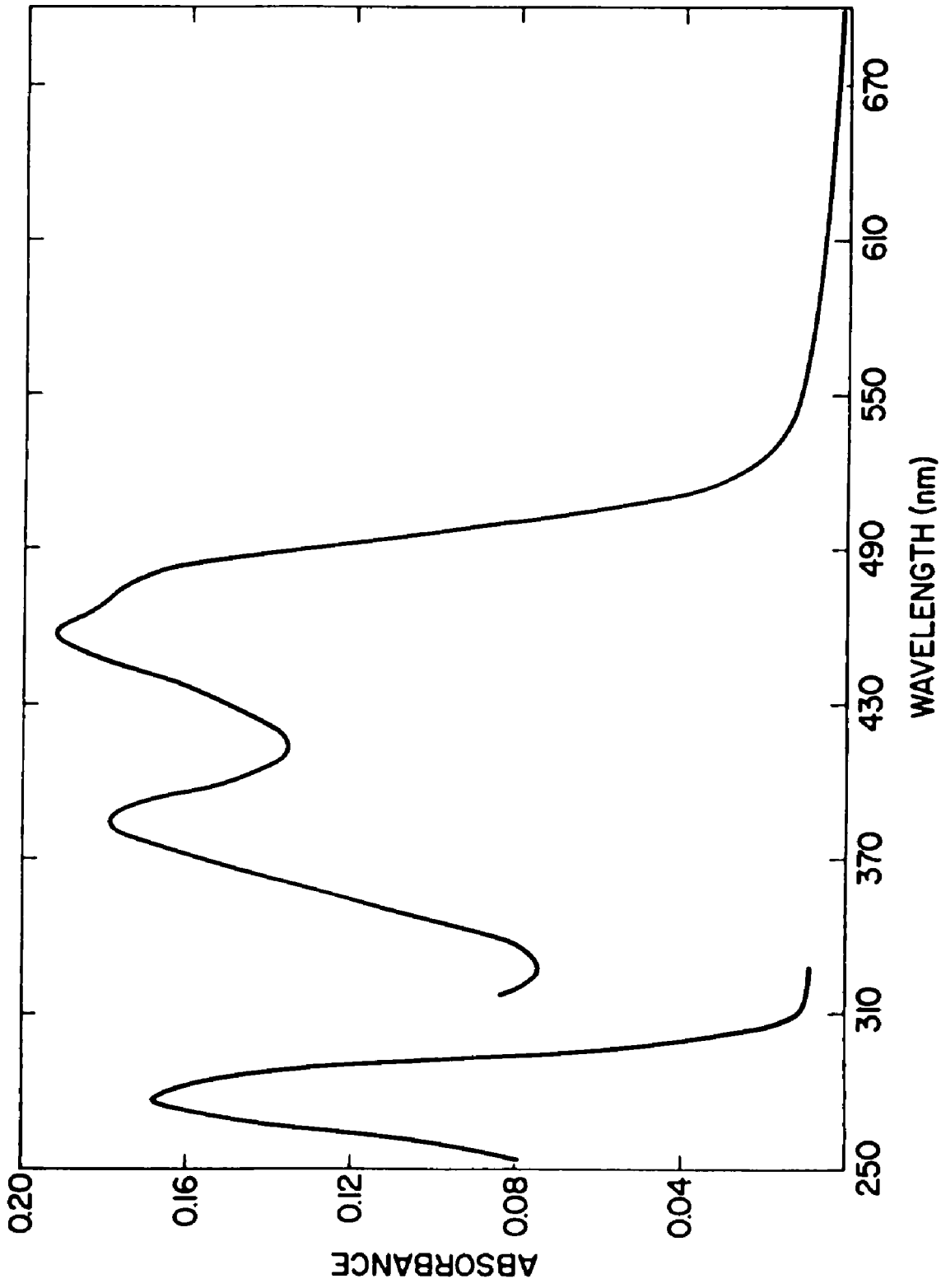

ABSORBANCE
0.20
0.16
0.12
0.08
0.04
250
310
370
430
490
550
610
670
WAVELENGTH (nm)

Fig. 5

Fig. 6. SDS-polyacrylamide gel electrophoresis of purified reductase. Four preparations of enzyme, designated as (a) through (d), were examined by electrophoresis of 0.5, 0.7, and 1.0 µg of protein, as described in Methods.

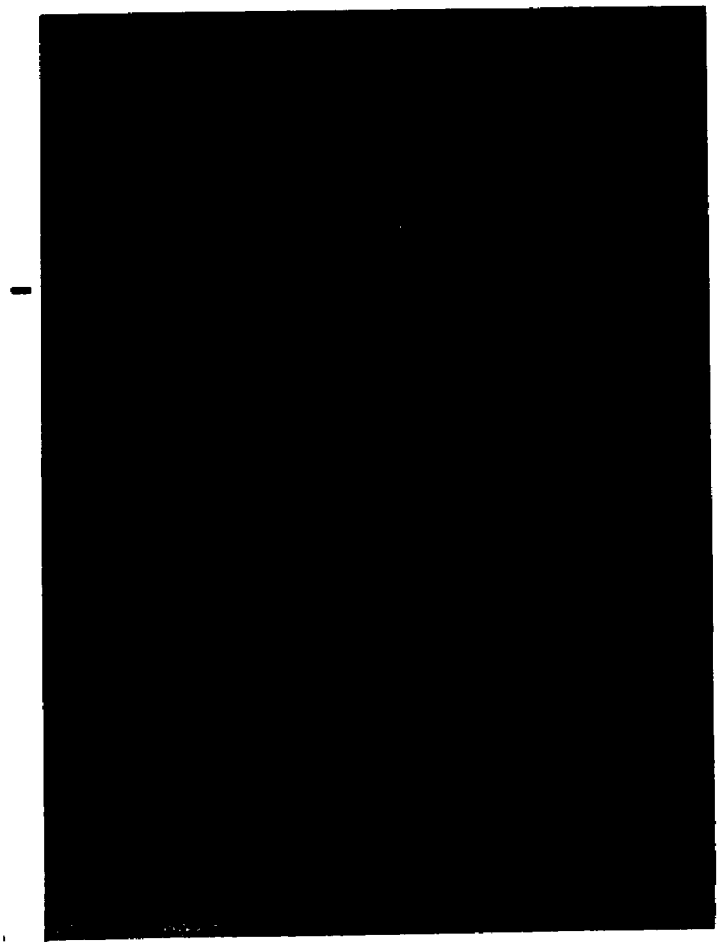


Fig. 6

in Fig. 6 also indicated that protein components other than these two were not present in significant amounts. An estimate of a minimal molecular weight of 76,000 was obtained by comparison of the relative mobility of the major band with the relative mobilities of standards of known molecular weight, as shown in Fig. 7. A similar analysis indicated an apparent molecular weight of 78,000 for the minor constituent.

Since the reductase obtained in the previous work was shown by similar methods to have an apparent molecular weight of 79,000 (70), approximately that of the upper (minor) band, enzyme prepared by the two different procedures was analyzed in the same electrophoretic experiment with the results shown in Fig. 8. Both enzyme preparations shown were purified over 100-fold with respect to the cytochrome P-450 and cytochrome c reductase activities of the microsomes, and both had similar spectral properties and flavin content. The mobility of the major band observed for the preparation obtained in the earlier studies corresponded to that of the minor band of the purified enzyme described here. When equal amounts of the two samples were mixed, two bands of roughly equal intensity were observed.

To ascertain if the difference in apparent molecular weight between these preparations was an artifact of the purification procedure, the enzyme obtained at successive stages in a single purification, as outlined in Table III,

Fig. 7. Estimation of minimal molecular weight of the reductase by SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed using the discontinuous system described in Methods. The molecular weight standards used were phosphorylase a (94,000), bovine serum albumin (69,000), catalase (58,000), glutamate dehydrogenase (53,000), ovalbumin (45,000), aldolase (40,000), lactate dehydrogenase (36,000), and chymotrypsinogen (25,700).

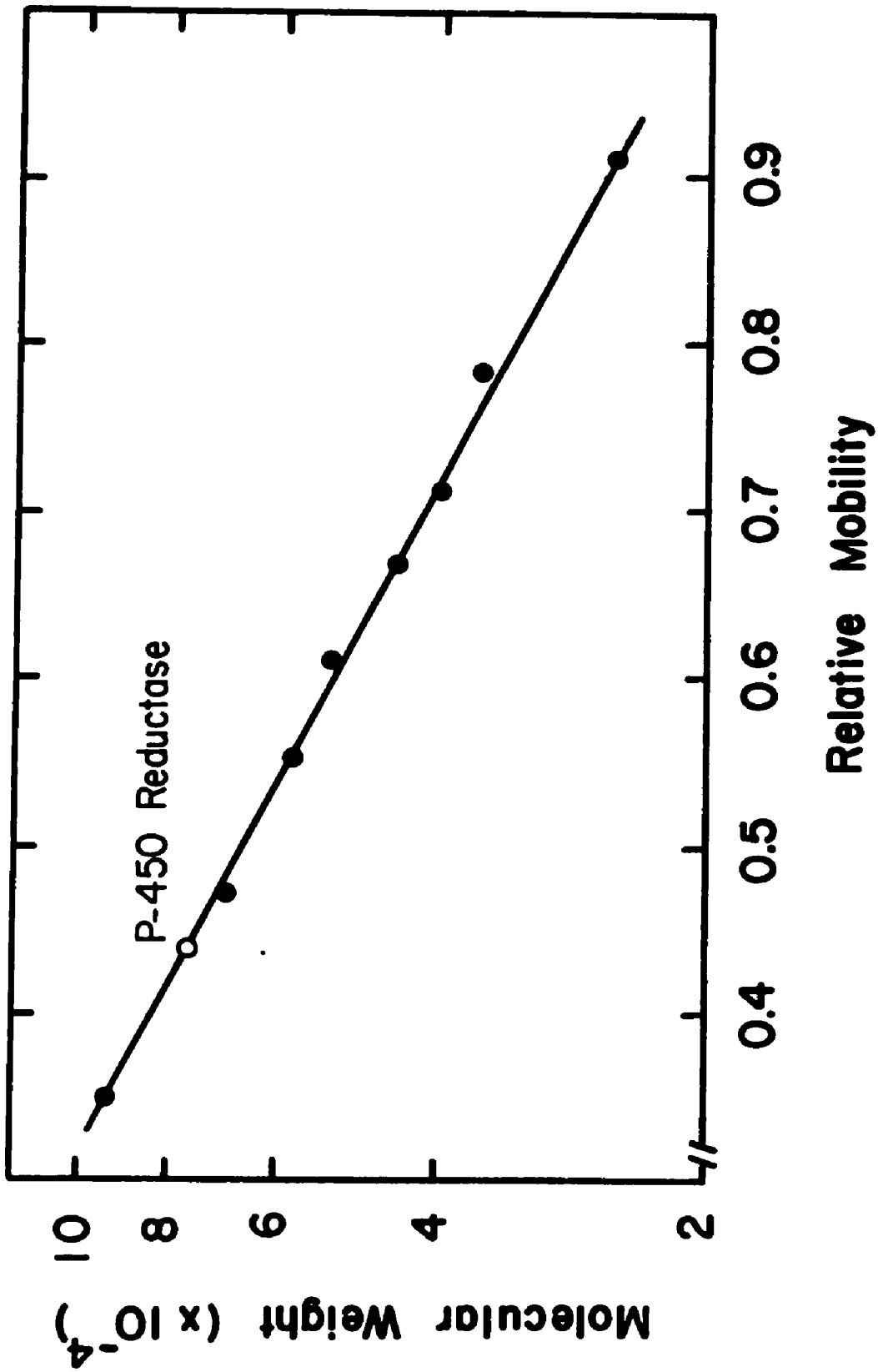

Molecular Weight (x 10⁻⁴)
10
8
6
4
2
P-450 Reductase
0.4
0.5
0.6
0.7
0.8
0.9
Relative Mobility

Fig. 7

Fig. 8. SDS-polyacrylamide gel electrophoresis of reductase prepared by different purification procedures. Sample (a), 0.7 µg of protein, was obtained by the procedure described in Methods, and sample (c), 1.0 µg of protein, was obtained by the procedure described elsewhere (70). Sample (b) was a mixture of (a), 0.35 µg of protein, and (c), 0.5 µg of protein. The upper electrode buffer contained 5 mM mercaptoethanol.

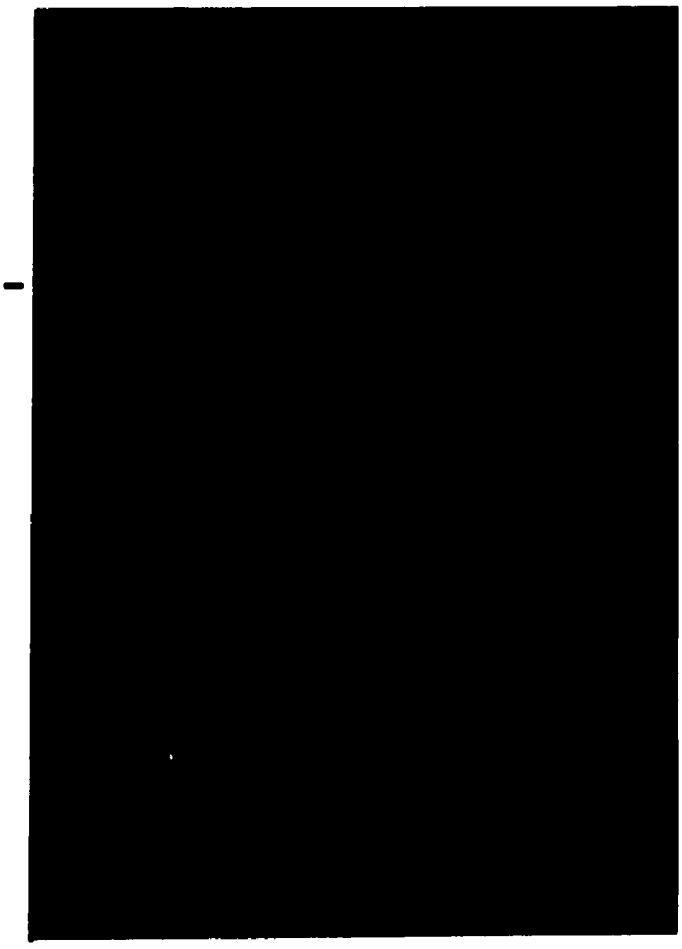


Fig. 8

was submitted to SDS-polyacrylamide gel electrophoresis. The results of this experiment are presented in Fig. 9. Two bands of approximately equal intensity with mobilities corresponding to those of the upper (minor) band and lower (major) band of the purified enzyme were observed in microsomes. Two bands of approximately equal intensity were also observed in the enzyme preparation purified 20-fold by the first chromatographic step on DEAE-cellulose. Only after the second chromatographic step did the relative proportions change in favor of the lower (major) band. These results suggest that two bands corresponding in mobility to the major bands of the two purified preparations are present in microsomes and copurify in the first stage of enzyme purification. The evidence presented thus far supports the conclusion that both the purified preparations represent microsomal forms of the reductase and that the use of two different purification procedures leads to the isolation of predominantly one or the other form.

To examine the possibility that the lower molecular weight form was a product of proteolytic degradation of the larger polypeptide chain and was produced during isolation of the microsomal fraction, the subcellular fractionation was carried out in the presence of the protease inhibitor, phenyl-methane sulfonyl fluoride (93). The electrophoretic pattern of microsomes prepared in the presence and absence of this inhibitor are compared in Fig. 10 (c and d). No

Fig. 9. SDS-polyacrylamide gel electrophoresis of enzyme obtained at various stages in a single purification, as follows: (a), phenobarbital-induced rat liver microsomes, 6 and 8 µg of protein; (b), enzyme obtained after chromatography on DEAE-cellulose in the presence of 0.5% Renex 690 and concentration with calcium phosphate, 4 and 6 µg of protein; (c), enzyme obtained after further chromatography on DEAE-cellulose in the presence of 0.1% deoxycholate and concentration with calcium phosphate, 1.0 and 1.2 µg of protein; and (d), purified enzyme obtained after the final chromatographic and concentration steps, 0.5 and 0.7 µg of protein.

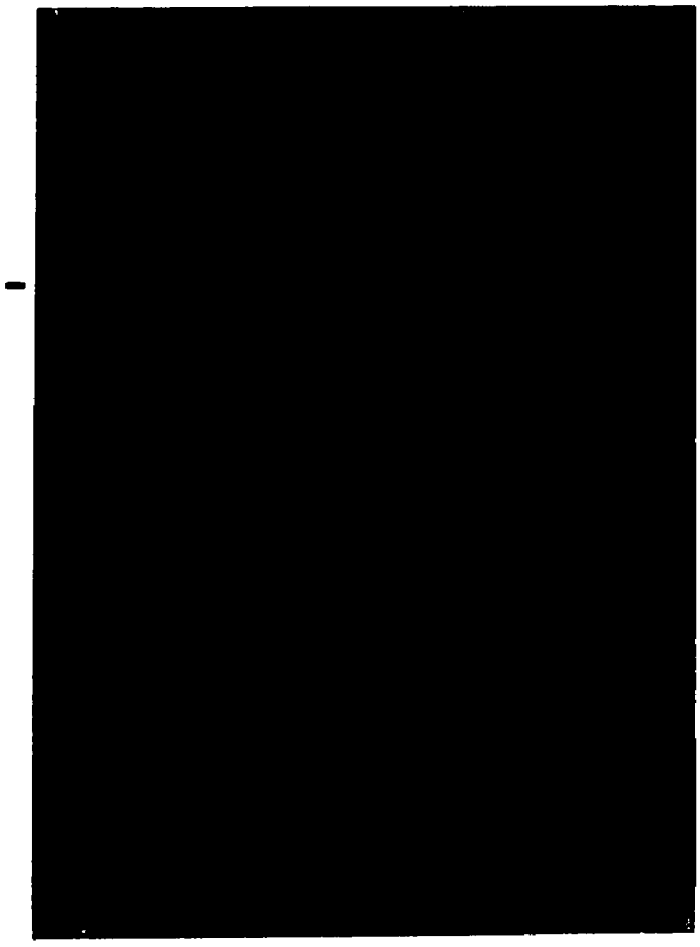


Fig. 9

Fig. 10. SDS-polyacrylamide gel electrophoresis of microsomes prepared as follows: (a), from untreated rats, 6 and 8 µg of protein; (b), from rats pretreated with phenobarbital and hydrocortisone (70), 6 and 8 µg of protein; (c), from rats pretreated with phenobarbital only in their drinking water, as described in Methods, 6 and 8 µg of protein; and (d), from the same animals described in (c) but prepared in the presence of the protease inhibitor, phenyl-methane sulfonyl fluoride (PMSF), 6 and 8 µg of protein. In the latter case, the homogenizing buffer was supplemented with 0.4 mM PMSF, and all other buffers used in the preparation of microsomes contained 0.1 mM PMSF. The 77,000 molecular weight region is indicated by the arrow.

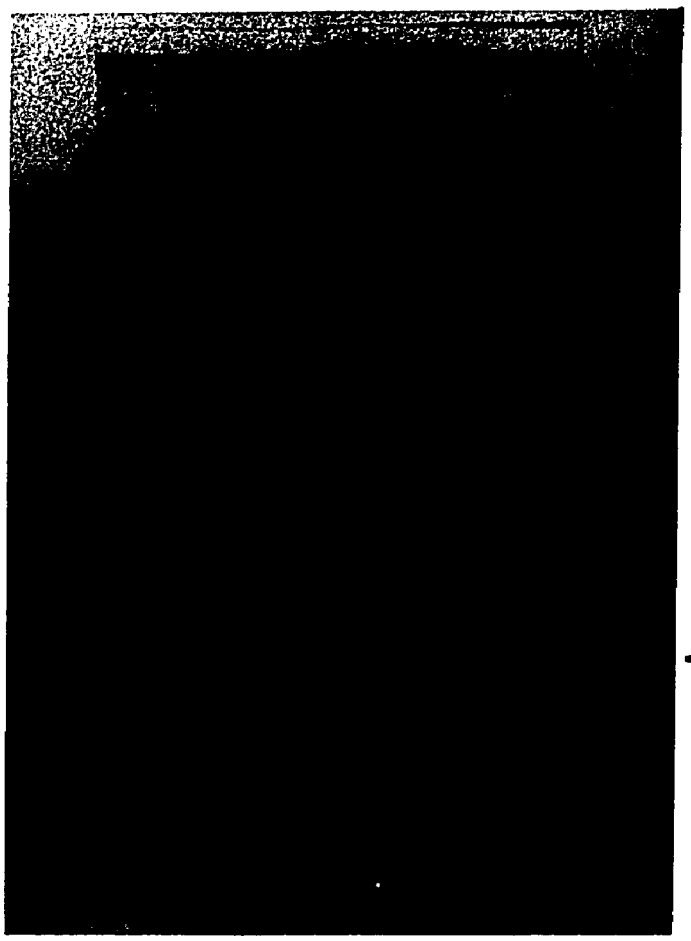


Fig. 10

significant difference between the relative proportions of the two bands in these preparations could be detected. It is difficult to entirely rule out the possibility that proteolysis of the larger polypeptide chain is responsible for the appearance of the 76,000 molecular weight form; however, this experiment provides little support for this hypothesis.

Although phenobarbital-induced microsomes were used as the starting material in both purification procedures, the method of administration differed in the two cases. In the earlier work (70), phenobarbital and hydrocortisone were administered via intraperitoneal injection, but in the present studies animals received phenobarbital only in their drinking water. To determine if the relative proportion of the two forms could be correlated with the physiological process of induction, microsomes were prepared from the livers of animals receiving no treatment, or phenobarbital pretreatment by one or the other of the two methods described above. These preparations were submitted to SDS-polyacrylamide gel electrophoresis and the results of the analysis are shown in Fig. 10 (a, b, and c). Two bands of approximately equal intensity were observed in the 77,000 molecular weight region in the microsomes of both untreated and induced animals, and regardless of the method of phenobarbital administration. Thus, no evidence for an obvious preferential induction of either band was obtained. These

studies should be extended in the future to include analysis of microsomes from animals pretreated with β-napthoflavone or 3-methylcholanthrene, compounds known to induce forms of P-450 distinct from that induced by phenobarbital(28,94).

The combined results of these experiments are consistent with the conclusion that two forms of this enzyme are present in rat liver microsomes. Additional evidence will be required to thoroughly establish how these forms differ. Unless otherwise noted, the characterization studies to follow were performed with enzyme preparations containing predominantly the 76,000 molecular weight form.

Native Molecular Weight: The molecular weight of the reductase under nondenaturing conditions was examined by gel filtration on Agarose A-1.5m, with the results shown in Fig. 11. The enzyme (0.25 mg per ml) was applied to a column (2.5 x 50 cm) along with standards of known molecular weight. The sample and the eluting buffer contained 0.02 M Tris-Cl, pH 7.5, 0.1 M KCl, 10% glycerol, and 0.1 mM EDTA, but no detergent was added. The behavior of the reductase under these conditions appeared to be that of a heterogeneous mixture of large molecular weight aggregates, as evidenced by a broad and asymmetric elution of enzyme activity. The peak fractions were eluted just after β-galactosidase and a semi-logarithmic plot of molecular weight versus elution volume give an approximate molecular weight of 520,000 for the predominant species.

Fig. 11. Estimation of molecular weight of the reductase by gel filtration on Agarose A-1.5m. The conditions of the experiment are described in the text. The enzymes used as standards were β-galactosidase, —o—o—o— , (540,000); glutamate dehydrogenase, —x—x—x— , (313,000); and catalase, —●—●—●— , (250,000). The elution profile was determined by assay of enzymatic activity. The reductase used in this experiment was purified by the procedure described earlier (70).

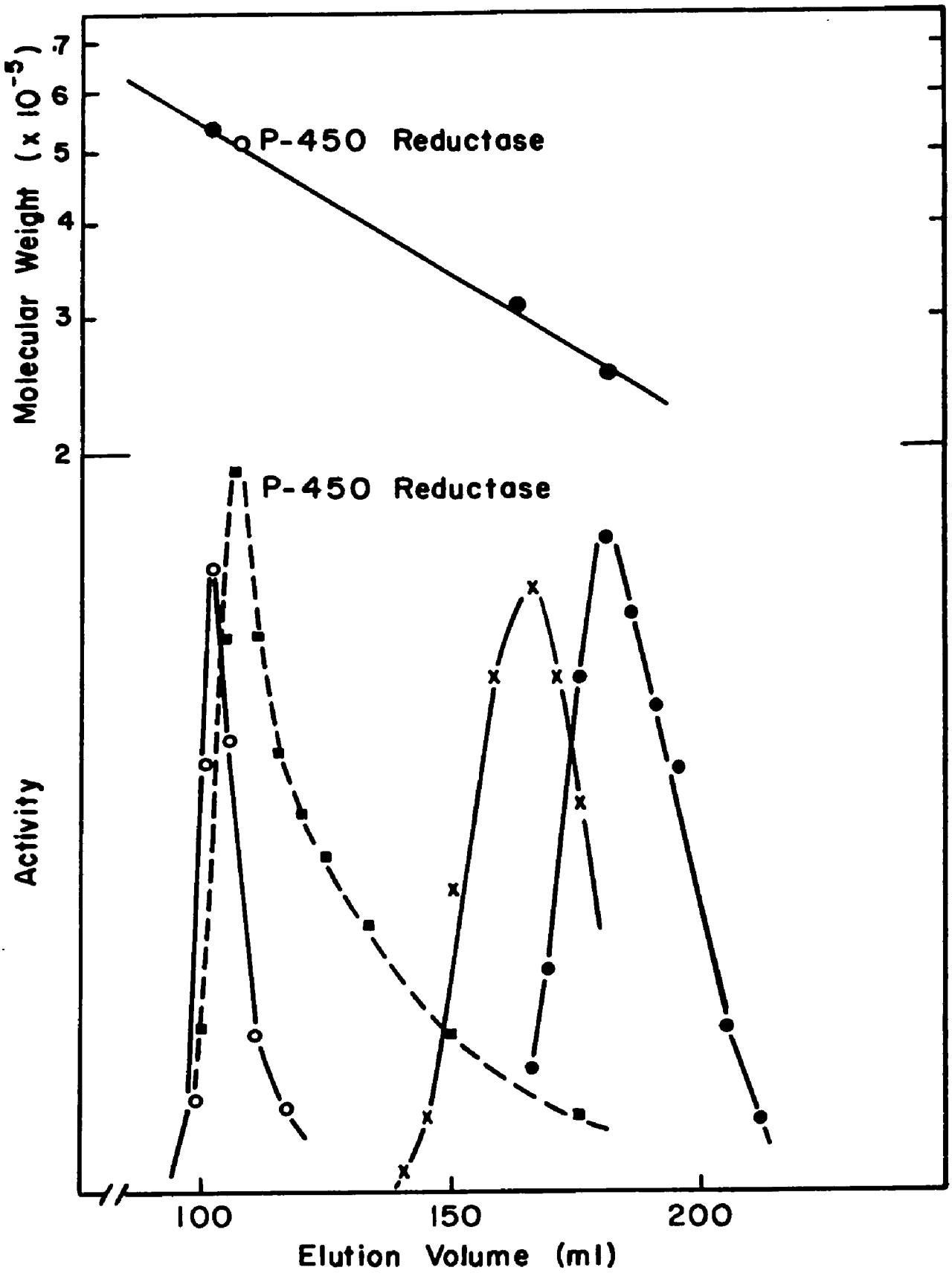

Molecular Weight (x 10^{-5})
7
6
5
4
3
2
P-450 Reductase
P-450 Reductase
Activity
100
150
200
Elution Volume (ml)

Fig. 11

Aggregation of purified detergent-solubilized membrane proteins is commonly observed (57,60,98,99), but since these preparations undoubtedly contain traces of remaining detergent it is difficult to assess the meaning of gel filtration results in terms of possible subunit structure without detailed knowledge of the interaction of the protein and detergent. Furthermore, it is possible that the reductase itself possesses sufficient amphipathic character to undergo polymerization, as has been shown to occur for the detergent-solubilized forms of both microsomal cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase (59,60). These enzymes contain a terminal sequence of primarily nonpolar amino acid residues which promote the formation of large polymers in aqueous solution.

SDS-polyacrylamide gel electrophoresis of the enzyme treated with mercaptoethanol indicated a minimal molecular weight of about 76,000. When electrophoresis was carried out under identical conditions but without mercaptoethanol treatment, a very broad band of protein was observed with approximately the same relative mobility as that of the treated enzyme. This result suggests that the native enzyme is not composed of subunits linked via interchain disulfide bonds. However, the poor resolution observed under non-reducing conditions may reflect the presence of intrachain disulfide bonds.

In the absence of amino acid analysis to confirm the subunit molecular weight as determined by SDS-polyacrylamide gel electrophoresis, it would be desirable to obtain a molecular weight estimate of disaggregated or monomeric enzyme by an alternate method, preferably one not involving the use of detergent. Spatz and Strittmatter (60) found that determination of the molecular weight by SDS-polyacrylamide gel electrophoresis gave an underestimation of the monomeric molecular weight of detergent-solubilized cytochrome $\underline{b}_5$ and NADH-cytochrome $\underline{b}_5$ reductase when compared to the value obtained from amino acid analysis. It was suggested that hydrophobic membrane proteins are capable of binding more SDS than are hydrophilic proteins, thereby increasing the charge to mass ratio of the protein-SDS complex. This would lead to an increased mobility during an electrophoretic experiment and result in an underestimation of the molecular weight by comparison to the behavior of more hydrophilic protein standards.

An earlier observation by Baggot and Langdon (91) in experiments with NADPH-cytochrome $\underline{c}$ reductase should receive attention in further studies on the detergent-solubilized enzyme. The results obtained from amino acid analysis and tryptic mapping suggested that the enzyme contained two nearly identical subunits of molecular weight approximately 40,000. Since there has been no evidence for the dissociation of the nearly 80,000 molecular weight polypeptide

into two subunits, it is possible that the reductase is composed of two similar units linked in continuous fashion via the covalent backbone.

Flavin Content of the Reductase: To identify the enzyme flavin, the chromatographic properties of flavin dissociated from the reductase were compared to those of FMN, FAD, and riboflavin on thin layers of cellulose or silica gel, and the resulting chromatograms are reproduced in Figs.12 and 13. Enzyme flavin was analyzed before and after treatment with Naja naja venom. The untreated sample showed two spots corresponding closely in mobility to those of FMN and FAD in both systems. Enzyme flavin treated with venom gave a single spot corresponding in mobility to that of FMN, a result consistent with the hydrolysis of FAD to FMN. Co-chromatography of the unhydrolyzed sample with a mixture of FMN and FAD gave only two spots and co-chromatography of the hydrolyzed sample with FMN gave only one spot.

The presence of both FMN and FAD in reductase samples was also established by quantitative fluorimetric determinations. Enzyme-bound flavin has fluorescence excitation maxima in the visible region at 375 and 450 nm and has an emission maximum at 535 nm. Emission was greatly enhanced by boiling the sample; bound flavin was only about 7% as fluorescent as free FAD. When the fluorescence of the dissociated flavin was measured at pH 7.7 and at pH 2.6, as described in Methods, a 1.05-fold higher value was obtained

Fig. 12. Identification of enzyme flavin by thin layer chromatography on cellulose. The reductase, concentrated to approximately 12 mg per ml using an Amicon A-75 Minicon concentrator, was boiled in the dark for 3 min to dissociate the flavin, and immediately cooled in an ice-water bath. A portion of this sample was incubated with a few crystals of Naja naja venom for 30 min at room temperature. Protein was precipitated from both the treated and the untreated sample by the addition of 4 volumes of cold methanol and removed by centrifugation. The flavin standards were prepared in sample buffer and diluted with methanol in a similar manner. Aliquots of each solution containing 0.5 to 1.0 nmol of flavin were submitted to thin layer chromatography using 5% Na_2HPO_4 as the developing solvent (85). Flavins were visualized by their fluorescence under long wavelength ultraviolet light. The average R_f values determined in this experiment are as follows: for FMN, 0.638; for FAD, 0.574; for riboflavin, 0.332; and for enzyme flavin, 0.575 and 0.632.

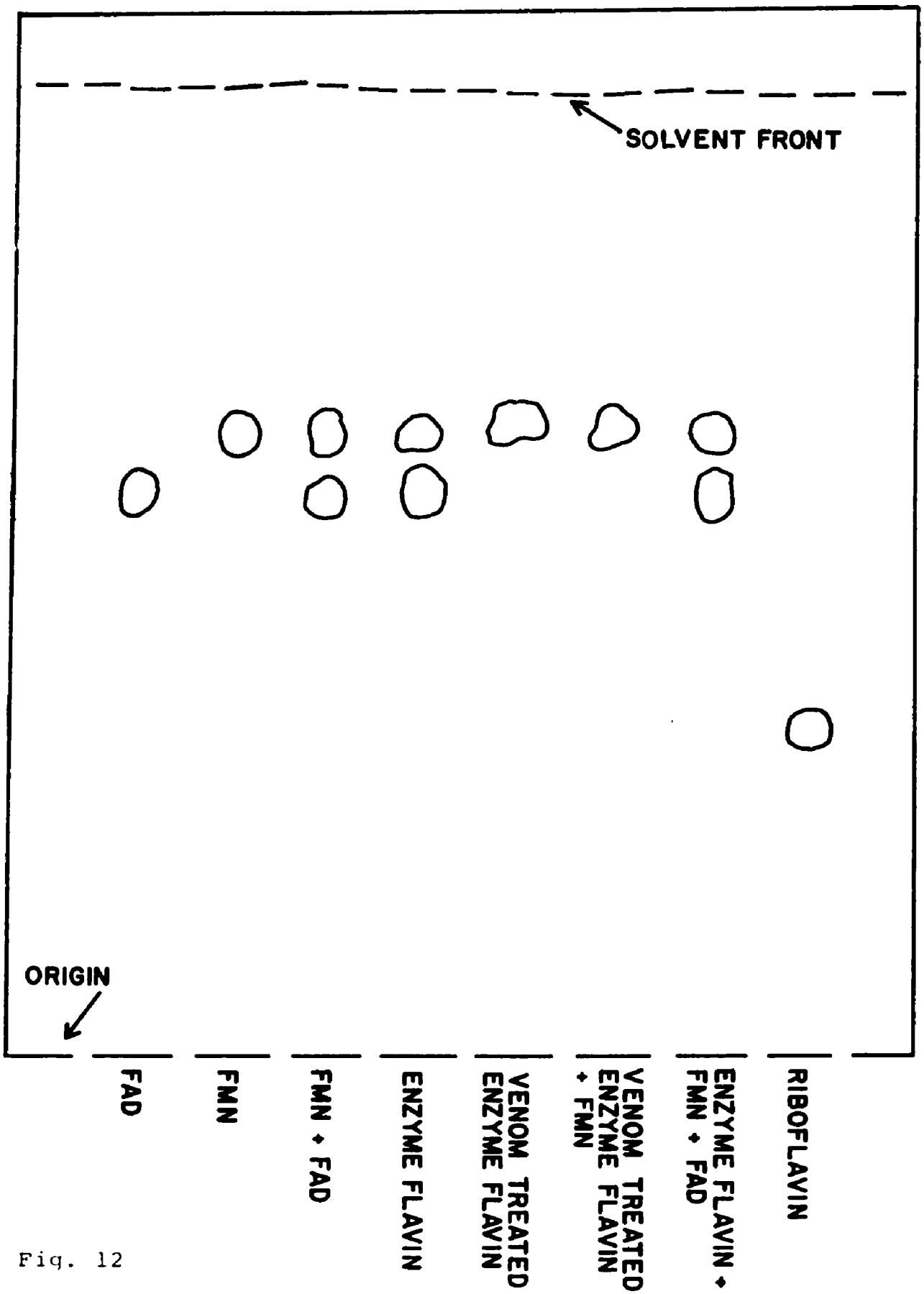

SOLVENT FRONT
ORIGIN
FAD
FMN
FMN + FAD
ENZYME FLAVIN
VENOM TREATED ENZYME FLAVIN
VENOM TREATED ENZYME FLAVIN + FMN
ENZYME FLAVIN + FMN + FAD
RIBOFLAVIN

Fig. 12

Fig. 13. Identification of enzyme flavin by thin layer chromatography on silica gel. Sample preparation was the same as described in Fig. 12, and the developing solvent was n-butanol-acetic acid-water (4:3:3) (85). The average R_f values determined in this experiment are as follows: for FMN, 0.567; for FAD, 0.484; for riboflavin, 0.674; and for enzyme flavin, 0.481 and 0.564.

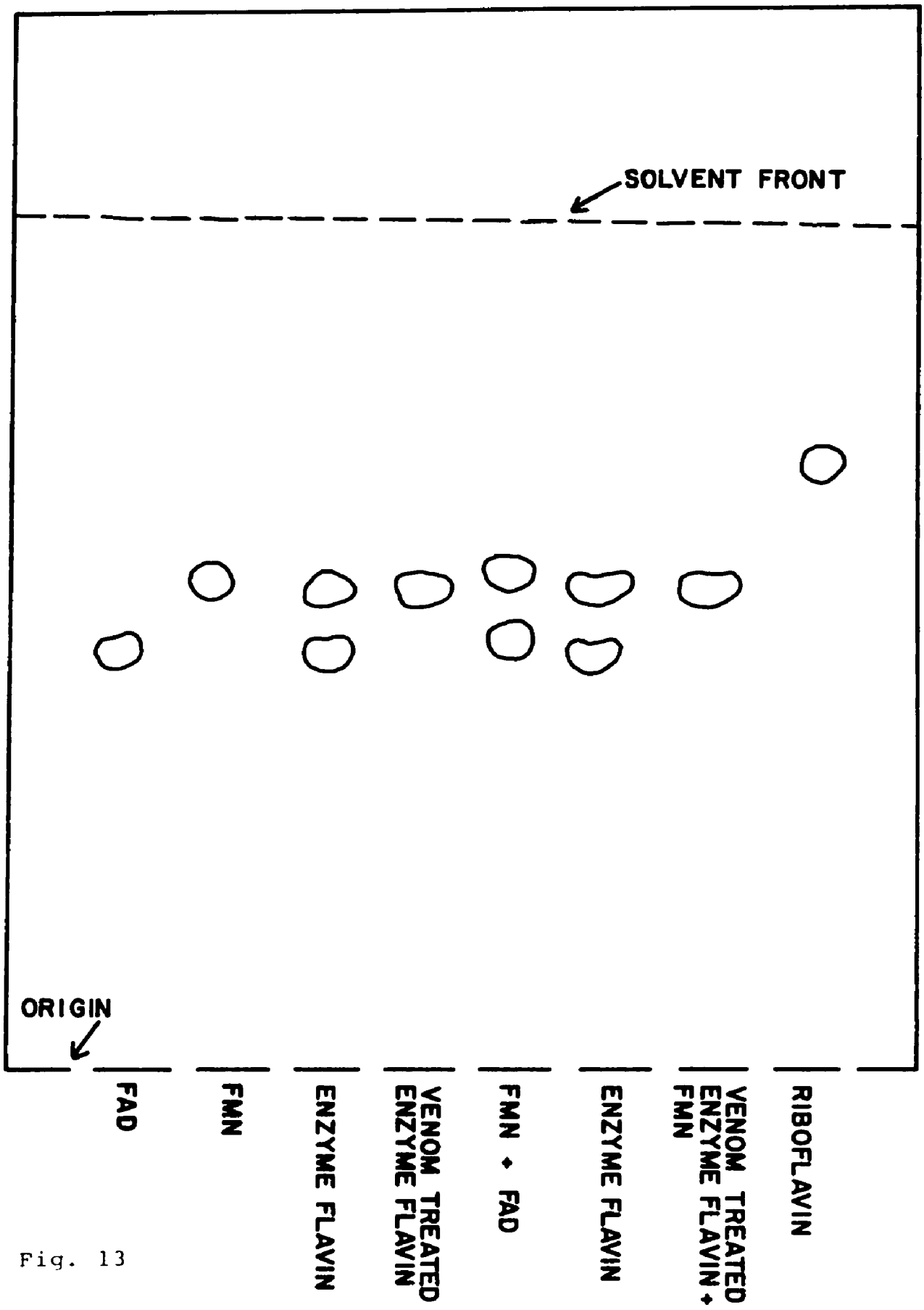

SOLVENT FRONT
ORIGIN
FAD
FMN
ENZYME FLAVIN
VENOM TREATED ENZYME FLAVIN
FMN + FAD
ENZYME FLAVIN
VENOM TREATED ENZYME FLAVIN + FMN
RIBOFLAVIN

Fig. 13

at the lower pH. Addition of a few crystals of Naja naja venom to a sample at pH 7.7 resulted in a 1.87-fold increase in fluorescence. These results confirm the presence of both FMN and FAD in samples of enzyme flavin. The amount of FMN and FAD per mg of protein was calculated for four different preparations of purified enzyme, and the data are presented in Table IV. All preparations analyzed contained greater than 47% of the total flavin as FMN. Calculations of the minimal molecular weight based on total flavin range from 41,500 to 49,200, and strongly suggest the presence of one molecule each of FMN and FAD per polypeptide chain of molecular weight 76,000. These data were also used to calculate an average extinction coefficient of 10.5 $m\underline{M}^{-1}cm^{-1}$ per flavin for the absorbance of the reductase at 456 nm.

The total flavin content of the reductase was also measured spectrophotometrically after dissociation of flavin from the enzyme by denaturation in guanidinium chloride. In this experiment, the spectrum of the enzyme was recorded before and after a 30 min incubation at 8° with 3.5 M guanidinium chloride, and the results are shown in Fig. 14. Dissociation of flavin is indicated by the shift in absorbence maxima to 450 and 376 nm and by the loss of the shoulder at 485 nm. Using an average extinction coefficient of 11.75 $m\underline{M}^{-1}cm^{-1}$ for an equimolar mixture of FMN and FAD, a flavin content of 22.2 nmol per mg of protein was calculated. An extinction coefficient of 10.6 $m\underline{M}^{-1}cm^{-1}$ for

TABLE IV

FLAVIN CONTENT OF THE REDUCTASE

Four preparations of the enzyme were analyzed for flavin by the method of Faeder and Siegel (86) and by enzymatic hydrolysis to FMN. The values represent the average result obtained in a determination employing both methods.

Preparation	nmol of FMN/mg	nmol of FAD/mg	nmol of total flavin/mg	FMN/FAD
1	11.0	12.1	23.1	0.92
2	9.7	10.6	20.3	0.92
3	10.3	10.6	20.9	0.97
4	11.5	12.6	24.1	0.91

Fig. 14. Spectrophotometric determination of the flavin content of the reductase. Spectrum A is that of the reductase in 0.05 M phosphate buffer, pH 7.6, containing 10% glycerol and 0.1 mM EDTA, at 8°, prior to dissociation of flavin. Spectrum B was obtained after a 30 min incubation of the enzyme at 8° with 3.5 M guanidinium chloride, and has been corrected for the volume change and for a slight absorbance in the near ultraviolet due to the addition of guanidinium chloride.

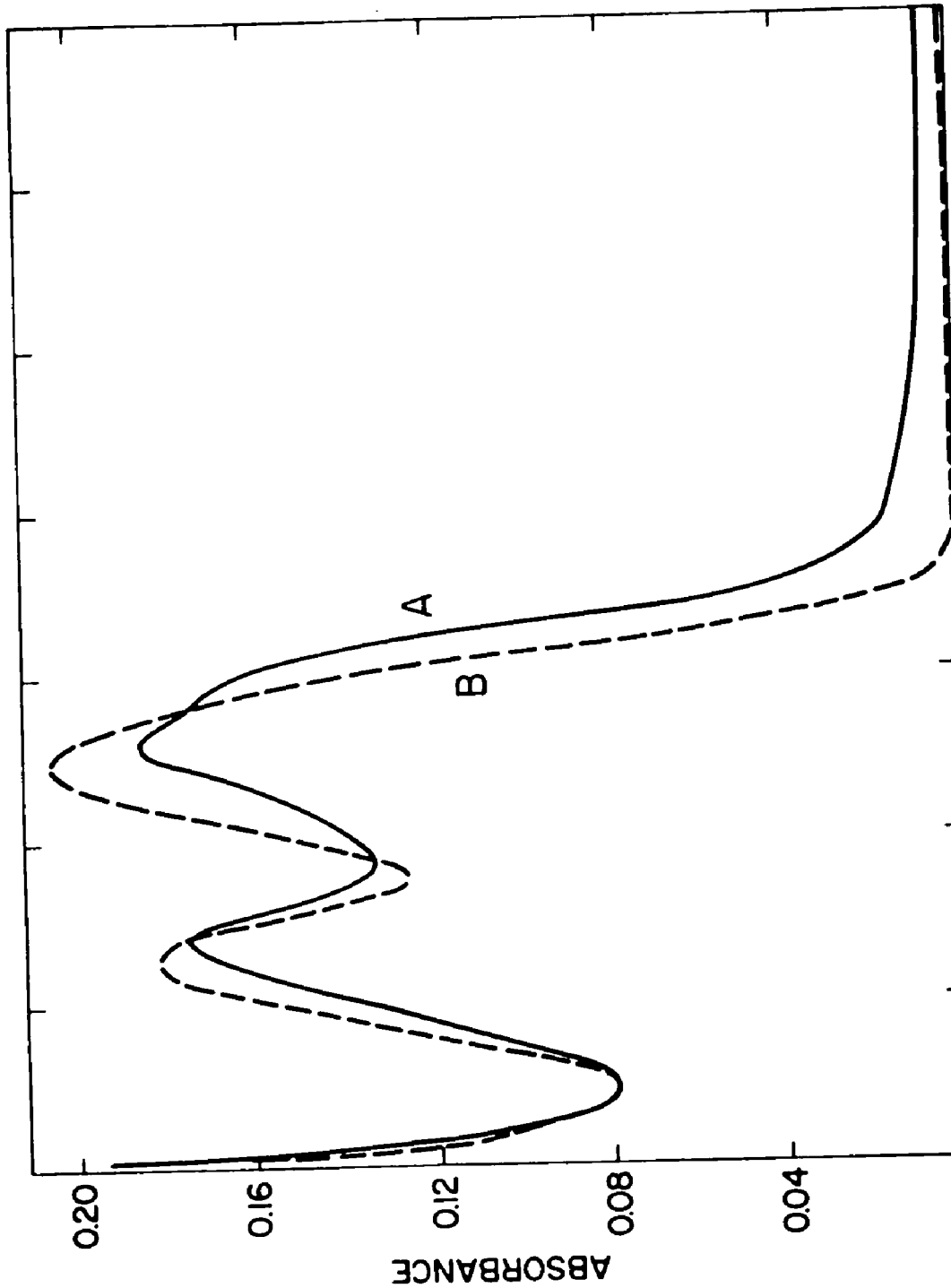

ABSORBANCE
0.20
0.16
0.12
0.08
0.04
300
360
420
480
540
600
660
WAVELENGTH (nm)
A
B

Fig. 14

enzyme-bound flavin at 456 nm was also calculated from this experiment and both values are in good agreement with those obtained by fluorimetric procedures. To verify the loss of flavin, the guanidinium chloride-treated enzyme was extensively dialyzed against buffer and the absorption spectrum was recorded (not shown). The absorbance maxima in the ultraviolet region was 280 nm and the spectrum was featureless in the visible region except for a small shoulder at about 408 nm. The significance of the latter observation is not known; an absorbance peak in this region may simply be due to a trace of tightly bound heme remaining in the sample.

In other experiments not shown, the activity of the enzyme toward P-450 or cytochrome c was enhanced by approximately 10% when FMN (4.2 μM) was included in the assay mixture. FAD (4.9 μM) was less effective and a mixture of the two flavins generally no more effective than FMN alone. These results also suggest that the enzyme preparations are only slightly FMN-depleted. If apoenzyme is present, it apparently cannot be reactivated simply by incubation with a mixture of FMN and FAD.

Requirements for P-450 Reductase Activity: The effects of various additions to or deletions from the usual assay on P-450 reductase activity are summarized in Table V. The apparent turnover of the enzyme in the complete assay system containing P-450LM$_2$, phospholipid, and benzphetamine,

TABLE V

REQUIREMENTS FOR P-450 REDUCTASE ACTIVITY

The activity of the enzyme was determined in the usual spectral assay and expressed relative to that observed in the complete assay mixture containing P-450 (0.44 μM, 11 nmol per mg), dilauroyl-GPC (30 μg) and benzphetamine. Detergents were added at a final concentration of 100 μg per ml, and rabbit liver cytochrome b_5 was present at a concentration of 0.28 μM. The apparent turnover of the reductase in the complete system was 266 mol of NADPH oxidized per min per mol of enzyme-bound FMN.

Conditions of the Assay	Reductase Activity (% of Complete)
Complete	100
- lipid	42
- P-450	< 1
- benzphetamine	21
+ deoxycholate	143
+ Renex 690	40
- lipid, + deoxycholate	48
+ cytochrome b_5	103
- reductase, + bromelain-solubilized NADPH-cyto-c reductase	< 1

is approximately 210, expressed as mol of NADPH oxidized per min per mol of enzyme-bound FMN; however, values as low as 190 or as high as 266 were observed with various preparations of P-450LM$_2$. In the absence of phosphatidylcholine, NADPH oxidation was decreased to less than one-half of the value obtained in the complete system. The role of lipid in the purified enzyme system is complex and is still under investigation. Previous studies showed that both lipid and substrate were required for the rapid reduction of P-450LM$_2$ by NADPH and the rabbit liver reductase (16,100). Evidence for a role in the formation of a complex between the reductase and P-450LM$_2$ was obtained by gel filtration and kinetic studies (30). The lipid does not, however, appear to promote the formation of large membrane-like structures (101).

When the reductase was assayed in the absence of P-450, a very low NADPH-oxidase activity was observed. When benzphetamine was omitted, the rate of NADPH oxidation was 21% of that observed in the complete system. The NADPH oxidation that occurs in the absence of substrate is almost completely inhibited by CO, which suggests that this activity is probably dependent on a reaction of P-450 with oxygen.

When 100 µg of deoxycholate were added to the complete reaction mixture, the rate of NADPH oxidation was enhanced approximately 1.4-fold; however, deoxycholate at this level

alone could not substitute for the phospholipid. Addition of deoxycholate is also known to have a stimulatory effect on cytochrome $\underline{b}_5$ reduction by NADH-cytochrome $\underline{b}_5$ reductase (60). In contrast to the effect of deoxycholate, the addition of 100 µg of the nonionic detergent, Renex 690, caused significant inhibition. These results emphasize the importance of minimizing the detergent content of the protein preparations prior to assay.

The addition of partially purified detergent-solubilized rabbit liver cytochrome $\underline{b}_5$ had no significant effect on the rate of NADPH oxidation when added in a 7- to 30-fold molar excess of cytochrome to flavoprotein. When the bromelain-solubilized NADPH-cytochrome $\underline{c}$ reductase (55) was substituted for the detergent-solubilized reductase at the same or at a higher concentration, no significant NADPH oxidation was observed.

It is not known if the rate of NADPH oxidation observed in the P-450 reductase assay represents the actual rate of P-450 reduction. If reduction is the rate-limiting step in the hydroxylation of substrate, or if the reductase dissociates after electron transfer to P-450, but prior to oxygen insertion, then the rate of NADPH oxidation observed under the conditions of this assay probably represents the rate of P-450 reduction. Alternatively, if the reductase does not dissociate from P-450 until substrate hydroxylation has occurred, then the rate of NADPH oxidation would reflect

the slowest step in the overall formation or reaction of the complex of P-450, reductase, phospholipid, and substrate with oxygen to produce product. It is not likely that the reduction of the flavoprotein with NADPH is rate-limiting, since it can be estimated from the rate of cytochrome c reduction at pH 7.4 in 0.1 M phosphate buffer, that the enzyme is capable of reacting with NADPH at a rate of at least 2000 per min, almost 10-fold faster than the turnover of the enzyme in the reconstituted system. In stopped flow experiments, performed under anaerobic conditions in the presence of CO, with an excess of NADPH, phospholipid, benzphetamine, and the rabbit liver reductase, Guengerich and Coon (100) determined that P-450LM$_2$ was reduced at a rate of 520 per min, however, this experiment has not yet been performed with the rat liver enzyme described here. In other kinetic studies (102), rate constants for the binding of reductase and oxidized P-450LM$_2$ and for the dissociation of this enzyme complex were also estimated. At the enzyme concentrations used in the reconstituted system, the rate of binding of the two proteins is slow relative to the rate of NADPH oxidation observed under these conditions. This result favors a mechanism of catalysis that does not involve dissociation and reassociation of the two proteins, and implies that a complex of reductase and P-450LM$_2$, once formed, functions in many catalytic cycles (102).

Dependence of Reductase Activity on the Substrate for the Hydroxylation Reaction: The activity of the reductase was determined in the presence of P-450LM$_2$ with several different substrates for the hydroxylation reaction. As shown in Table VI, NADPH oxidation occurred at different rates when benzphetamine, ethylmorphine, cyclohexane, and aminopyrine were used as the substrate. In each case, the rate of NADPH oxidation was greater than that observed in the absence of substrate. Since the nature of the rate-limiting reaction under the conditions of the reductase assay is still not certain, at least two possible explanations may be considered for the effect of substrate on reductase activity. Either reduction of P-450 is dependent on the particular substrate bound to P-450, or the differences observed are due to reactions which involve the interaction of P-450 with substrate, reactions which presumably occur subsequent to electron transfer. If further studies establish that reduction of P-450 is not rate-limiting under these reaction conditions, the dependence of reductase activity on the substrate for the hydroxylation reaction may provide additional support for the involvement of a complex of the two enzymes in the entire catalytic event.

Analysis of the reaction mechanism is complicated by the finding that not all of the reducing equivalents

TABLE VI

DEPENDENCE OF REDUCTASE ACTIVITY ON THE SUBSTRATE FOR THE HYDROXYLATION REACTION AND THE FORM OF P-450 USED AS THE ELECTRON ACCEPTOR

The activity of the reductase, expressed as mol of NADPH oxidized or mol of product formed per min per mol of enzyme-bound FMN, was determined under the conditions described in Methods with the indicated substitutions. The P-450LM_2 used in this experiment contained 16.9 nmol per mg of protein, and was present at a concentration of 0.5 μM. Substrate concentrations were as follows: ethylmorphine, 10 mM; cyclohexane (added in 0.01 ml of acetone), 1 mM; aminopyrine, 10 mM; and aniline, 5 mM. Product formation in the assay with aniline as substrate was measured by determination of radioactive p-hydroxy aniline (103), under conditions similar to those used for the spectral assay, except that 0.5 μmol of NADPH was used. Product formation was not linear with added reductase over as great a range as generally observed with other substrates, and the values presented were obtained from experiments using only 0.01 nmol of reductase. The control reaction in this determination contained no reductase. P-450LM_4 was used at a concentration of 0.5 μM.

Cytochrome P-450	Substrate	Apparent Turnover Number	
		NADPH Oxidized	Product Formed
LM_2	Benzphetamine	235	92
LM_2	Ethylmorphine	137	--
LM_2	Cyclohexane	131	--
LM_2	Aminopyrine	72	--
LM_2	None	57	--
LM_2	Aniline	--	106
LM_4	Aniline	--	56

transferred from NADPH were utilized for the hydroxylation of benzphetamine. With this substrate, the rate of NADPH oxidation was 2.5-times the rate of formaldehyde formation. If the rate of NADPH oxidation is corrected for the amount observed in the absence of substrate, the ratio of NADPH oxidized to product formed is 1.9, still considerably higher than the theoretical ratio of 1.0 expected for a mixed function oxidation reaction. In a recent study, Nordblom and Coon (104) examined the stoichiometric relationship between NADPH and oxygen consumption and product formation in the reconstituted enzyme system. It was shown that NADPH oxidation also occurred in excess of product formation when the enzyme system was studied with P-450LM$_2$ as the rate-limiting component. The experiments demonstrated that hydrogen peroxide production accounted for the NADPH and oxygen uptake observed in excess of that required for substrate hydroxylation. Hydrogen peroxide production was also reported to occur in the presence of substrates other than benzphetamine, and the proportion of NADPH utilized for hydrogen peroxide formation varied with the particular substrate used. Since a significant proportion of reducing equivalents from NADPH were not utilized for benzphetamine hydroxylation in the P-450 reductase assay described here, it is possible that hydrogen peroxide formation is a competing reaction under these conditions as well.

The ability of the reductase to transfer electrons to the β-napthoflavone-induced form of P-450 (P-450LM$_4$) was demonstrated in an experiment using aniline as the substrate. As shown in Table VI, with P-450LM$_4$, the rate of product formation was approximately one-half of that observed under similar conditions with P-450LM$_2$ as the electron acceptor and aniline as the substrate. It is not known if the difference in the rate of product formation in the two cases is due to a difference in the interaction of the reductase with the two forms of P-450, or if it is due to a difference in the interaction of the two forms of P-450 with the substrate aniline.

Dependence of Reductase Activity on pH and Ionic Strength: The pH dependence of reductase activity with P-450LM$_2$ as the electron acceptor is shown in Fig. 15. The rate of NADPH oxidation was maximal at pH 7.2 to 7.4, a slightly lower range than that found maximal for cytochrome c reduction (Fig. 4). The effect of increasing the buffer concentration on P-450 reductase activity is shown in Fig. 16. Activity was enhanced somewhat by increasing the concentration of phosphate buffer from 0.01 to 0.1 M, but at higher concentrations, the rate of NADPH oxidation decreased. As will be shown later, maximal rates of reduction of other electron acceptors, such as cytochrome c and DCPIP, are obtained in 0.3 M phosphate buffer, pH 7.7.

Fig. 15. Rate of NADPH oxidation in 0.1 M phosphate buffer as a function of pH. P-450 (0.44 μM, 11 nmol per mg), dilauroyl-GPC (30 μg), and reductase (0.07 nmol) were present, and cyclohexane (1mM), added in 0.01 ml of acetone, was used as the substrate.

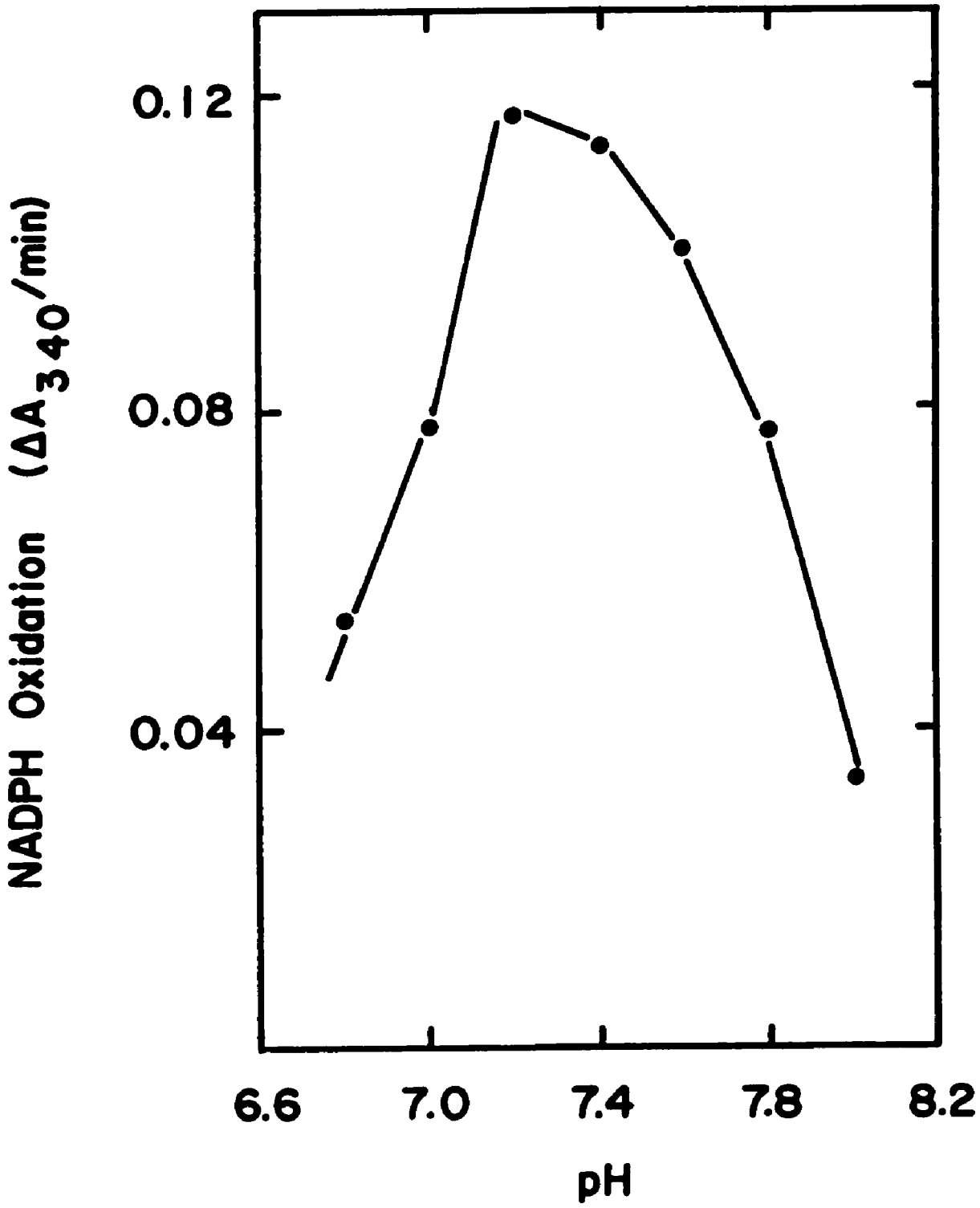


Fig. 15

Fig. 16. Rate of NADPH oxidation as a function of phosphate buffer concentration at pH 7.4. P-450 (0.44 uM, 11 nmol per mg) and dilauroyl-GPC (30 µg) were present, and benzphetamine was used as the substrate.

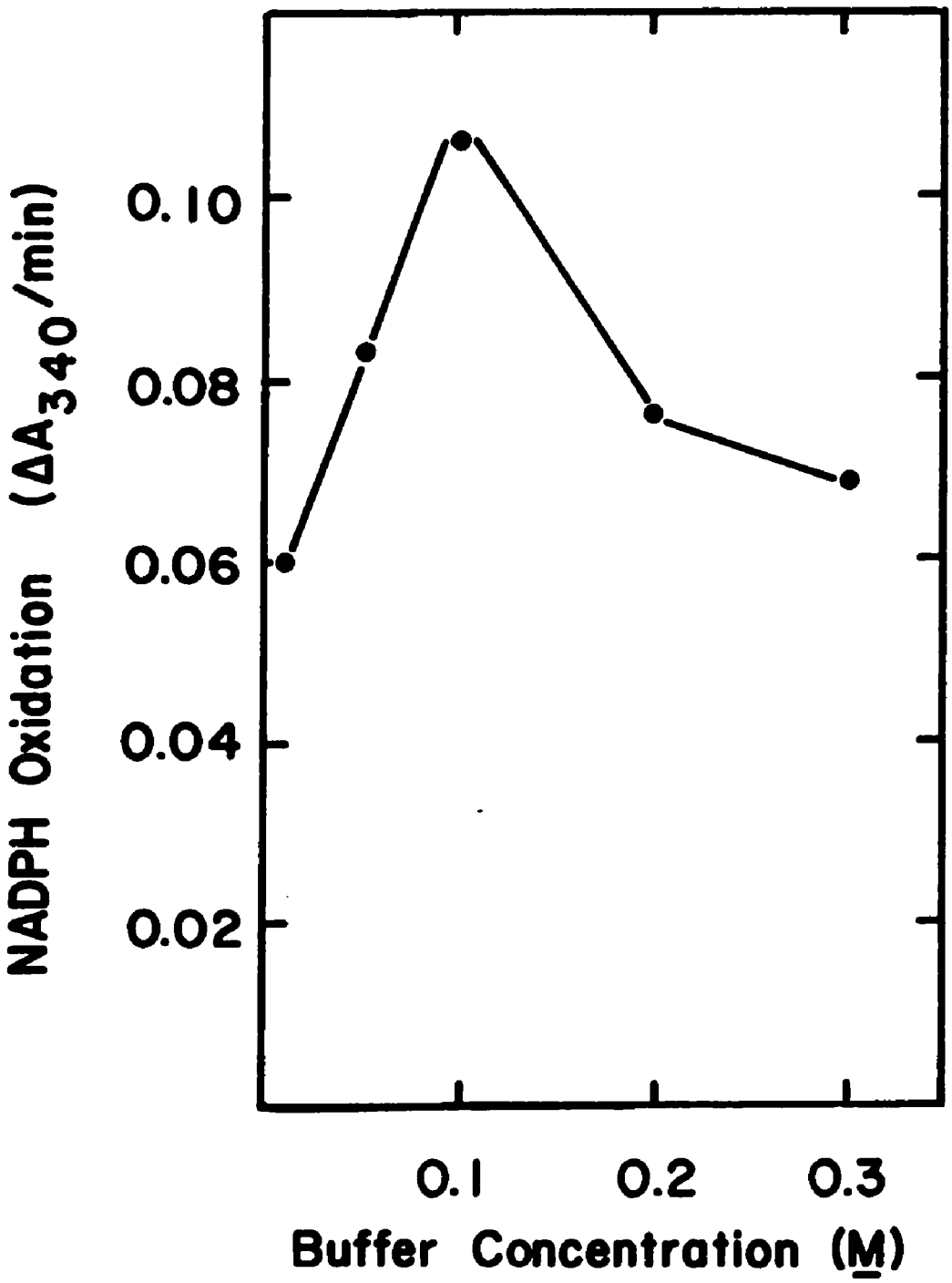

NADPH Oxidation (ΔA340/min)
0.10
0.08
0.06
0.04
0.02
0.1
0.2
0.3
Buffer Concentration (M)

Fig. 16

K_m of NADPH and Inhibition by NADP: The dependence of P-450 reductase activity on the concentration of NADPH is shown in Fig. 17, in the form of a Lineweaver Burk plot. An apparent K_m of 2 μM was obtained from this experiment, and a turnover number for the enzyme of 192 mol of NADPH oxidized per min per mol of enzyme-bound FMN was calculated for an infinite concentration of NADPH. The kinetic plot obtained when the reaction rates were measured in the presence of NADP suggests a competitive inhibition by the oxidized pyridine nucleotide with NADPH. A value for the K_i of NADP of 6 μM was calculated from the change in slope of the lines. Inhibition of the reactions catalyzed by NADPH-cytochrome c reductase by NADP is also competitive with respect to NADPH (46,47).

Reduction of Other Electron Acceptors: In addition to P-450, the detergent-solubilized flavoprotein is capable of reducing several artificial electron electron acceptors, as shown in Table VII. The apparent turnover of the enzyme, in terms of the total number of electrons transferred per min, is very similar for the four acceptors cytochrome c, ferricyanide, DCPIP, and menadione. This observation was made in earlier studies on the lipase-solubilized reductase, and it was suggested that these four reactions share a common rate-limiting step (46,53). As is also the case with NADPH-cytochrome c reductase, the rate of reduction of the above electron acceptors is dependent on the ionic

Fig. 17. Determination of the apparent K_m of NADPH and inhibition by NADP. Cytochrome P-450LM_2 (14.5 nmol per mg) was present at a concentration of 0.4 μM and reductase at 0.014 μM. Dilauroyl-GPC (30 μg) was present and benzphetamine was used as the substrate. Curve A was obtained in the absence of NADP, and curves B and C in the presence of 26.8 and 53.5 μM NADP, respectively. The concentration of NADP solutions was determined following conversion to NADPH in the presence of glucose-6-phospate and glucose-6-phosphate dehydrogenase. Velocity is expressed as nmol of NADPH oxidized per min per ml. Reductase concentration is expressed as enzyme-bound FMN.

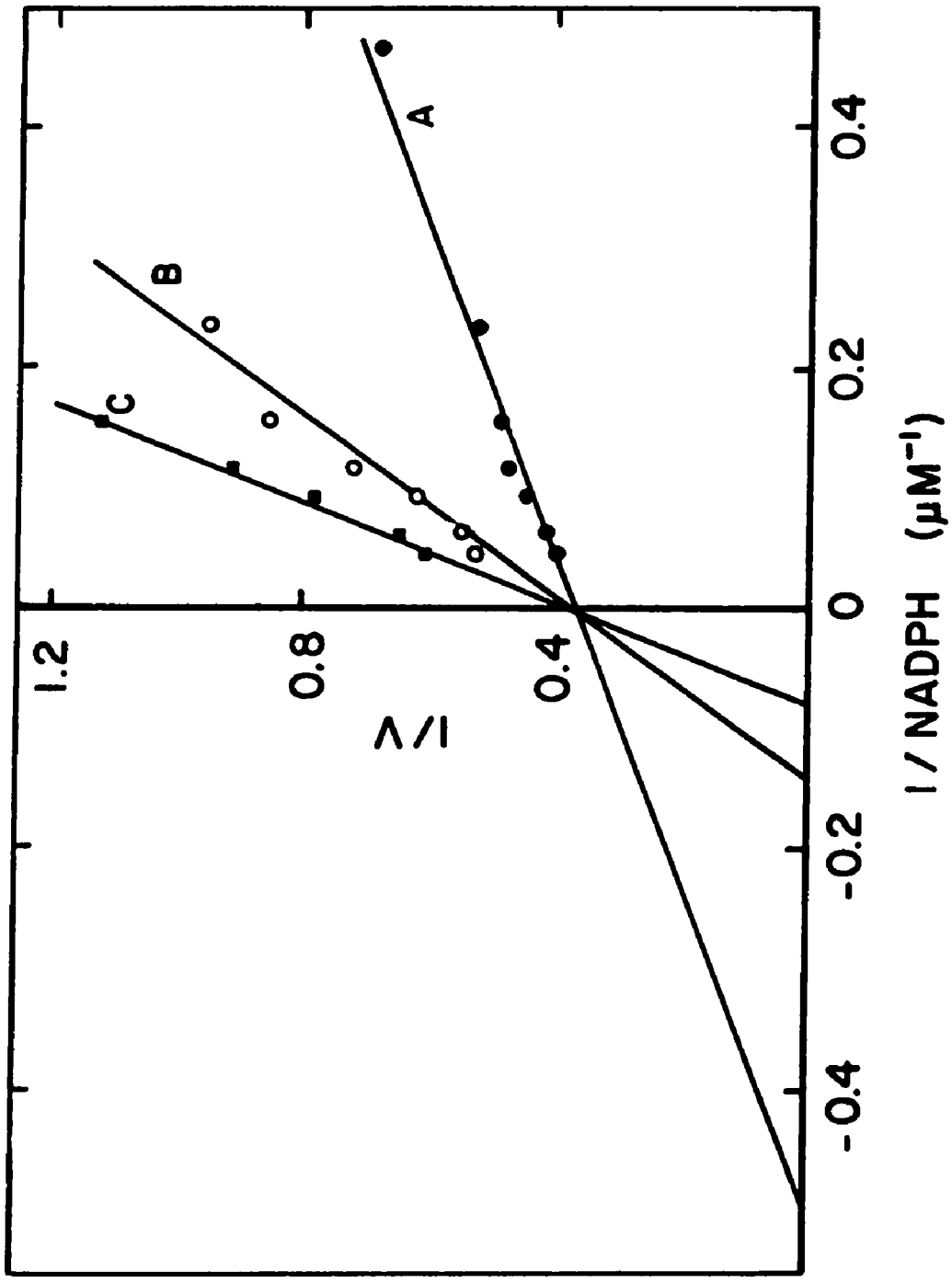


Fig. 17

TABLE VII

REDUCTION OF OTHER ELECTRON ACCEPTORS BY NADPH-CYTOCHROME P-450 REDUCTASE

Reaction rates were measured at 30° in 0.1 M potassium phosphate, pH 7.7, containing 0.1 mM EDTA, as described in Methods. Apparent turnover numbers are expressed as mol of acceptor reduced per min per mol of enzyme-bound FMN, except in the case of menadione reductase and NADPH oxidase activities which are given as mol of NADPH oxidized per min per mol of enzyme-bound FMN.

Electron Acceptor	Apparent Turnover Number
Cytochrome c	4010
Ferricyanide	5310
Dichlorophenol-indophenol	1960
Menadione	2370
Nitro Blue Tetrazolium	216
3-Acetyl Pyridine Adenine Dinucleotide Phosphate	121
None (Oxygen)	5

strength of the assay buffer. The dependence of the rate of DCPIP reduction on buffer molarity at pH 7.7 is shown in Fig. 18. For conditions of ionic strength of one or less, Phillips and Langdon (47) showed that the effect on the reaction catalyzed by the protease-solubilized enzyme was independent of the nature of the added electrolyte, and due strictly to ionic strength. It was also shown that the rate enhancement observed by increasing ionic strength was similar for both DCPIP and cytochrome c reduction. In the range studied, increases of nearly two orders of magnitude in the K_m and K_i of NADPH and NADP, respectively, also accompanied the increases in velocity.

However, in studies where much higher salt concentrations were used, Bilimoria and Kamin (105) reported significant differences in the rates of cytochrome c reduction when various anions were tested over a range of ionic strength from 1 to 9. At these high salt concentrations the rate versus ionic strength profiles for the reaction with cytochrome c and with menadione were not identical. In addition, the reaction of the enzyme with cytochrome b_5 or with Fe-EDTA, activities barely detectable at an ionic strength of less than one, became significant at very high salt concentrations. The exact mechanism by which high salt concentration increases the turnover, or otherwise modifies the reactivity of the enzyme toward certain acceptors is not yet clearly understood.

Fig. 18. Rate of DCPIP reduction as a function of phosphate buffer concentration at pH 7.7. All buffers contained 0.1 m$\underline{M}$ EDTA.

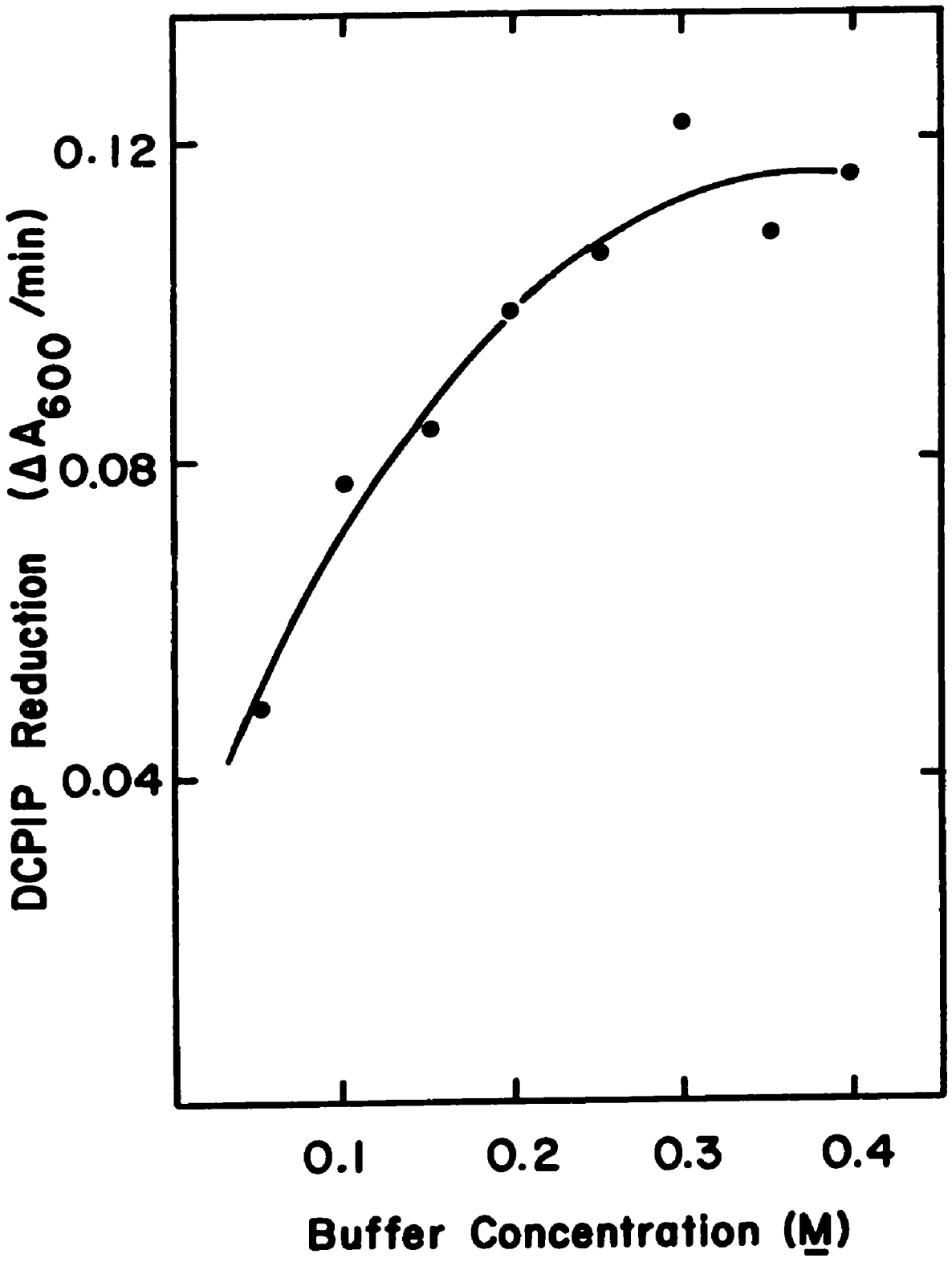

0.12
0.08
0.04
DCPIP Reduction (ΔA_{600}/min)
0.1
0.2
0.3
0.4
Buffer Concentration (M)

Fig. 18

The detergent-solubilized reductase also catalyzed the reduction of nitro blue tetrazolium, although at a slower rate than that observed with cytochrome c. This reaction shows an almost complete dependence on the presence of lipid , as previously demonstrated by Roerig et al. (81) for NADPH-cytochrome c reductase. The rate of reduction is enhanced nearly 20-fold by the addition of 250 µg of dilauroyl-GPC to the reaction mixture. As previously shown, P-450 reductase activity is also enhanced by phospholipid; however, the amount of lipid required for near maximal rates of nitro blue tetrazolium reduction is approximately 10-times the amount required in the reaction with P-450. As is also the case for P-450 reductase activity, the high ionic strength buffers in which maximal rates of cytochrome c reduction are observed, inhibit nitro blue tetrazolium reductase activity. The enzyme catalyzed hydrogen transfer from NADPH to 3-AcPyADP at a slow, but significant rate. In the absence of a suitable electron acceptor, the enzyme catalyzed NADPH oxidation at a rate only about 0.2% of that observed with cytochrome c.

The ability of the enzyme to reduce detergent-solubilized rabbit liver cytochrome b_5 was examined in preliminary experiments summarized in Table VIII. The rate of cytochrome b_5 reduction was very slow in the absence of added detergent, phospholipid, or salt. The initial rates of reduction were linear with time for only about 30 sec, and

TABLE VIII

REDUCTION OF CYTOCHROME b_5 BY NADPH-CYTOCHROME P-450 REDUCTASE

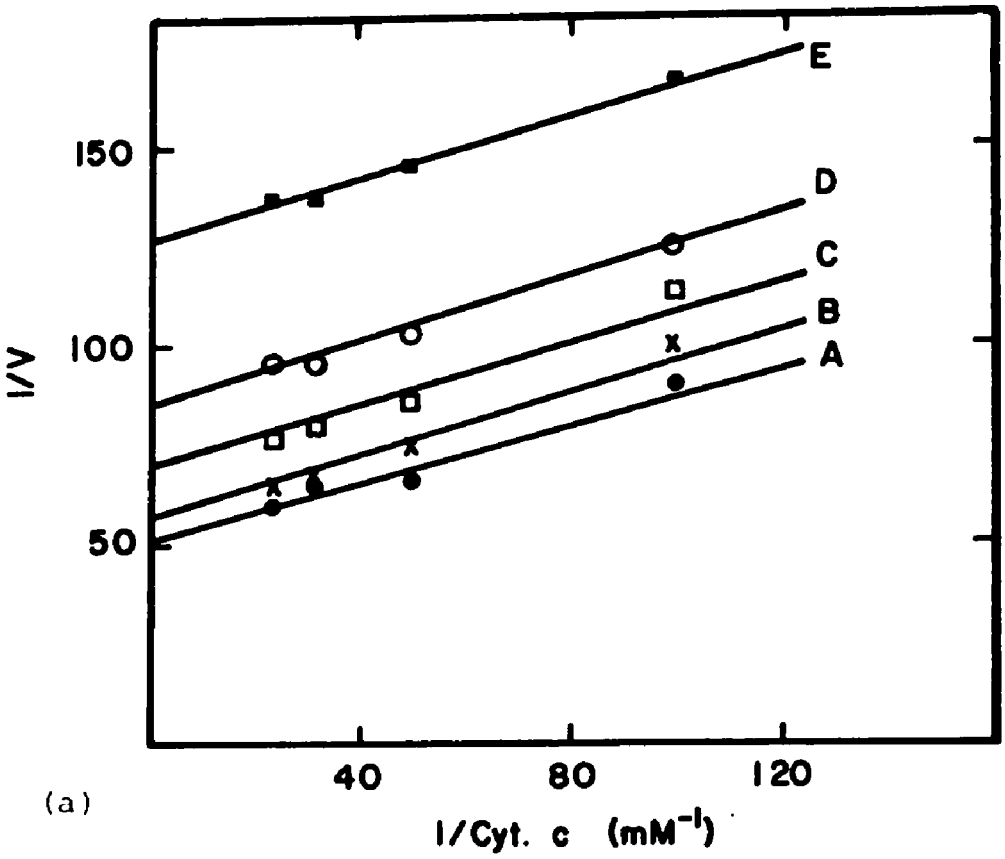
Cytochrome b_5 reduction was measured at 30° in 0.1 M potassium phosphate buffer, pH 7.4. The reaction mixture contained 5.4 μM partially purified detergent-solubilized rabbit liver cytochrome b_5, 45 nM reductase, and where indicated, 500 μg per ml of deoxycholate, 100 μg per ml of dilauroyl-GPC, or 0.8 M KCl. The percent of complete reduction was calculated by comparing the absorbance at the end of the reaction to that of a dithionite-reduced sample. Rates are expressed as mol of cytochrome b_5 reduced per min per mol of reductase. For the anaerobic experiment, the gas phase was replaced with nitrogen by alternately evacuating and flushing a suitable reaction vessel (106). The nitrogen was treated to remove traces of residual oxygen by passage through a column of BASF catalyst heated to 100°. NADPH was added from a side arm of the sealed vessel.

Reaction Conditions	Initial Rate	Steady State Level of Reduction (% Complete Reduction)
No additions	6	11
+ lipid	> 65	43
+ KCl	37	27
+ deoxycholate	52	42
+ deoxycholate, $-O_2$	42	95

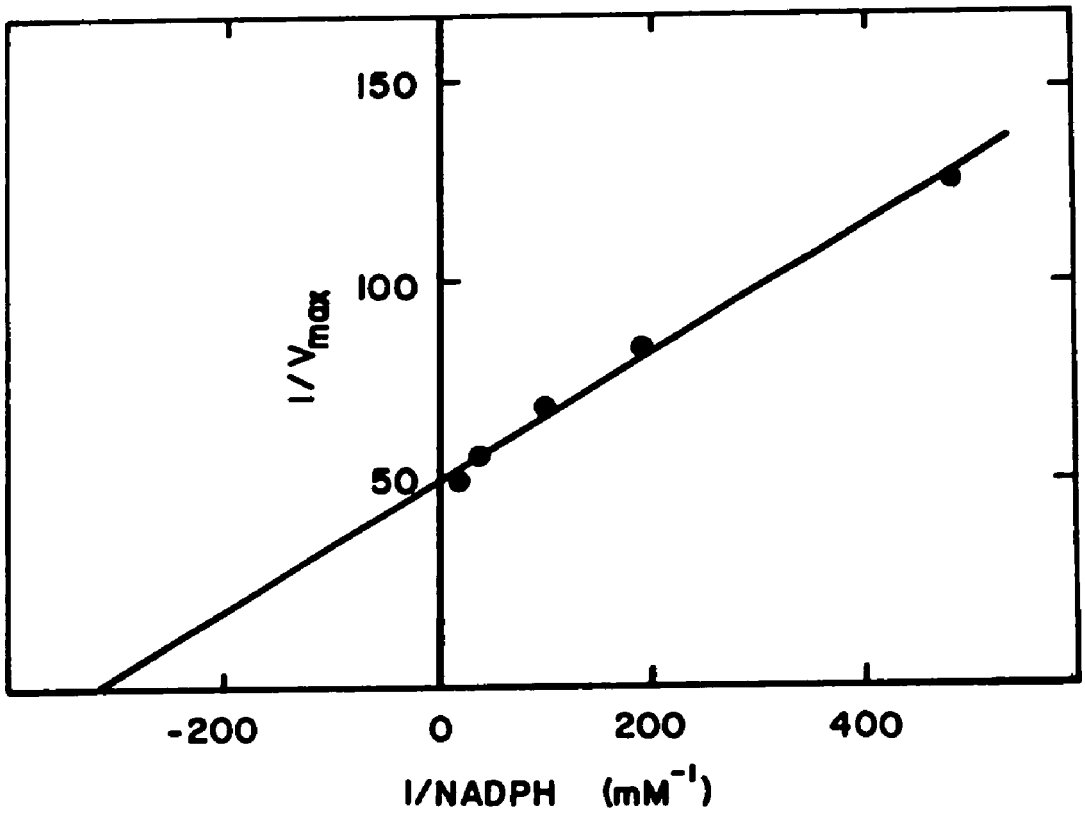
in the presence of dilauroyl-GPC, a rapid initial reaction was followed by a much slower approach to the steady state. Both the initial rates and the steady state level of reduced cytochrome b_5 attained are presented in Table VIII. Complete reduction was observed in the presence of deoxycholate under anaerobic conditions, but is probably not observed in the aerobic experiments due to a competing reaction of reduced cytochrome b_5 with oxygen. The stimulatory effect of KCl has also been reported by others (107).

Kinetic Analysis of the Reaction with Cytochrome c and Pyridine Nucleotide Specificity: The reaction with cytochrome c was studied at 30° in 0.1 M phosphate buffer, pH 7.7. Initial reaction rates were measured at several fixed concentrations of NADPH with varying concentrations of cytochrome c. A Lineweaver Burk plot of the data obtained yielded a series of parallel lines, as shown in Fig. 19a. A double reciprocal plot of maximal velocities versus cytochrome c concentration is shown in Fig. 19b and was used to obtain a value for the K_m of NADPH of 3 μM. A double reciprocal plot of the original data as velocity versus NADPH concentration also gave a series of parallel lines (not shown); a plot of these intercepts versus reciprocal cytochrome c concentration was used to obtain a value for the K_m of cytochrome c of 8 μM. A maximal velocity of 5780 mol of cytochrome c reduced per min per mol of enzyme-bound FMN was calculated for these reaction conditions.

Fig. 19. (a) Determination of the K_m of NADPH with cytochrome c as the electron acceptor. Reaction rates were measured in 0.1 M phosphate buffer, pH 7.7, containing 0.1 mM EDTA, and 3.4 pmol of reductase was used. Curves A through E were obtained at the following concentrations of NADPH: A, 52 µM; B, 26 µM; C, 10 µM; D, 5.2 µM; and E, 2.1 µM. The Km of NADPH at infinite concentrations of cytochrome c and NADPH was determined from the graph shown in (b). Velocity is expressed as µmol of cytochrome c reduced per min per ml.


E
D
C
B
A
150
100
50
1/V
40
80
120
1/Cyt. c (mM⁻¹)

(a)


150
100
50
1/V_max
-200
0
200
400
1/NADPH (mM⁻¹)

(b)

Fig. 19

The simplest interpretation of the kinetic plots obtained in this study and by others with the lipase-solubilized enzyme (64,108), is that the reductase utilizes an ordered addition or "ping pong" mechanism (109) in its reaction with NADPH and cytochrome c.

The value for the K_m of NADPH obtained in this experiment is nearly the same as that of 2 μM obtained at pH 7.4 with P-450 as the electron acceptor (Fig. 17). Both constants are within the range of values of 2 to 5 μM reported by others for NADPH-cytochrome c reductase under reasonably similar conditions (46,47,110). The value for the K_m of cytochrome c is also within the range of values from 2 to 10 μM reported in these earlier studies.

The activity of the enzyme with NADH as the electron donor was studied using cytochrome c as the acceptor. The Lineweaver Burk plot obtained by varying the concentration of NADH at a constant level of cytochrome c is shown in Fig. 20. The reaction conditions were similar to those described above for the experiments using NADPH. The apparent K_m of NADH is approximately 20 mM, almost 7000-fold higher than that determined for NADPH. The extrapolated value for the maximal velocity with NADH as the electron donor indicated a turnover for the enzyme under these conditions of about one-tenth of that determined above with NADPH as the electron donor.

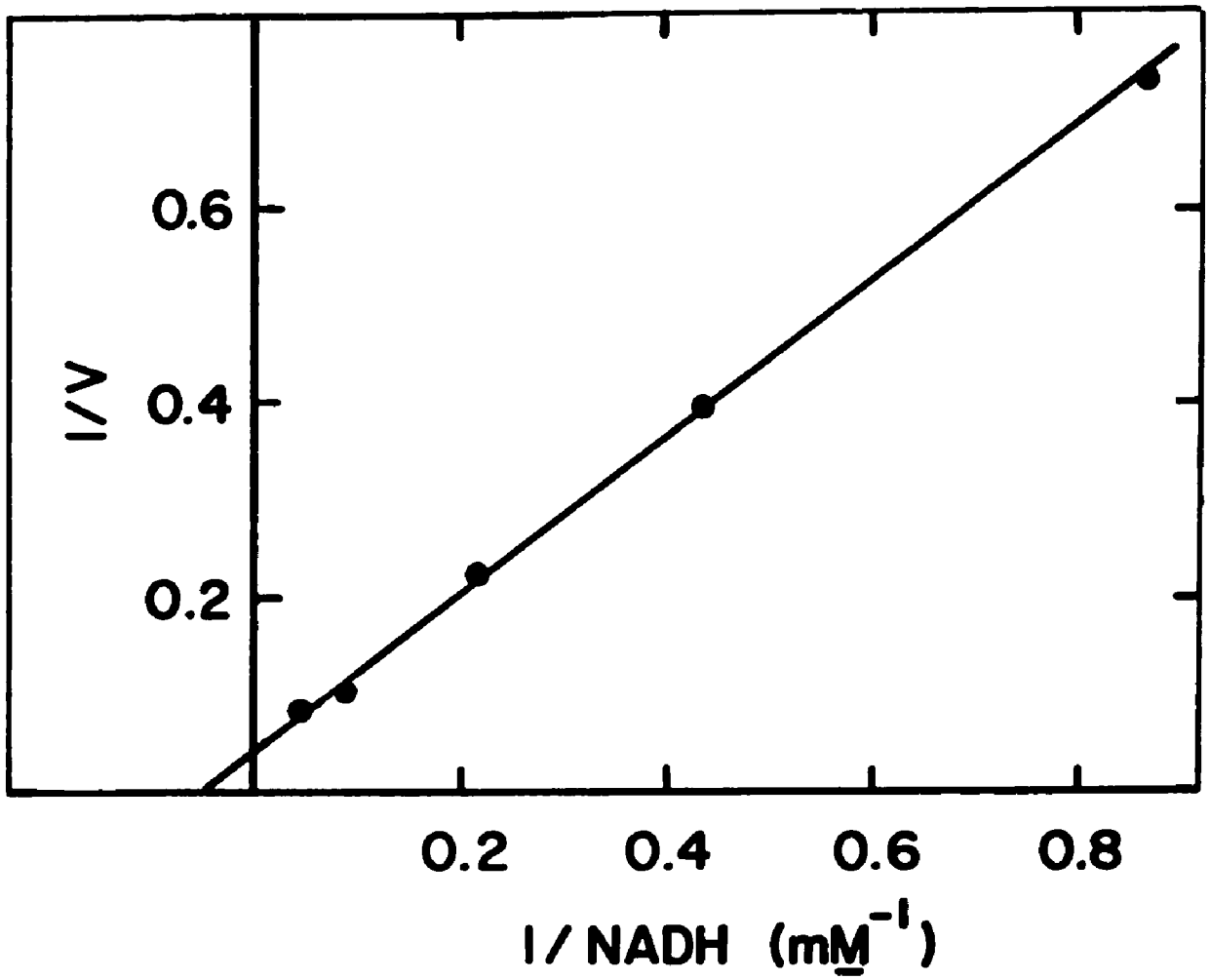


Fig. 20. Determination of the apparent K_m of NADH with cytochrome c as the electron acceptor. Reaction rates were determined in 0.1 M phosphate buffer, pH 7.7, containing 0.1 mM EDTA, and 35 pmol of reductase was used. Velocity is expressed as nmol of cytochrome c reduced per min per ml, and all values were corrected for the nonenzymatic rate observed in the absence of reductase. The concentration of cytochrome c was 40 μM.

Properties of the Air-Stable Semiquinone Form of the Reductase: The spectral intermediates observed by reduction of the enzyme with NADPH under aerobic conditions are shown in Fig. 21. Addition of 1 mol of NADPH per mol of flavin resulted within a few minutes in the formation of an air-stable spectrum (Spectrum B) with the characteristic long wavelength absorbance of a neutral or "blue" flavin semiquinone (111). This spectrum is very similar to that previously observed for NADPH-cytochrome c reductase (56, 64). The ratio of absorbance at 456 nm to that at 585 nm is 2.9, considerably higher than that of the semiquinone form of single-flavin enzymes such as flavodoxin (112). As previously discussed by Iyanagi and Mason (56), the spectral properties of this form of the enzyme are consistent with those expected for a mixture of oxidized flavin and flavin semiquinone. Since one molecule of enzyme contains a molecule each of FMN and FAD, the possibility that the air-stable form of the enzyme contains one of the two flavins in the oxidized state and the other as a flavin semiquinone will be discussed below. Further reduction of the air-stable form of the enzyme with a 5-fold molar excess of NADPH to flavin resulted in the transient formation of a spectral intermediate also characterized by semiquinone-type absorbance, but with maximal absorbance at 592 nm and decreased absorbance at 456 nm (Spectrum C). This spectrum reverted within several minutes to that of the air-stable

Fig. 21. Spectral intermediates observed upon aerobic reduction of the enzyme with NADPH. Spectrum A is that of the oxidized enzyme. Spectrum B was obtained after the addition of 1 mol of NADPH per mol of flavin. Flavin concentration was determined using an extinction coefficient of 10.6 mM^{-1} cm^{-1} per flavin for enzyme absorbance at 456 nm. Spectrum C was recorded immediately after further addition of a 5-fold excess of NADPH to flavin.

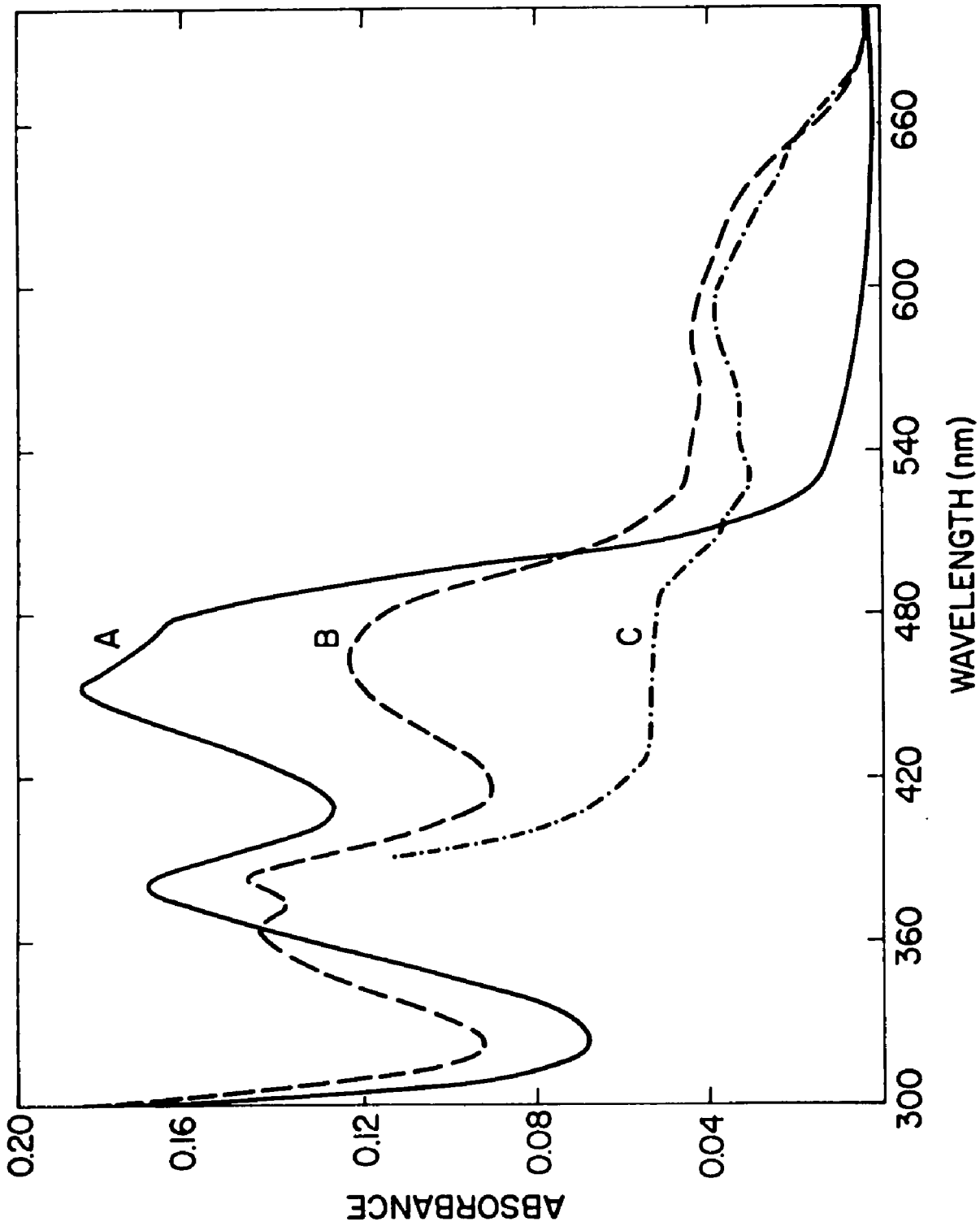

ABSORBANCE
0.20
0.16
0.12
0.08
0.04
300
360
420
480
540
600
660
WAVELENGTH (nm)
A
B
C

Fig. 21

form (Spectrum B). The air-stable form of the enzyme is further oxidized extremely slowly; at 4° complete reoxidation occurs in approximately 2 weeks. Enzyme existing in the air-stable form is fully active when assayed with either cytochrome P-450 or cytochrome c as the electron acceptor.

Spectrophotometric titrations of NADPH cytochrome c reductase have led to some controversy regarding the oxidation state of the air-stable form of the enzyme. This question is of importance, since previous studies by Masters et al. (64,107,108) indicated that reoxidation of reduced enzyme by several artificial electron acceptors yields this form of the enzyme and not the fully oxidized enzyme. Although this result has not yet been confirmed for the reaction of the reduced detergent-solubilized enzyme with oxidized P-450, should it also be true in this case, correct assignment of the oxidation state of the air-stable semiquinone form will assist in the understanding of the mechanism of electron transfer.

On the basis of spectral properties, quantitative EPR measurements, and back titration with ferricyanide, Iyanagi and Mason (56) concluded that the air-stable form of the enzyme represents an oxidation state one reducing equivalent below that of the fully oxidized enzyme. Reduction of the oxidized enzyme under anaerobic conditions with either dithionite or NADPH was shown to require 0.25 mol of

reductant per mol of flavin or one electron per molecule of enzyme for the formation of this spectral intermediate (113). It was proposed that the oxidation state of the two flavins in the air-stable form of the enzyme is as follows:

F
|
FH·

where F represents a fully oxidized flavin and FH· represents a flavin semiquinone.

On the other hand, Masters et al. (114) suggest that this form of the enzyme is a two-electron reduced state, which they represent as follows:

FH·
|
FH·

where FH· represents a flavin semiquinone. This conclusion was based on aerobic and anaerobic titrations of oxidized enzyme which required 0.5 mol of NADPH per mol of flavin or two electrons per molecule of enzyme to produce the spectrum of the air-stable form. In their experiments, back titration of the air-stable form of the enzyme required two electrons from ferricyanide for complete reoxidation of the enzyme.

An aerobic spectrophotometric titration of oxidized enzyme with NADPH is shown in Fig. 22. Linear absorbance changes upon the addition of aliquots of reductant occurred only in the early stages of the titration. The isosbestic

Fig. 22. Spectrophotometric titration of oxidized enzyme with NADPH under aerobic conditions. Enzyme (1.0 ml, 17.5 μM flavin, determined as described in Fig. 21), in 0.05 M phosphate buffer, pH 7.4, containing 10% glycerol, 0.1 mM EDTA, and 1 mg per ml of BSA, was titrated with a solution of 0.81 mM NADPH at 10°, until no further absorbance changes were observed. The total volume change due to the addition of NADPH was less than 2%, and volume changes were taken into account in the calculation of enzyme and NADPH concentrations. The molar ratio of NADPH added to flavin present was 0, 0.19, 0.37,, 0.56, and 0.74 in Spectra A through E, respectively. Several intermediate spectra have been ommitted for the sake of clarity. The inset shows a plot of absorbance changes at 456 and 585 nm as a function of NADPH added, and was used to estimate the endpoint of the titration.

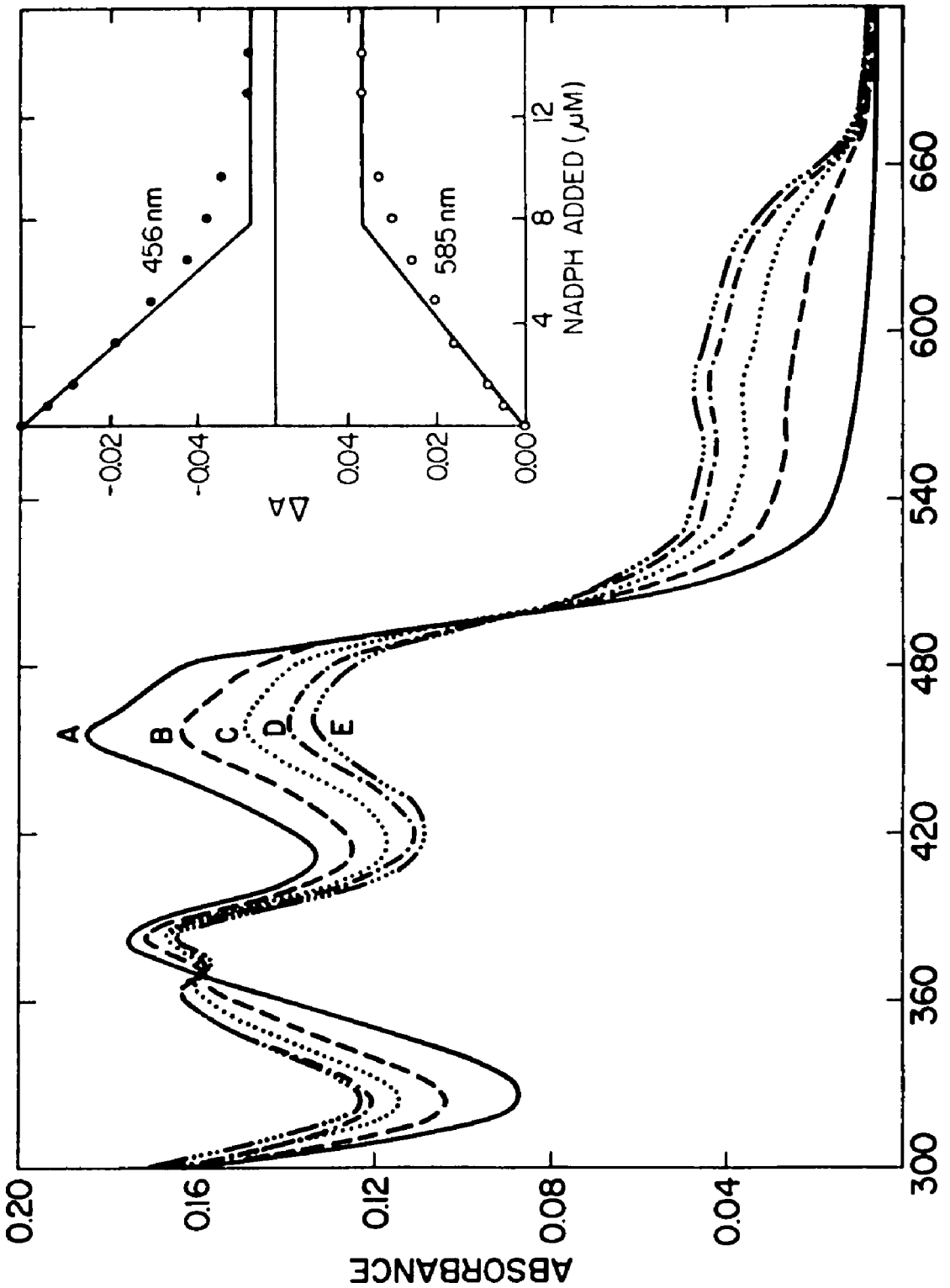

ABSORBANCE
0.20
0.16
0.12
0.08
0.04
300
360
420
480
540
600
660
WAVELENGTH (nm)
A
B
C
D
E
ΔA
-0.02
-0.04
0.04
0.02
0.00
456 nm
585 nm
4
8
12
NADPH ADDED (μM)

Fig. 22

points observed during reduction were at 369 and 501 nm. Extrapolation of the initial linear changes at 456 and 585 nm (Fig. 22, inset) suggests that a total of 0.45 mol of NADPH per mol of flavin, or 1.8 electrons per molecule of enzyme are required for the formation of the air-stable species.

For the back titration of the air-stable form of the enzyme with ferricyanide, oxidized enzyme was first reduced aerobically with 1 mol of NADPH per mol of flavin, and the titration was begun immediately after a stable spectrum was obtained. As shown in Fig. 23, stepwise oxidation proceeded with spectral changes similar to those observed in the previous experiment, but with isosbestic points at 363 and 502 nm. It is believed that the differences between the two titrations below 380 nm is a result of turbidity due to the precipitation of the enzyme during the NADPH titration. Detergent was present in the ferricyanide experiment and was of some help in maintaining the solubility of the enzyme during this titration. A plot of absorbance changes versus the amount of ferricyanide added (Fig. 23, inset) showed that 0.55 mol per mol of flavin, or 1.1 electron per molecule of enzyme was required to produce the spectrum of the oxidized enzyme.

The result of the ferricyanide back titration is in agreement with that of Iyanagi and Mason (56), and indicates that reoxidation of the air-stable form of the enzyme

Fig. 23. Spectrophotometric titration of the air-stable semiquinone form of the enzyme under aerobic conditions. Enzyme (1.1 ml, 17.4 μM flavin, determined as described in Fig. 21), in 0.15 M phosphate buffer, pH 7.4, containing 10% glycerol, 0.1 mM EDTA, and 0.1% Renex 690, was reduced with 1 mol of NADPH per mol of flavin, and when no further spectral changes were observed, spectrum A was recorded. Spectra B through G were obtained after the addition of 0.10, 0.21, 0.32, 0.42, 0.52, 0.64, and 0.74 mol of ferricyanide per mol of flavin, respectively. The total volume change due to the addition of ferricyanide was less than 2%, and volume changes were taken into account in the calculation of enzyme and ferricyanide concentrations. The temperature was 8°. The inset shows a plot of absorbance changes at 456 and 585 nm as a function of ferricyanide added, and was used to estimate the endpoint of the titration.

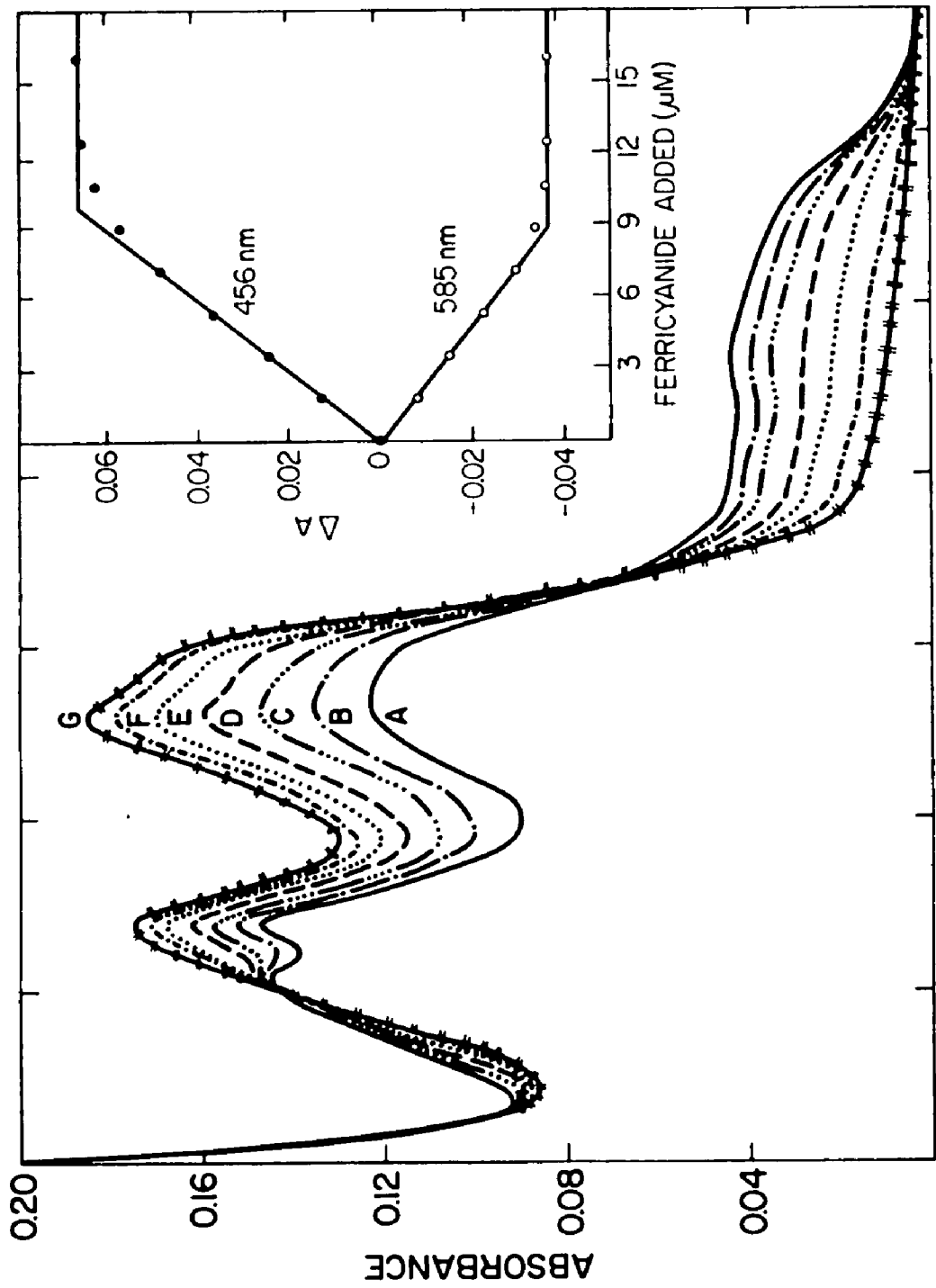

ABSORBANCE
0.20
0.16
0.12
0.08
0.04
300
360
420
480
540
600
660
WAVELENGTH (nm)
A
B
C
D
E
F
G
ΔA
0.06
0.04
0.02
0
-0.02
-0.04
456 nm
585 nm
3
6
9
12
15
FERRICYANIDE ADDED (μM)

Fig. 23

requires only one electron. However, the titration of oxidized enzyme to the air-stable state required nearly two electrons from NADPH. Possible explanations for the contradictory results obtained with the detergent-solubilized enzyme are as follows: Since NADPH is a two-electron reductant, it is very likely that initially both electrons are transferred to a single molecule of flavin on the enzyme. If a rapid one-electron oxidation of the enzyme by air can occur more rapidly than intermolecular equilibration, when the enzyme is titrated aerobically with NADPH only one electron from NADPH would remain with the enzyme and the second would be lost in a reaction of the enzyme with oxygen. The site of oxidation by air could be (a) the reduced flavin initially formed in the reaction with NADPH; (b) the second flavin, providing intramolecular equilibration can occur; or (c) an unidentified invisible electron acceptor other than flavin. If, indeed, an electron acceptor other than flavin exists, the contradictory results could also be explained if this group is unable to donate its electron to ferricyanide in the back titration. This possibility could be excluded if titration of the ferricyanide-oxidized enzyme with NADPH required only one electron from NADPH to produce the spectrum of the air-stable form of the enzyme. However, Iyanagi et al. (113) indicated that equilibration in this particular reductive phase under anaerobic conditions was so slow that the presence of very high levels of a mediator

was required during potentiometric measurements. Apparently the intermolecular equilibration required to bring the system to its final potential was quite slow, therefore, a competing reaction of enzyme flavin with oxygen may be significant under aerobic conditions. The possibility that air-oxidation of flavin in the aerobic NADPH titration is responsible for the additional uptake of one electron could be eliminated by performing this experiment under anaerobic conditions. Anaerobic titration of the enzyme to the fully reduced state should also establish the presence or absence of electron acceptors other than flavin.

Studies on FMN-Depleted Enzyme: Selective removal of FMN was accomplished by irradiation of the reductase in the presence of ammonium sulfate, or by dialysis against 2 M KBr at pH 8.5, as outlined in Methods. Irradiation in the presence of ammonium sulfate was previously used by Baggot and Langdon (91) to remove flavin from NADPH-cytochrome c reductase. At the time these earlier studies were carried out, it was not known that the enzyme contained both FMN and FAD, and the selective nature of the treatment was apparently not realized. This procedure was also used for the selective removal of FMN from the sulfite reductase by Seigel and coworkers (115,116). Removal of FAD from D-amino acid oxidase by dialysis against high concentrations of KBr has previously been described by Massey and Curti (90), and removal of FMN from flavodoxin, by Mayhew (84).

Treatment with high concentrations of KI has also been used to prepare flavin-depleted xanthine oxidase and xanthine dehydrogenase (117,118).

The data presented in Table IX summarize the various activities and flavin content of the native enzyme and FMN-depleted enzyme prepared by KBr treatment. The enzyme used in this study was prepared by the purification procedure described elsewhere (70), and was approximately 15% FMN-depleted, as judged by the ratio of FMN to FAD, and by the stimulation of activity toward P-450, cytochrome c, and DCPIP by preincubation with FMN. However, in contrast to the above activities, with ferricyanide as the electron acceptor, reductase activity was not stimulated by the addition of FMN. After treatment of the enzyme with KBr, only 30% of the theoretical amount of FMN was still present, but very little loss of FAD occurred. The treated enzyme had decreased activity toward P-450, cytochrome c, and DCPIP, and the residual activity corresponded fairly well to the amount of FMN still remaining in the sample. Ferricyanide activity, on the other hand, was not significantly altered by the treatment. Preincubation of the depleted enzyme with FMN restored cytochrome c activity to nearly the maximal value. Slightly less, but still significant restoration of activity toward P-450 and DCPIP was also demonstrated.

TABLE IX

EFFECT OF FMN REMOVAL BY KBr TREATMENT OF REDUCTASE

Reductase Preparation	Flavin Content[a]		Activities toward Acceptors[b]			
	FMN	FAD	Cyt. P-450	Cyt. c	DCPIP	ferri-cyanide
Native	84	100	81	81	89	102
Native + FMN			100	100	100	100
KBr-treated	30	93	22	24	28	106
KBr-treated + FMN			74	97	81	103

[a] % relative to FAD content of native enzyme

[b] % relative to activities of FMN-supplemented native enzyme

That participation of FMN is apparently not required for ferricyanide reduction was also confirmed in studies with FMN-depleted enzyme prepared by irradiation in the presence of ammonium sulfate. The data regarding cytochrome c and ferricyanide reduction by such a preparation are shown in Table X. The activity of the treated enzyme was determined before and after dialysis to remove ammonium sulfate. Before dialysis, the treated enzyme showed very little cytochrome c reductase activity, but the ability to reduce ferricyanide was essentially unchanged. Addition of FMN to the treated enzyme restored over 80% of the maximal activity toward cytochrome c but had little effect on the activity toward ferricyanide. As also reported by Baggot and Langdon (91), dialysis of the treated enzyme invariably results in some loss of FMN-dependent activity. In this experiment, 25% of the FMN-dependent activity toward cytochrome c observed prior to dialysis was lost, and approximately 20% of the activity toward ferricyanide was lost. Analysis of the dialyzed sample for flavin confirmed the removal of over 90% of the maximal amount of FMN. Compared to the initial value, 83% of the FAD remained. If some loss of FAD occurs on dialysis, this may account for the failure to recover all of the FMN-dependent activity after removal of ammonium sulfate. In other experiments now shown, loss of FAD was always accompanied by a loss of activity toward all four acceptors studied.

TABLE X

EFFECT OF FMN REMOVAL BY IRRADIATION OF REDUCTASE IN THE PRESENCE OF AMMONIUM SULFATE

The enzyme was treated with ammonium sulfate and light, as described in Methods, for a period of 8.5 hrs at pH 8.5.

Additions	Activities before dialysis[a]		Activities after dialysis[b]		Flavin content[b] after dialysis	
	Cyt. c	ferricyanide	Cyt. c	ferricyanide	FMN	FAD
None	7	96	6	77	8	83
FMN	81	103	60	78		

[a] % relative to activity of FMN-supplemented native enzyme

[b] % relative to FAD content of native enzyme

Flavin binding to FMN-depleted enzyme prepared by ammonium sulfate and light treatment was studied in an experiment in which the concentration of flavin in the preincubation mixture was varied, and cytochrome c reduction was used as a measure of the amount of reactivated enzyme present. A double reciprocal plot of velocity versus flavin concentration is shown in Fig. 24, and gave an apparent K_m for FMN of 0.02 μM. To determine if flavins other than FMN were as effective in restoring activity to the depleted enzyme, the experiment was performed with FAD in the preincubation mixture, and a double reciprocal plot of the data obtained is also shown in Fig. 24. The similarity of the values obtained for the maximal velocity with either FMN or FAD suggests that FMN-depleted enzyme may be reconstituted to full activity toward cytochrome c with either of these flavins. The apparent K_m for FAD was 5 μM, considerably higher than that obtained for FMN. This result indicates that FMN, as expected, is bound more tightly by the depleted enzyme than is FAD.

Fig. 24. Reactivation of FMN-depleted enzyme with FMN or FAD. The depleted enzyme was prepared by treatment with ammonium sulfate and light at pH 8.5, as described in Methods. The assay buffer contained 0.3 M phosphate, pH 7.7, and 0.1 mM EDTA. Enzyme and flavin were preincubated in the assay mixture for 4 min prior to the addition of NADPH. Reaction rates were corrected for a slight amount of activity observed in the absence of flavin. Curve A was obtained when the concentration of FMN was varied, and Curve B when the concentration of FAD was varied. Velocity is expressed as nmol of cytochrome c reduced per min per ml.

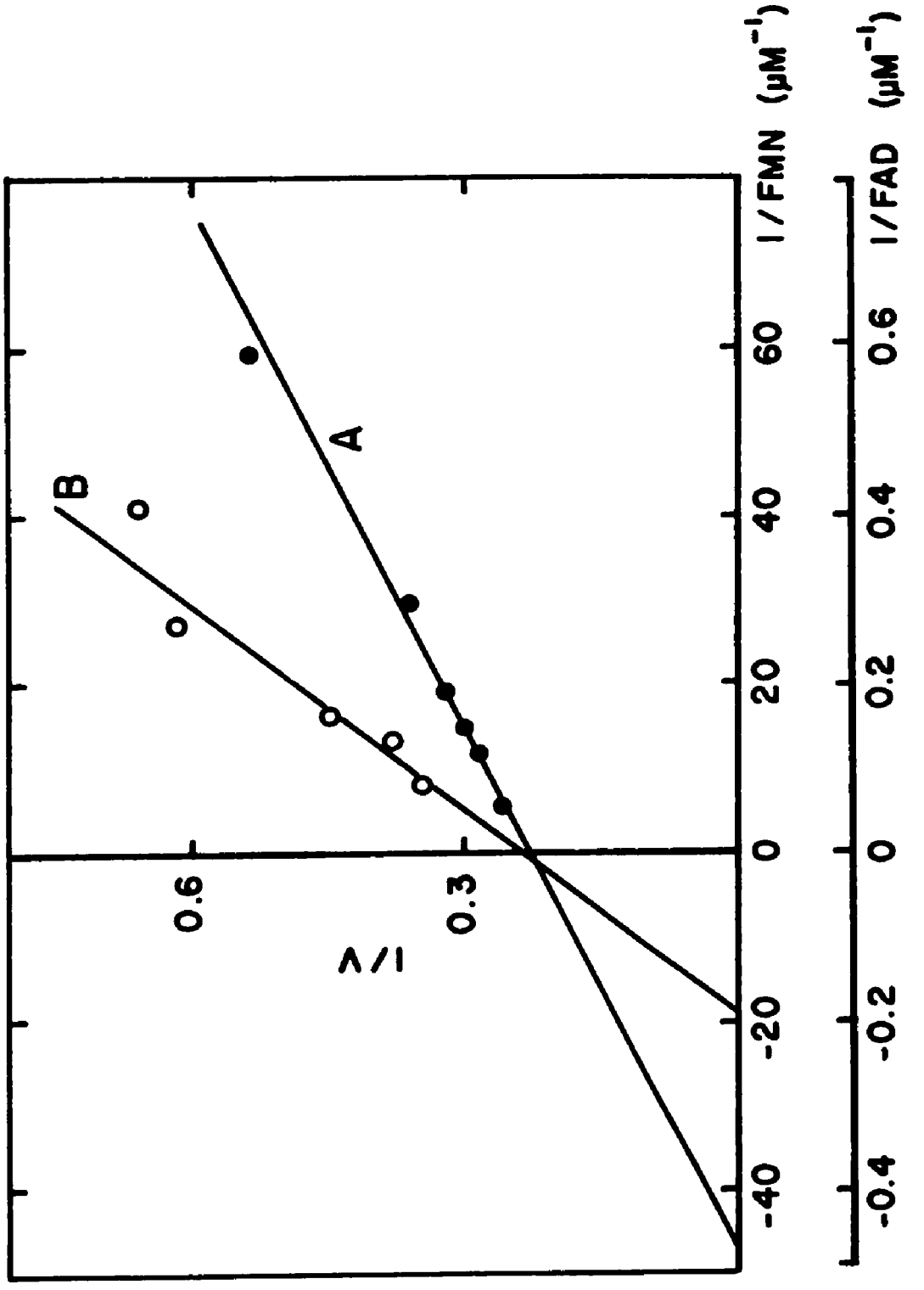


Fig. 24

DISCUSSION

The purification of the protein components of the microsomal P-450-containing electron transport system has been a difficult task and has involved the contributions of numerous researchers over a period of several years. With the use of glycerol as a stabilizing agent and the modification of conventional fractionation and chromatographic procedures to include the use of detergents as solubilizing agents, significant progress was made. In the present work, a procedure was developed for the purification of P-450 reductase from rat liver microsomes which involves the use of nonionic and ionic detergents during chromatography on DEAE-cellulose.

At the time these studies were undertaken, there was substantial evidence for the participation of microsomal NADPH-cytochrome c reductase in electron transfer to P-450. However, protease- or lipase- solubilized preparations of the purified enzyme were unable to function in P-450 reduction. The finding of Lu et al. (15), that a reductase fraction obtained by detergent-solubilization retained the ability to reduce P-450, raised the possibility that the catalytic activity of the NADPH-cytochrome c reductase was altered during isolation of the enzyme. Since

purification of the detergent-solubilized P-450 reductase was required to clearly establish the relationship between the P-450 and cytochrome c reductase activities of microsomes these initial studies were extended in the present work. The enzyme was isolated in highly purified form from phenobarbital-induced rat liver microsomes and characterization was undertaken.

The specific activity of the enzyme with cytochrome c and with P-450 as the electron acceptor was determined at each stage of the purification to provide evidence that these two activities were associated with a single protein. The ability of the preparations to reduce P-450 was determined indirectly by measuring NADPH oxidation in the presence of saturating amounts of P-450LM$_2$ and phospholipid, with benzphetamine as the substrate, and the reductase as the rate-limiting component. To determine the P-450 reductase activity of the microsomes, it was assumed that the reductase is also the limiting component of the membrane-bound hydroxylating system, and the rate of product formation from benzphetamine was used as an estimate of microsomal P-450 reductase activity. It was found that the ratio of the specific activity with cytochrome c to that with P-450 as the electron acceptor, when the latter was determined as described above, did not vary significantly throughout the enzyme purification. These data provide

strong evidence that the same enzyme is responsible for the NADPH-dependent reduction of P-450 and cytochrome c.

Based on cytochrome c reductase activity, a 150-fold purification was achieved by isolation of the enzyme from phenobarbital-induced microsomes. Since the molecular weight of the enzyme is known, this observation permits calculation of a reductase content in induced microsomes of about 0.08 nmol per mg of protein. This value is in good agreement with previous estimates by others (13,56), and on a molar basis, is 20 to 30 times lower than the P-450 content of induced microsomes. This result is consistent with the initial assumption that the reductase is the limiting component of the microsomal drug hydroxylation system. Based on this estimate of the reductase content, the turnover number of the flavoprotein in microsomes, which metabolize benzphetamine at a rate of 0.014 μmol per min per mg of protein, is at least 175 per min with this substrate for the hydroxylation reaction. The turnover number of the purified enzyme, as measured by NADPH oxidation in the reconstituted system with benzphetamine as the substrate, is approximately 210 per min, and compares favorably to the above estimate of the activity of the enzyme in microsomes. The similarity in the activity of the microsomal and reconstituted system, when calculated on the basis of the reductase content, suggests that further study of the purified enzymes under conditions where the

reductase is the rate-limiting component may provide information directly applicable to the function of this system in the microsomal membrane.

Product formation from benzphetamine and aniline was demonstrated with the purified enzymes, strongly indicating that the reductase, P-450, and phosphatidylcholine are the only components required for hydroxylation activity. However, with benzphetamine as the substrate, the rate of NADPH oxidation was at least twice the rate of product formation. Since a one-to-one stoichiometry of NADPH oxidized to product formed was previously demonstrated for microsomal P-450-dependent reactions such as aminopyrine demethylation (94) and cyclohexane hydroxylation (95), the purified enzyme system appears to be somewhat less efficient than the microsomal system in substrate hydroxylation. Certain compounds, such as perfluorinated hydrocarbons, are known to uncouple electron flow and product formation (119). These compounds stimulate NADPH and oxygen uptake by the formation of a P-450-substrate complex, but are not hydroxylated by P-450. Since the stoichiometry of benzphetamine demethylation has not been studied in rat liver microsomes, it is not known whether the reaction of P-450 with benzphetamine is partially uncoupled in the manner described above, or if, for other reasons, the purified enzyme system is less efficient in substrate hydroxylation than is the microsomal system. The recent studies of Nordblom and

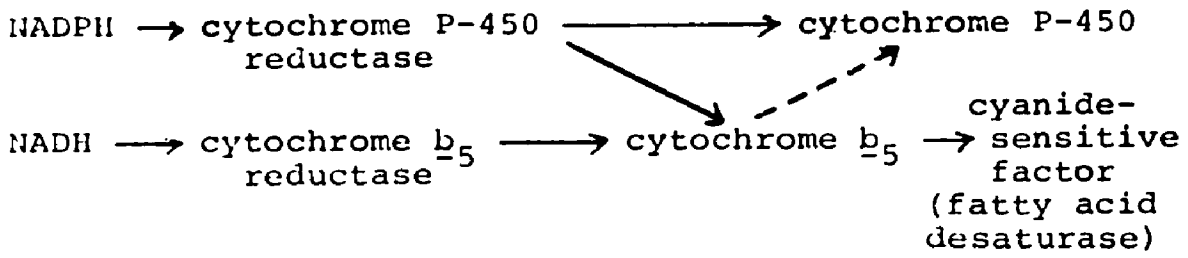
Coon (104) provide evidence that, in the reconstituted enzyme system, reducing equivalents from NADPH that are not used in the hydroxylation of substrate are used in the formation of hydrogen peroxide.

The data obtained in this work were not sufficient to identify the rate-limiting step in the reaction of the reductase with P-450 under the conditions of the P-450 reductase assay. It is not likely that NADPH oxidation in this system is limited by the rate of reduction of the flavoprotein with NADPH. The rate of cytochrome c reduction under similar conditions of pH and ionic strength indicates that the enzyme is capable of reacting with NADPH at a rate almost 10-fold faster than the observed turnover of the enzyme in the P-450 reductase assay. However, the rate of NADPH oxidation by the reductase was shown to depend on the particular substrate for the hydroxylation reaction. This observation suggests that the rate-limiting step in the reaction of the reductase with P-450 is effected by the substrate bound to P-450, however, the mechanism by which substrate effects reductase activity is not known. If reduction of P-450 is rate-limiting, then the rate of transfer of electrons from the reductase to P-450 apparently varies, and depends on the substrate bound to the cytochrome. Alternatively, the rate of NADPH oxidation may reflect a step in the reaction which is dependent on the interaction of P-450 and a particular substrate. This could

occur if a complex of P-450 and reductase catalyzes the hydroxylation reaction. In this case, the rate of NADPH oxidation would reflect the slowest step in the overall formation and reaction of the protein complex.

Since reduction of cytochrome b_5 by NADPH is observed in microsomes, although at a rate slower than that seen with NADH (120), the ability of the reductase to reduce cytochrome b_5 was examined. Complete reduction of partially purified rabbit liver cytochrome b_5 was demonstrated under anaerobic conditions. However, the rate of reduction was very slow unless phospholipid, detergent, or salt was present. Since P-450 reductase activity is enhanced by lipid, the stimulation of cytochrome b_5 reductase activity by phosphatidylcholine was not totally unexpected. Further studies will be required to provide more detailed information concerning the interaction of the two purified enzymes and to determine the role of lipid in this reaction.

The probable interactions of the NADH- and NADPH-dependent electron transport systems of liver microsomes are summarized in the following scheme:



Reduction of cytochrome $\underline{b}_5$ by the P-450 reductase provides a pathway for electron transfer from NADPH to the cyanide-sensitive factor, and is generally believed to account for the NADPH-dependent activity of microsomes in fatty acid desaturation (121).

It is unlikely that the P-450 reductase has a significant role in the NADH-dependent reactions of liver microsomes. It was found that the apparent K_m for NADH was approximately 20 m$\underline{M}$, and was nearly four orders of magnitude greater than the K_m for NADPH. These results suggest that the enzyme is quite specific for NADPH as the electron donor.

Studies on the reconstituted enzyme system presented here and by others (122-124) showed that cytochrome $\underline{b}_5$ is not an obligatory component of the NADPH-dependent electron transfer system. However, the possible role of cytochrome $\underline{b}_5$ in electron transfer to P-450 in microsomes is still a controversial matter, and has received attention primarily because the NADPH-dependent hydroxylation activities of microsomes are enhanced by the addition of NADH (13,125). Studies on the stimulatory effect of NADH on microsomal drug demethylation by Estabrook and coworkers (126-129) led these authors to propose that one of the two electrons required for the hydroxylation reaction may be supplied by cytochrome $\underline{b}_5$. On the other hand, Staudt $\underline{et\ al}$. (95) suggest that the synergistic action of NADH represents an

"electron sparing effect," whereby electrons supplied by cytochrome b_5 are used when electron flow from NADPH and product formation are partially uncoupled. According to the mechanism of Estabrook and collaborators (12,126-129), electrons from NADH supplied via cytochrome b_5 are used in the hydroxylation of substrate by P-450, but the mechanism of Staudt et al. (95) suggests that electrons supplied from NADH are used in reduction of the activated oxygen to water when substrate hydroxylation has not occurred.

In characterization studies on the purified reductase, evidence was obtained for the existence of two forms of the reductase in rat liver microsomes. The two forms, isolated by different purification procedures, were obtained relatively free of cross contamination, and are distinguished by a difference in apparent molecular weight of 2000. The preparation and preliminary characterization of the enzyme with an apparent molecular weight of 78,000 was previously described (70), and the preparation of enzyme containing predominantly the 76,000 molecular weight polypeptide was described in detail here. Both forms of the enzyme contain FMN and FAD, and both have similar activities when cytochrome c or rabbit liver P-450LM_2 is used as the electron acceptor. The specific activities are also similar to those determined by Dignam and Strobel (71) for a purified P-450 reductase with an apparent molecular weight of 80,000 with cytochrome c or

rat liver P-450 as the electron acceptor.No evidence was obtained for an obvious preferential induction of either form by phenobarbital; the experiments suggest that the two forms are present in approximately equal quantities in microsomes from both untreated and phenobarbital pretreated animals.

Previous experiments in which reductase, lipid, and P-450 fractions prepared from animals induced by different pretreatments were interchanged showed that the substrate specificity of the microsomal hydroxylase system resides in the cytochrome component (18,20, 65). The existence of multiple forms of P-450 with different catalytic activities (28,30,94) provides a reasonable explanation for the broad substrate specificity of the microsomal system. However, if more than one form of the reductase is present in microsomes, it is possible that there are discrete electron transport systems, involving the interaction of a particular reductase with only one or a few forms of P-450. In studies by Lu et al. (18) with enzyme preparations obtained from rat liver microsomes, it was found that the activity of partially purified reductase obtained from phenobarbital-induced microsomes with P-450 prepared from this source was twice that observed for a reductase preparation from 3-methylcholanthrene-induced microsomes. On the other hand, a similar experiment was performed by Fujita and Mannering (65) but with a different substrate for the hydroxylation

reaction, and very little difference in the activity of the two reductase fractions was observed. More conclusive evidence regarding the possible specificity of different forms of the reductase for different forms of P-450 is clearly required, since as the limiting component of the microsomal enzyme system, the reductase may have an important role in determining the overall rate of metabolism of various compounds by this enzyme system.

Although immunochemical studies have aided in the identification of multiple forms of P-450 (30,43), no evidence has yet been obtained for the existence of immunochemically distinct forms of the reductase in liver microsomes. In studies with antibodies prepared to the protease-solubilized enzymes no differences among the forms of the reductase present in microsomes from untreated, phenobarbital- and 3-methylcholanthene-induced animals were detected by immunochemical techniques (49,130,131). Welton and Aust (131) used SDS-polyacrylamide gel electrophoresis to analyze the immunoprecipitate obtained by treatment of rat liver microsomes with antibody, and reported that a single band with an apparent molecular weight of 79,000 was observed regardless of the inducing agent administered to the animals. These workers previously demonstrated that their antibody preparations inhibit over 90% of microsomal cytochrome c reductase activity. However, the electrophoretic system used by these authors differs from that described here, and

it is possible that this is reason the two forms were not resolved in these earlier studies.

The existence of two forms of the enzyme was unfortunately not discovered until this work was nearly completed, and more detailed comparative studies were not performed. Thus, further experiments will be required to determine exactly how the two forms differ, and if they have physiologically distinct roles in microsomal electron transport. An observation by Golf et al. (66) may be useful in future experiments to distinguish between the two forms on the basis of differences in physical properties. In experiments with detergent-solubilized rat liver reductase, isoelectric focusing was used to separate the sample into two fractions, both containing low, but detectable amounts of cytochrome c reductase activity. These findings must be interpreted with some caution, however, since no information regarding P-450 reductase activity was reported.

The majority of the characterization studies summarized here, with the exception of those on the FMN-depleted enzyme, were performed with enzyme preparations containing predominantly the 76,000 molecular weight form. These preparations were judged to be of slightly higher purity on the basis of SDS-polyacrylamide gel electrophoretic and spectral properties than the enzyme obtained in the earlier work (70).

The flavin dissociated from the enzyme was found to consist of approximately equimolar quantities of FMN and FAD. Estimates of the minimal molecular weight based on total flavin ranged from 41,500 to 49,200, and strongly indicate the presence of one molecule each of FMN and FAD per polypeptide chain of molecular weight 76,000. The spectrum of the oxidized enzyme was characterized by absorbance maxima at 276, 384, and 456 nm, with a shoulder of 485 nm, and appeared to be that of a simple flavoprotein. However, the possibility that prosthetic groups other than flavin are also present has not yet been ruled out. The extinction coefficient for enzyme absorbance at 456 nm is 21.2 $m\underline{M}^{-1}$ cm^{-1}, or 10.6 $m\underline{M}^{-1}$ cm^{-1} per flavin.

The flavin content and spectral properties of the reductase described above are similar to those of the trypsin-solubilized NADPH-cytochrome $\underline{c}$ reductase characterized by Iyanagi and Mason (56). The presence of FMN in other preparations of lipase -solubilized reductase, previously thought to contain only FAD, was recently confirmed (114), and the bromelain-solubilized enzyme used in the studies presented here also contained equimolar quantities of FMN and FAD. Therefore, the difference in the activity of detergent-solubilized and the other enzymes with P-450 is apparently not due to a difference in the flavin content of the two preparations. However, as previously demonstrated by Welton and Aust (63), the molecular weight of the

detergent-solubilized reductase, as determined in SDS-polyacrylamide gel electrophoresis experiments, is significantly greater than the values of 68,000 (56) to 71,000 (63) obtained by similar techniques for the cytochrome *c* reductase. In addition, the cytochrome *c* reductase does not appear to aggregate (46,47,53,56,132), but gel filtration of the detergent-solubilized reductase showed that the enzyme behaved as a heterogeneous mixture of large molecular weight aggregates with an approximate molecular weight of 520,000.

Similar differences in the minimal molecular weight and state of aggregation were noted in comparative studies on the protease- and detergent-solubilized forms of cytochrome b_5 and NADH-cytochrome b_5 reductase (57-60). By analogy to these extensively studied enzymes, it is possible that the P-450 reductase is also an amiphipathic protein, and contains a terminal sequence of primarily hydrophobic amino acid residues which is susceptible to proteolytic cleavage. Experiments communicated in preliminary form by Satake *et al.* (69) provide further support for this proposal, and suggest that trypsin treatment of the detergent-solubilized reductase yields a smaller, soluble enzyme which is no longer active in P-450 reduction. These experiments imply that the portion of the polypeptide chain removed by trypsin treatment is required for the enzyme to function in P-450 reduction, but the mechanism by which such treatment alters the catalytic activity of the enzyme is still uncertain. However,

experiments by Coon et al. (30) involving gel filtration of mixtures of P-450, phospholipid, and reductase provide evidence that the physical interaction of the detergent- and protease-solubilized enzymes with P-450 is not identical.

Although the detergent- and protease-solubilized forms of the enzyme differ dramatically in their catalytic activity with cytochrome P-450 or cytochrome b_5 as the electron acceptor, the characteristics of the reactions of the detergent-solubilized enzyme with nonphysiological electron acceptors, such as cytochrome c, have thus far been found to be similar to those previously reported by numerous workers (46,47,53,81,65,105) for NADPH-cytochrome c reductase. The reaction with cytochrome c showed a similar dependence on the pH and ionic strength of the assay buffer, and a kinetic analysis of this reaction suggested that the mechanism of catalysis is probably the same for both enzymes. In addition to cytochrome c, both enzymes catalyze the reduction of ferricyanide, DCPIP, and menadione, and the rate of reduction of nitro blue tetrazolium is enhanced by the addition of phospholipid. It was also found that the P-450 reductase catalyzes hydrogen transfer to the 3-acetyl pyridine analogue of NADP, a reaction not previously reported for the cytochrome c reductase.

The spectral changes observed after the addition of NADPH to oxidized enzyme showed that semiquinone formation occurred during reduction of enzyme flavin. It was also shown that the enzyme may exist in a partially reduced state

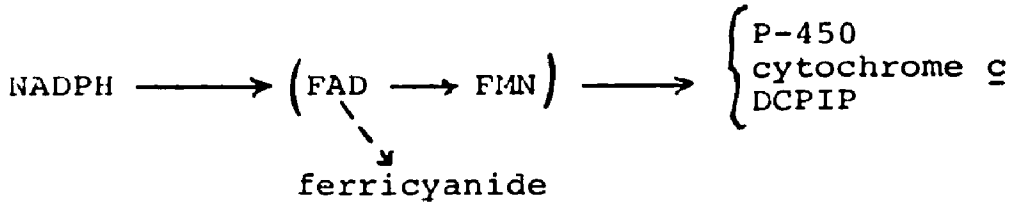
that is stable in air. The absorption spectrum of this form of the enzyme has the characteristic long wavelength absorbance of a neutral flavin semiquinone, and is similar to that previously observed for trypsin- or lipase-solubilized forms of the enzyme (56,64). Stabilization of a neutral flavin semiquinone to further oxidation by air would appear to be a fairly unique property of the reductase; to our knowledge, only in the case of Azotobacter vinelandii flavoprotein, an enzyme containing a single molecule of FMN per polypeptide chain, has a similar resistance of a neutral semiquinone to air oxidation been reported (133). Iyanagi and Mason (56) suggest that the spectral properties of this form of the reductase are consistent with those expected if one of the two flavins exists in the fully oxidized state and the other as a flavin semiquinone. Further evidence for this proposal was sought, since, in the case of the cytochrome c reductase, more extensive studies by Mason and coworkers (56, 113) and by Masters et al. (114) have led to an as yet unresolved controversy regarding the oxidation state of this form of the enzyme.

In the present work, spectrophotometric titration of the air-stable form of the enzyme with ferricyanide showed that one electron per molecule of enzyme was required for the reoxidation of flavin. On the other hand, titration of the oxidized enzyme with NADPH under aerobic conditions suggested that nearly two electrons per molecule of enzyme

are required for the formation of the air-stable species. Possible explanations for these conflicting results were presented earlier, and it was suggested that interpretation of the NADPH titration may be complicated by difficulties inherent in the aerobic nature of the experiment. Further studies under anaerobic conditions will be required to completely resolve this matter, and to exclude the possibility that electron carriers other than flavin are present on the enzyme. In the case of the detergent-solubilized enzyme, the results of the ferricyanide back titration are in agreement with those of Iyanagi and Mason (56) and are consistent with their conclusion that the air-stable form of the enzyme contains one fully oxidized flavin and one flavin semiquinone.

Since the enzyme contains both FMN and FAD, it is necessary to determine the role of each flavin in catalysis to understand in detail the mechanism by which the reductase transfers electrons from NADPH to suitable acceptors. This problem was approached by the study of the catalytic properties of enzyme from which FMN was removed. It was found that removal of FMN caused a corresponding decrease in enzyme activity in P-450, cytochrome c and DCPIP reduction. Incubation of the depleted enzyme with FMN restored a significant amount of P-450 and DCPIP reductase activities, and cytochrome c reductase activity was almost completely restored. On the other hand, it was found that removal of FMN

had no significant effect on the ability of the enzyme to reduce ferricyanide. From these results, it can be concluded that FMN is required for electron transfer to P-450, cytochrome c, and DCPIP, but is not required for electron transfer to ferricyanide. The ability of the FMN-depleted enzyme to reduce ferricyanide suggests that electrons from NADPH may enter the enzyme without participation of FMN. Therefore, the possibility may be considered that the two flavins have distinct roles in catalysis by the enzyme as follows:



Electrons enter FAD and must be transferred to FMN before acceptors such as P-450, cytochrome c, and DCPIP can be reduced. Ferricyanide, on the other hand, is capable of accepting electrons from enzyme containing only FAD; however, at the present time, it is not possible to exclude participation of FMN in the reaction of the native enzyme with ferricyanide. Demonstration of transhydrogenase activity by the FMN-depleted enzyme would lend further support to the postulated role of FAD as the site for the reaction of the enzyme with reduced pyridine nucleotide, however, these studies have not yet been performed.

The proposed scheme for transfer of electrons by the reductase implies that the two flavins are not functionally

equivalent and also that they must interact. Studies by Iyanagi et al. (113) on the trypsin-solubilized reductase provide additional evidence that the two flavins are not equivalent, since they have different oxidation-reduction potentials. On the basis of potentiometric and spectrophotometric titrations and a computer fit of the data, midpoint potentials were assigned as follows:

(1)	$F_1 \rightleftharpoons F_1H\cdot$	E = -0.110 V
(2)	$F_1H\cdot \rightleftharpoons F_1H_2$	E = -0.270 V
(3)	$F_2 \rightleftharpoons F_2H\cdot$	E = -0.290 V
(4)	$F_2H\cdot \rightleftharpoons F_2H_2$	E = -0.365 V

The data did not provide information as to the identity of the two flavins, which are represented simply as F_1 and F_2, designated by the order in which they are titrated. If these data are considered in conjunction with the scheme for intramolecular transfer proposed above, it might be considered that F_1, or the first flavin to be reduced, represents FAD, and that the lower potential flavin, F_2, represents FMN. Since the oxidation-reduction potential for reactions (2) and (3) are similar, electron transfer between the two flavins is thermodynamically possible.

Studies on the sulfite reductase flavoprotein by Siegel and coworkers (115,116) give precedent to the idea that one flavin of a two-flavin pair may serve as an "acceptor" for electrons from reduced pyridine nucleotide

from which they are transferred to the second or "donor" flavin, and subsequently to the substrate serving as the final electron acceptor. The sulfite reductase flavoprotein also contains FMN and FAD, and functions in electron transfer in bacteria (134). The enzyme also reduces several nonphysiological electron acceptors such as cytochrome c, ferricyanide, and DCPIP. However, in this case, the FMN-depleted enzyme shares only a transhydrogenase-type activity in common with the native enzyme; ferricyanide, as well as the physiological and other electron acceptors are reduced via an FMN-dependent pathway (116). The experiments presented by these authors provide evidence for a sequence of intramolecular transfer which involves the initial reaction of reduced pyridine nucleotide with FAD, and subsequent transfer of electrons to the oxidized acceptor via FMN.

The FMN-dependence of the P-450 reductase-catalyzed reactions with certain electron acceptors could be explained if electron transfer from FAD to FMN permitted the direct involvement of partially or fully reduced FMN with acceptors, as implied by the proposed scheme presented earlier. It is also possible that FMN functions simply as an electron sink, and is required only for the intramolecular disproportionation of a two-electron reduced form of the enzyme to produce the semiquinone state, which could be the form required for reaction with acceptors such as cytochrome c and P-450.

Flavoproteins also function as electron carriers in electron transfer systems similar to the NADPH-dependent hydroxylating system of liver microsomes. The flavin content of some of these reductases and their respective electron acceptors are summarized in Table XI. In each of these systems the overall transfer of two electrons from reduced pyridine nucleotide to the terminal electron acceptor is required for the oxidation of one molecule of substrate. It is apparent that of this group of flavoproteins, the P-450 reductase is unusual in that it contains two mol of flavin per mol of enzyme, and is also the only reductase which catalyzes electron transfer from reduced pyridine nucleotide directly to the terminal oxidase. In the other systems, the flavoprotein reduces an intermediate one-electron acceptor such as putidaredoxin, rubredoxin, or cytochrome b_5. The current understanding of the bacterial P-450-containing enzyme system indicates that the final transfer of electrons to the terminal oxidase requires two successive one-electron transfer steps, performed in this case by the reduced iron-sulfur protein, putidaredoxin (141). It is tempting to speculate that the unique properties of a multiflavin enzyme provide an alternate mechanism for the two one-electron transfer steps which are apparently carried out in other systems by additional electron carriers. As previously discussed by Hemmerich *et al.* (142), flavin interaction via semiquinone formation in a two-flavin enzyme

TABLE XI

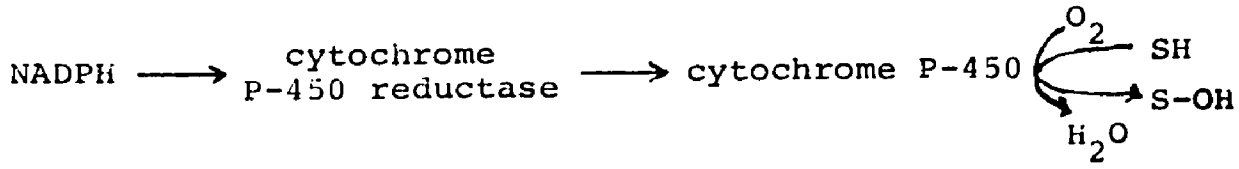
COMPARISON OF PROPERTIES OF FLAVOPROTEINS FUNCTIONING IN ELECTRON TRANSFER

Flavoprotein	Source and Function of Electron Transfer System	Molecular Weight	Flavin Content	Nature of Electron Acceptor	Terminal Oxidase
NADPH-cytochrome P-450 reductase	Liver microsomes; hydroxylation of fatty acids, steroids, drugs and other foreign compounds (15,29)	76,000	1 FAD 1 FMN	P-450	P-450
NADPH-adrenodoxin reductase (135)	Adrenal mitochondria; 11β-hydroxylation of steroids (7,8)	54,000	1 FAD	Iron-sulfur protein	P-450
NADH-putidaredoxin reductase (136)	Pseudomonas putida; camphor hydroxylation (6)	43,500	1 FAD	Iron-sulfur protein	P-450
NADH-cytochrome b_5 reductase (60)	Liver microsomes; fatty acid desaturation (120,121,137)	43,000	1 FAD	Cytochrome b_5	Fatty acid desaturase (nonheme iron protein)
NADH-rubredoxin reductase (140)	Pseudomonas oleovorans; ω-hydroxylation of fatty acids and hydrocarbons (138,139)	55,000	1 FAD	Iron-sulfur protein	ω-hydroxylase (nonheme iron protein)

would provide a simple mechanism for the successive transfer of two electrons at the same potential. The results of the studies on the FMN-depleted P-450 reductase are consistent with a mechanism of electron transfer which involves interaction of the two flavins. It is hoped that the availability of purified enzyme will allow further studies to test the validity of the proposed mechanism.

SUMMARY

NADPH-cytochrome P-450 reductase was obtained in highly purified form from phenobarbital-induced rat liver microsomes. The enzyme was solubilized by treatment of the microsomes with the nonionic detergent, Renex 690, and purified by column chromatography in the presence of detergents and glycerol. The enzyme functions in electron transfer in the NADPH-dependent hydroxylating system of liver microsomes as follows:



where S represents substrate.

P-450 reductase activity was determined by NADPH oxidation in a reconstituted enzyme system containing purified $P\text{-}450LM_2$ and phospholipid, with benzphetamine as the substrate and the reductase as the rate-limiting component.

Evidence was obtained for the existence of two forms of the reductase in rat liver microsomes. The two forms were obtained relatively free of cross contamination by different purification procedures, and are distinguished by a difference in apparent molecular weight of 2000, as

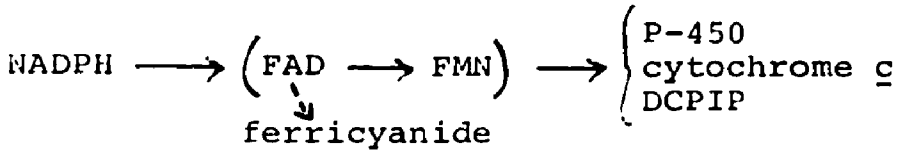
determined by their relative mobility when submitted to SDS-polyacrylamide gel electrophoresis. The minimal molecular weights of the two forms are 76,000 and 78,000. The flavin content and spectral properties of both forms are similar and both have similar activities when P-450LM$_2$ is used as the electron acceptor with benzphetamine as the substrate. The experimental results suggest that both forms of the enzyme are present in approximately equal amounts in microsomes from untreated and phenobarbital-pretreated rats. The 76,000 molecular weight form was characterized in further detail.

The enzyme contains one molecule of FMN and FAD per polypeptide chain, and has the spectral properties of a simple flavoprotein. The oxidized spectrum is characterized by absorbance maxima at 276, 384, and 456 nm, with a shoulder at 485 nm, and the extinction coefficient for enzyme absorbance at 456 nm is 21.2 mM^{-1} cm^{-1}. The spectra observed following addition of NADPH to oxidized enzyme under aerobic conditions were characterized by the long wavelength absorbance typical of a neutral flavin semiquinone. It was found that the enzyme may exist in a partially reduced semiquinone form that is stable in air.

In addition to P-450, the enzyme catalyzes the reduction of detergent-solubilized liver microsomal cytochrome b_5, as well as the reduction of the nonphysiological electron acceptors cytochrome c, ferricyanide, DCPIP, menadione,

nitro blue tetrazolium, and 3-AcPyADP. Reductase activity with P-450, cytochrome b_5, and nitro blue tetrazolium is enhanced in the presence of phospholipid. The enzyme is quite specific for NADPH as the electron donor; The K_m of NADH was nearly 10,000-fold higher than that of NADPH. NADP was found to inhibit reductase activity and this inhibition was competitive with respect to NADPH.

FMN was selectively removed from the enzyme by dialysis against 2 M KBr at pH 8.5 or by irradiation in the presence of 40% ammonium sulfate. Removal of FMN caused a loss of enzyme activity in P-450, cytochrome c, and DCPIP reduction. Incubation of the depleted enzyme with FMN restored a significant amount of P-450 and DCPIP reductase activity, and cytochrome c reductase activity was almost completely restored. Removal of FMN did not alter the ability of the enzyme to reduce ferricyanide. These findings are consistent with a scheme for electron transfer in which FMN and FAD have distinct roles in overall catalysis by the enzyme as follows:



The proposed mechanism involves interaction of FAD and FMN for the transfer of electrons from NADPH to electron acceptors such as P-450; ferricyanide reduction may occur via an FMN-independent pathway.

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