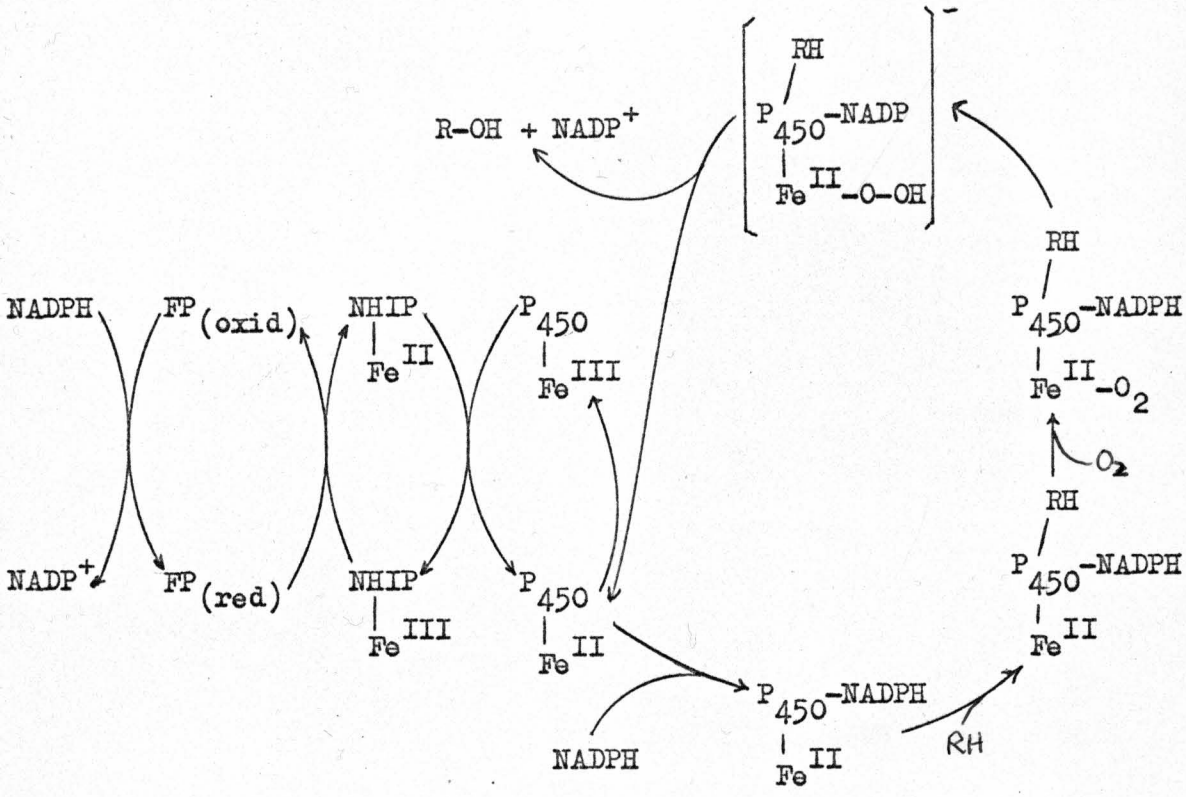
ENZYMATIC MECHANISM OF STEROID HYDROXYLATION

by Yun-Yen Tsong

(Under the supervision of Professor Charles J. Sih)

Based on analog, kinetic and stereochemical studies, a new mechanism for steroid hydroxylation is proposed which may be portrayed as shown below.



Inherent in this mechanism is the dual function of NADPH. NADPH serves in an accessory capacity keeping the autooxilizable P_{450} in the reduced state via the NADPH-Cytochrome-P_{450} reduction system, a sequence which is

steroid independent. Secondly, NADPH is directly involved in the steroid hydroxylation reaction to generate the highly reactive hydroperoxo complex, $[Fe^{II}-O-OH]^-$. There is no overall volume change of the P_{450} on completion of the stoichiometric cycle of the reaction. The alternative mechanism involving oxidation of Cytochrome-P_{450} during hydroxylation is difficult to reconcile with the experimental results herein described.

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Professor of Pharmaceutical Biochemistry

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by

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INTRODUCTION

Oxygen is one of the most abundant elements on earth and is essential for all forms of life. Therefore the mechanism by which molecular oxygen is utilized by living organisms has been a popular subject among biochemists. Enzymes which incorporate molecular oxygen into substrates have been termed oxygenases (1). These enzymes are ubiquitously distributed in nature since they have been found in animal tissues, plants and microorganisms. For convenience oxygenases have been divided into two groups. Those which catalyze the incorporation of one atom of molecular oxygen into substrates are termed monooxygenases (1) or mixed function oxidases (2) while those enzymes which incorporate two atoms of oxygen per molecule of organic substrates are referred to as diooxygenases according to the following reactions:

$$\text{Sub} + O_2 \longrightarrow \text{Sub-}O_2$$

Diooxygenases

$$\text{Sub} + O_2 + H_2A \longrightarrow \text{Sub-O} + H_2O + A$$

Monooxygenases

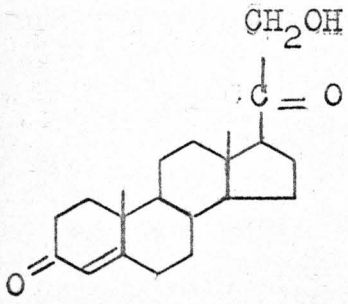
Here Sub is the substrate and H_2A is the reductant. The importance of oxygenases in cellular metabolism is evident

from the wide variety of substrates they attack such as carbohydrates, lipids, amino acids, vitamins, drugs, hormones, and so forth.

Mainly due to the efforts of Hayaishi and his coworkers (3,4) several diooxygenases have been obtained in crystalline form and the enzymatic mechanism has been studied in some detail. In contrast, very little is known concerning the enzymatic mechanism of monooxygenases primarily because of the complexity of the system, their particulate nature and their relative instability. However, in recent years considerable progress has been made with the steroid 11β-hydroxylase system of adrenal cortex mitochondria with respect to the resolution of the enzyme components. Since steroid hydroxylases fall under the definition of monooxygenases or mixed function oxidases, we selected this enzyme system to serve as a probe into the mechanism of monooxygenases. The purpose of this investigation is to examine the steroid 11β-hydroxylase of beef adrenal cortex in relation to the functional role of the electron donor, H_2A, the nature of the so-called "active form of oxygen," when oxygen is activated by such enzymes, and the chemical events in enzymic oxygenation.

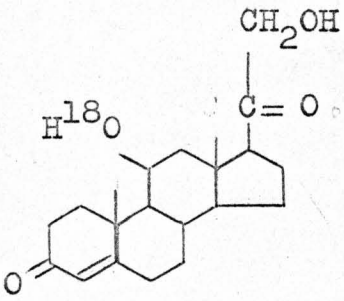
LITERATURE

The C-11β hydroxylation of desoxycorticosterone (DOC) by adrenal cortex mitochondria requires reduced triphosphopyridine nucleotide (NADPH) (5) as the electron donor, and incorporates ^{18}O from molecular oxygen into the substrate molecule, DOC (6). Although the stoichiometry for NADPH in the reaction has yet to be established, there is nearly a one to one correlation between oxygen consumed and DOC hydroxylated (7). Therefore, the steroid 11β-hydroxylase of adrenocortical mitochondria resembles classical mixed function oxidation.



Desoxycorticosterone

$$\text{NADPH} + \text{H}^+ + {}^{18}\text{O}_2 \longrightarrow$$



Corticosterone

$$+ \text{NADP}^+ + \text{H}_2\text{O}$$

Enzymatic hydroxylations of steroids have been found to proceed with retention of configuration (8-10) and appear to follow the rule of Bloom and Shull (11).

Therefore it is likely that enzymatic hydroxylation may be favored by the electrophilic nature of the displacing reagent such as OH^+ species. Although the requirement of several enzyme components for hydroxylation of steroids was first reported by Tomkins (12,13) and subsequently by others (14,15), the functional role of these components remained unknown until the elegant studies of Cooper, Estabrook and coworkers (16-18) on the one hand, and Kimura and coworkers (19-21) on the other. The mitochondrial 11β-hydroxylase has now been resolved into three components: Flavoprotein (Adrenodoxin-reductase), non-heme iron protein (NHIP) or Adrenodoxin, and hemoprotein (Cytochrome-P_{450}).

Adrenodoxin-reductase.--The enzymatic reduction of Adrenodoxin by NADPH is catalyzed by the enzyme, adrenodoxin-reductase. This enzyme is associated with mitochondrial particles and could be partially solubilized by sonification (18). The extremely low concentration of this enzyme in the adrenal cortex mitochondria has hampered its purification. From one kilogram of beef adrenal cortex, one could obtain approximately 4-5 mg of this flavoprotein with a specific activity of about 500 (mμmoles of dichlorophenol indophenol (DCPIP) reduced/min/mg protein) (18). The absorption spectrum of the most purified preparation is typical of a flavoprotein as characterized by the decrease in absorbance was maximal at 450 to 460 mμ upon reduction by dithionite. Adrenodoxin-reductase has a molecular weight of about

60,000 as determined by gel-filtration, and contains one mole of flavin adenine dinucleotide (FAD) as the prosthetic group, with no apparent metal component (21). Although either NADPH or NADH could serve as the electron donor, the rate of reduction of adrenodoxin with NADPH was about 400 times faster than with NADH. Attempts to replace the adrenodoxin-reductase by photosynthetic pyridine nucleotide reductase (22), or NADPH-cytochrome c reductase from pig liver (23) were unsuccessful, indicating that the NADPH-adrenodoxin reductase system is quite specific.

Adrenodoxin.--Adrenodoxin has been isolated from adrenal cortex mitochondria of beef and pig (17,21) and recently, this non-heme iron protein has also been purified from testis (24). Like all non-heme iron proteins, adrenodoxin binds tightly to diethylaminocellulose (DEAE), facilitating its isolation. The yield of adrenodoxin is about 7-15 mg from 1 kilogram of beef adrenal cortex (25). The purified brown adrenodoxin is homogeneous upon ultracentrifugation with a sedimentation constant (S-20, w) of 1.55S; it has a molecular weight of about 13-15,000 (26) and contains two atoms of iron and two moles of labile sulfide per mole of protein. The optical spectrum of oxidized adrenodoxin displays three broad absorption maxima at 455 mμ, 414 mμ, and 320 mμ with a small maximum at about 280 mμ. Upon reduction by NADPH and adrenodoxin reductase, the absorption in the visible spectrum is bleached about

50 per cent; the original oxidized spectrum can be slowly restored by the introduction of air, indicating that reduced form of adrenodoxin is autooxidizable. Electron spin resonance spectroscopy reveals that the reduced adrenodoxin has a prominent axially symmetric signal with g = 1.94 and g = 2.01. Substitution of iron and sulfur in adrenodoxin with isotopes Fe^{57} and S^{33}, it was shown that the signal at g = 1.94 is due to sulfur as a ligand of iron (27,28). Quantitative evaluation of the ESR signal by double integration indicates that about 50 per cent of the iron, as determined by chemical analysis is enzymatically reducible, although titrations of adrenodoxin with NADPH and adrenodoxin reductase or potentiometrically reveal that adrenodoxin is a 2 electron acceptor. The oxidation-reduction potential of adrenodoxin, E'_o at pH 7.4 is +164 mV. Optical rotatory dispersion spectra showed marked multiple Cotton effects with three distinct maxima. Characteristics of the principal effect are as follows: an inflection point at 440 mμ, a peak at 480 mμ, and a trough at 400 mμ. The amplitude is about 800 degrees and the sign is positive. Upon reduction the multiple effect is markedly changed. Circular dichroism studies of the oxidized and reduced non-heme iron proteins from adrenal cortex and spinach show remarkable similarities, indicating that the environment of the iron chromophores is essentially identical in both proteins (26). The changes in optical rotatory dispersion during the oxidation-reduction may be

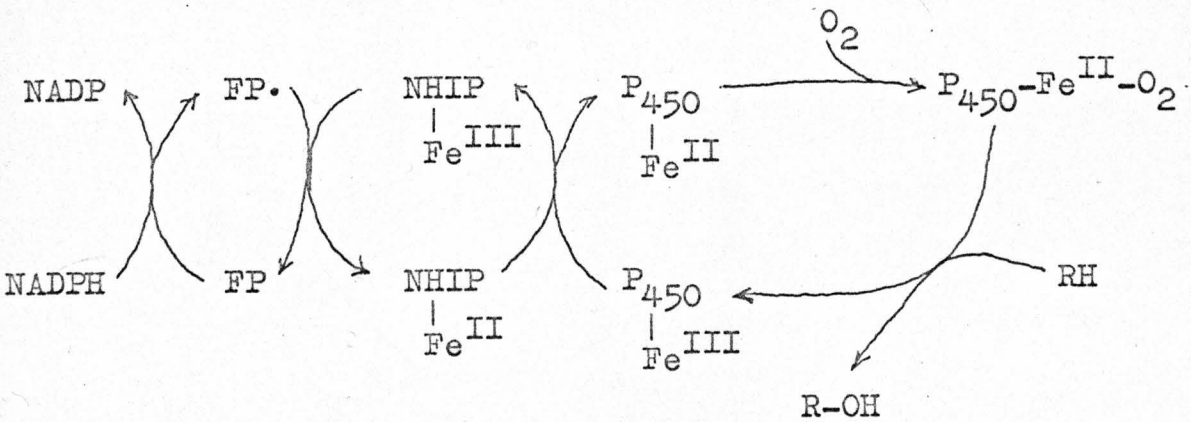
related to the catalytic function of the protein resulting from an alteration in the structure of the chromophore (21). The chemical structure of the iron environment has been examined by titration with p-chloromercuribenzoate (PCMB). Determination of the decolorization at 414 mμ, due to the decomposition of the iron coordination structure concomitant with the increment of absorbance increase at 250 mμ due to the formation of the PCMB mercaptide, indicates the presemce of 4 mercurial reactive groups per iron atom. This suggests the possible involvement of two cysteinyl sulfhydryl and one labile sulfide. Proton relaxation measurements (29) indicates the iron is relatively inaccessible to the water environment. Acid denaturation or PCMB treatment partially exposes the iron to water, but the enhancement of proton relaxation is still markedly less than ferric ion in solution. These results suggest that the iron atoms reside in the matrix of the protein coordinated by nitrogen and sulfur ligands with an absence of water molecules in the coordination sphere of the iron.

Cytochrome P_{450}.--This carbon monoxide-binding pigment was first demonstrated in liver microsomes (30,31) and subsequently in adrenal microsomes as the terminal oxidase in mixed function oxidative type reactions such as drug metabolism and steroid 21-hydroxylation (32). Harding, et al., (33-35), first showed that a type of Cytochrome-P_{450} also occurs in adrenal cortex mitochondria, where it may

serve as the terminal oxidase for the 11β-hydroxylation of DOC. Using the photochemical action spectrum technique of Warburg (36), the rate of conversion of DOC into corticosterone, in the presence of light was compared with the rates obtained in the darkness in the presence and absence of CO. It was observed that light reversal of the CO-inhibition was maximal at 450 mμ (37). Furthermore, half inhibition by CO of DOC hydroxylation occurred when $CO/O_2 = 1$. These findings led to the assignment of "Cytochrome-P_{450}" to the CO-binding pigment of adrenal cortex mitochondria as the oxygen-activating enzyme in 11β-hydroxylation of steroids. Cytochrome-P_{450} has resisted purification primarily due to its particulate nature and its facile conversion into an inactive form, "P_{420}", with an absorption maximum at 420 mμ under an atmosphere of CO. A variety of agents have been shown to catalyze the conversion of Cytochrome-P_{450} into P_{420}. These include: ureas, amides, ketones, nitriles, monohydric alcohols, guanidium salts, cholate, sodium dodecyl sulfate and phospholipase (38-40). However, P_{420} can now be converted back into P_{450} by treatment with polyols such as glycerol or reduced glutathione (41,42). Also, P_{450} can now be stabilized for more than one week in the presence of 20 per cent (v/v) glycerol. As a rule, two types of change in the difference spectrum of Cytochrome-P_{450} are produced by various substrates: "Type I" is characterized by a trough at about 420 mμ and a peak at about 388 mμ; "Type II"

is characterized by a peak at about 430 mμ and a trough at about 394 mμ (43,44). Upon the addition of DOC to adrenal cortex mitochondrial preparations, Type I spectral change was observed in the difference spectrum (45). These spectral shifts were explained in terms of an alteration in ligand binding to the heme of Cytochrome-P_{450} or allosteric transitions of the hemoprotein (46). Evidence seems to suggest that Cytochrome-P_{450} is largely in the oxidized form during the aerobic steady state of hydroxylation but no conclusive evidence is available as to whether DOC combines with the oxidized or reduced form of Cytochrome-P_{450}. The nature of the prothetic group of Cytochrome-P_{450} from adrenal cortico-mitochondria is unknown but protoporphyrin IX has been identified as the prosthetic group of Cytochrome-P_{450} from Pseudomonas (47).

On the basis of reconstitution experiments, the following roles of these three components of adrenal cortex mitochondria in electron transfer and steroid hydroxylation have been proposed (14,18,21) (Scheme I).

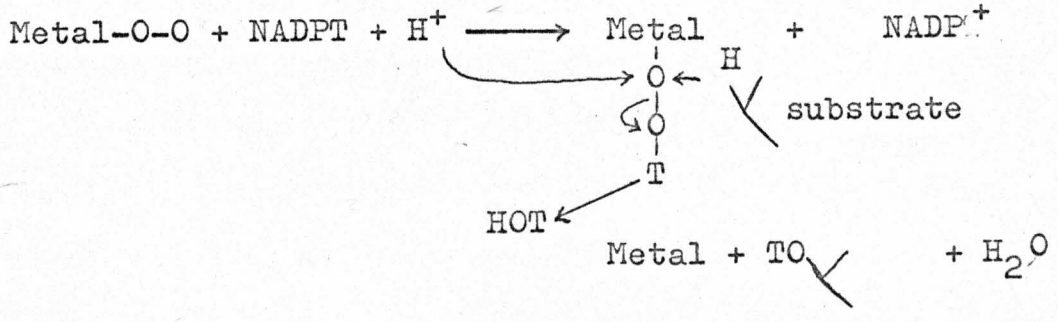


Scheme I. Currently accepted model for steroid hydroxylation

According to the above scheme, electrons from NADPH are transferred to Cytochrome-P_{450} through adrenodoxin reductase and adrenodoxin. The reduced Cytochrome-P_{450} thus generated reacts with DOC and molecular oxygen to yield an activated complex, DOC-Cytochrome-P_{450}-Fe^{II}-O_2, which is then converted into corticosterone and H_2O. In the hydroxylation process Cytochrome-P_{450}-Fe^{II} is oxidized to Cytochrome-P_{450}-Fe^{III}; the function of NADPH in this mechanism is <u>solely</u> to provide the reducing equivalents for Cytochrome-P_{450}-Fe^{III}.

The steroid 18-hydroxylase, cholesterol-20 and 22-hydroxylases also reside in the adrenal cortex mitochondria and have been shown to require the NADPH-Cytochrome-P_{450} reductase system for activity (14,48,49). However, the nature of Cytochrome-P_{450} among different hydroxylase appear to be different (49), even though mechanistically they may be identical.

An alternative enzymatic mechanism for steroid hydroxylation was proposed by Hayano (50) which may be portrayed as follows (Scheme II):



Scheme II. Hayano's hydroxylation mechanism

This model may be visualized as one where oxygen, NADPH and substrate are brought together to a single active site on the enzyme. The oxygen is activated by fixation to the metal followed by NADPH reduction generating a hydroperoxo complex. Finally, the species OH^+ displaces the H of the substrate to complete the formation of the hydroxylated steroid; the non-utilized atom of oxygen accepts the H of the substrate and together with a proton is liberated as water. Thus, the role of NADPH here is to generate the hydroperoxo complex of the oxygen activating enzyme. Although Kadis (51) reported that when microsomes of sow ovaries was incubated with progresterone and NADPT, substantial quantity (10 per cent) of the tritium label appeared on the hydrogen of the 17-hydroxyl group, in view of the fact that the hydroxyl proton at C-17 does undergo rapid exchange with the medium (52), this finding should be more critically examined.

The steroid C-21 hydroxylase of adrenal microsomes has been studied in considerable detail. The stoichiometry of the C-21 hydroxylation has been carefully established. It was found that 1 mole of oxygen and 1 mole of NADPH were consumed per mole of 17α-hydroxyprogesterone hydroxylated (53). The presence of a microsomal Cytochrome-P_{450} as the oxygen activating enzyme in C-21 hydroxylation was also established (54). However, there is no evidence supporting the presence of a non-heme iron component, similar to adrenodoxin, in microsomes. In fact, three components have

been separated from rabbit microsomal extract and shown to be required for the conversion of laurate to ω-hydroxy-laurate. These have been identified as Cytochrome-P_{450} (the CO-binding pigment of microsomes), NADPH-Cytochrome c reductase, and a heat stable factor which is ether extractable suggesting lipoidal in nature (55).

MATERIALS AND METHODS

Diethylaminoethyl cellulose (DEAE, 0.03 meq/gm); glycylglycine, horse heart Cytochrome c (type III), D-glucose-6-phosphate (disodium salt, hydrate, mol. wt. = 358), glucose-6-phosphate dehydrogenase (type X, from yeast), tris(hydroxymethyl)aminomethane (Sigma 7-9) were products of Sigma Chemical Company. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), and thionicotinamide adenine dinucleotide phosphate (thio-NADP) were purchased from the Boehringer Mannheim Corporation. Desoxycorticosterone (DOC) and corticosterone were obtained from the Mann Research Laboratories. D-glucose-1-^{3}H (specific activity = 3.46 curies/m mole), desoxycorticosterone-4-^{14}C (specific activity = 35 mc/m mole), and water-^{3}H (specific activity = 5 curies/g) were purchased from the New England Nuclear Corporation. Dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene and PPO (2,5-diphenyoxazole) were products of the Packard Instrument Company. Sephadex G-100 was obtained from the Pharmacia Company. 2,6-Dichlorophenol indophenol was a product of Fischer Scientific Company. Hydroxyapatite was purchased from the Clarkson Chemical Company (capacity = 93 mg bovine albumin adsorbed per gram of hydroxyapatite).

NADP was estimated enzymatically by the use of glucose-6-phosphate dehydrogenase (56), and NADPH was assayed by its absorbancy at 340 mμ ($\epsilon = 6.22 \times 10^6$). Adrenodoxin was measured by its absorbancy at 415 mμ. NADPH-adrenodoxin reductase activity was assayed by the reduction of 2,6-dichlorophenol indophenol at 600 mμ (18). Protein was determined by the Biuret method (57) or by the ratio of absorbancy at 260/280 mμ (58). The paper chromatographic system consisted of toluene-propylene glycol (59) with a 3 hour development. Under the condition, DOC has a R_f of 0.7 whereas corticosterone has a R_f value of 0.15. Radioactive chromatographic strips were quantitatively assayed by the Vanguard automatic scanner, equipped with a automatic data system. Radioactivity was determined in a Packard Tricarb, model 524. This counter has a counting efficiency of 72 per cent for carbon 14 and 22 per cent for tritium. The scintillation fluid contained 100 g of naphthalene, 10 g of PPO and 250 mg of dimethyl-POPOP per liter of dioxane.

Preparation of Tetrahydro-NADP and Hexahydro-NADP.--To a solution of barium chloride, maintained at 70°C was slowly added over a period of two hours an equivalent quantity of sodium carbonate as a 5 per cent solution. After decantation, the barium carbonate was collected and 37 per cent formaldehyde was added to an extent of 2 per cent of the barium carbonate by weight. An aqueous solution of

palladium chloride was then added until the weight of the added palladium is 1 per cent that of barium carbonate. After stirring the mixture for 16 hours at room temperature, the palladium suspended on barium carbonate was collected by filtration, washed with acetone, ether and dried under vacuum.

Commercial NADP was further purified by chromatography on Sephadex G-25; the purified fractions were collected and lyophilized. Catalytic hydrogenation of NADP was carried out under 1 atm of hydrogen pressure with a suspension of palladium-barium carbonate in water containing 100 mg of $(NH_4)_2SO_4$ per millimole of NADP at 0°C. The reaction was terminated when 2 moles of hydrogen per mole of NADP was consumed (20 hours). The yield of tetrahydro-NADP was nearly quantitative.

The hexahydro-NADP was prepared in a similar manner except that the reaction was terminated after the consumption of 3 moles of hydrogen per mole of substrate (72 hours at room temperature).

Either tetrahydro-NADP or hexahydro-NADP, after removal of the catalyst, was chromatographed over a DEAE-cellulose column (2.6 x 10 cm), equilibrated with 0.01 M glycylglycine buffer, pH 7.5. The column was eluted with a gradient consisting of 0.01 M glycylglycine and 0.6 M NaCl in 0.01 M glycylglycine. The emergence of tetrahydro-NADP was followed by the absorbancies at 291 mμ (ϵ = 13,600) and 263 mμ (ϵ = 16,300) while that of hexahydro-NADP was

detected by the absorbancy at 260 mμ (ϵ = 15,400). Fractions containing the desired tetrahydro- or hexahydro-NADP were combined, lyophilized and stored at 0°C. The contents of tetrahydro-NADP and hexahydro-NADP in lyophilized form were 0.127 μmole and 0.133 μmole per mg of lyophilized powder, respectively. See reference (60) for details.

Enzymatic Reduction of Thionicotinamide Adenine Dinucleotide Phosphate.--The reaction mixture contained 40 mg (30 μmoles, 60 per cent purity) of thio-NADP, 60 μmole of glucose-6-phosphate, 120 μmole of $MgCl_2$ and 0.3 mg of glucose-6-phosphate dehydrogenase in a total volume of 25 ml of 0.01 M glycylglycine buffer, pH = 7.5. Periodically, an aliquot (0.1 ml) of the reaction mixture was diluted tenfold and its absorbancy at 399 mμ was followed. The reaction was terminated when no further increase in absorbancy (1.278, 92 per cent completion) was noted.

The mixture was then chromatographed over a DEAE-cellulose column (2.2 x 12 cm). The column was eluted with a gradient system consisting of 250 ml of 0.01 M glycyl-glycine buffer, pH=7.5 and 250 ml of 0.01 M glycylglycine, pH = 7.5, containing 0.6 M NaCl and 6 ml fractions were collected. The presence of thio-NADPH was detected by the absorbancy at 399 mμ (AmM = 11.7) and 260 mμ (AmM = 15.8). Fractions (57-69) were combined and diluted with six volumes of 0.01 M glycylglycine and passed over a small DEAE-cellulose column (2 x 5 cm). Elution of the column with

0.4 M NaCl in 0.01 M glycylglycine, pH = 7.5 afforded thio-NADPH in fractions 6-12 (3 ml per fraction). These fractions were pooled and lyophilized to yield 316 mg (22 μmoles) of thio-NADPH. Under identical conditions, the rate of reduction of thio-NADP by glucose-6-phosphate dehydrogenase was 2.3 times faster than NADP (61).

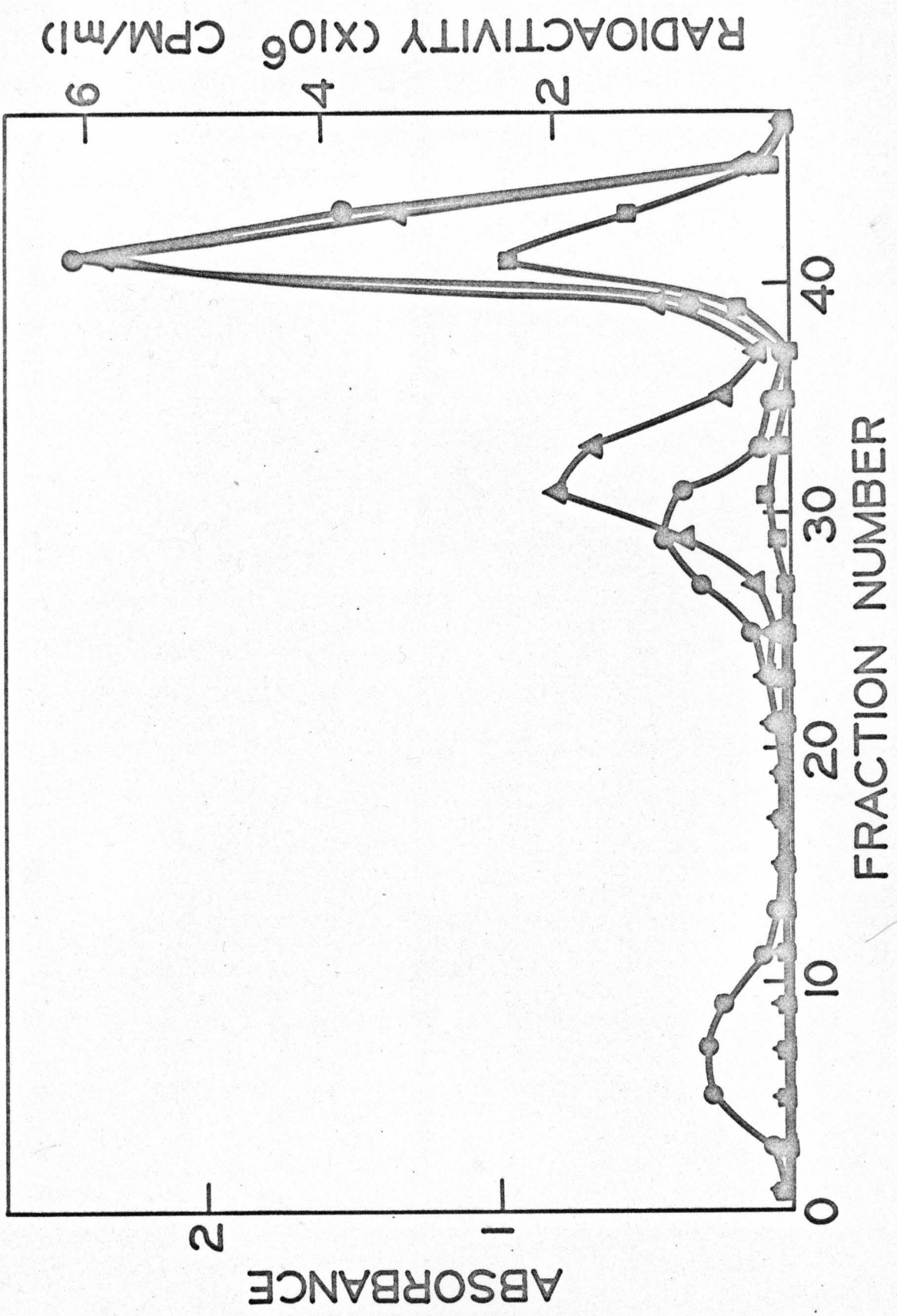
Preparation of $NADP^3H_B$.--The system contained 10 μmole of $MgCl_2$, 5 μmole of creatine phosphate, 5 μmole ATP, 1 μmole NADP, 0.5 mg hexokinase, 0.5 mg of creatine phosphokinase, 0.3 mg of glucose-6-phosphate dehydrogenase, and D-glucose-1-^{3}H (1 mc, 0.29 μmole) mixed with 0.41 μmole of non-radioactive glucose as carrier in a total volume of 5 ml of 0.05 M glycylglycine buffer, pH = 7.5. When the absorbance at 340 mμ reached a constant, the reaction was terminated. Under these conditions at 25°C the reaction is generally complete after 30 minutes (62). The reaction mixture was cooled to 0°C, diluted with 30 ml of distilled water, containing 1 μmole of NADPH as carrier and chromatographed over a DEAE-cellulose column (2.2 x 8.5 cm). The nucleotides were separated by linear gradient elution with sodium chloride. The mixing chamber contained 200 ml of 0.01 M glycylglycine, pH = 7.5 and the reservoir contained 200 ml of 0.6 M NaCl in 0.01 M glycylglycine buffer, pH = 7.5 (63). The flow rate was 1 ml per minute and 4 ml fractions were collected; all operations were performed at 4°C. $NADP^3H_B$ was detected by virtue of its absorbancy at

340 mμ and 260 mμ and by liquid scintillation analysis of the fractions (0.05 ml aliquots). Figure 1 shows the elution profile for NADP (fractions 27-37) and NADPH (fractions 39-45). Two other radioactive peaks were residual glucose-1-^{3}H (fractions 3-11) and glucose-6-phosphate-1-^{3}H (fractions 23-35). The recovery of radioactivity from the column was 65 per cent. Fractions 39-45 containing NADP^{3}H were combined and diluted with 6 volumes of 0.01 M glycylglycine buffer, pH = 7.5 and poured over a small DEAE-cellulose column (2 x 5 cm). The adsorbed NADP^3H_B was eluted from the column with 0.01 M glycylglycine, pH = 7.5 containing 0.3 M NaCl and 2 ml fractions were collected. The total recovery of NADP^3H_B was 65 per cent, with a specific activity of 2.36 x 10^7 cpm/μmole.

Preparation of [4-$^3H_2$]-NADPH.--NADP (50 mg) and sodium bicarbonate (36 mg) were placed in the main compartment of a Warburg flask and sodium dithionite (25 mg) was placed in the side arm. Tritiated water (1 ml, 5 curies) was added to the main compartment; then, after putting in a 25° bath, the apparatus was flushed with carbon dioxide. The sodium dithionite was washed into the main compartment to give a yellow solution. After carbon dioxide had stopped being evolved (about 30 minutes) the contents of the flask were removed, diluted with a further 1 ml of water and freeze dried. The lyophilized material was dissolved in water (2 ml) and acetone (30 ml) at -15° was added to give a

Figure 1. Chromatographic separation of NADP$^{3}H_{B}$ from reaction mixture on DEAE-cellulose column.

The details of isolation are described in the text. -O-O- = radioactivity cpm/ml; -△-△- = absorbancy at 260 mμ; -□-□- = absorbancy at 340 mμ. Fractions (3-11) = unreacted glucose-1-^{3}H; (23-35) = residual glucose-6-phosphate-1-^{3}H; (39-45) = NADP$^{3}H_{B}$.


ABSORBANCE
2
1
0
10
20
30
40
FRACTION NUMBER
RADIOACTIVITY (x10⁶ CPM/ml)
2
4
6

white precipitate. After keeping at -15° for 45 minutes, the solution was centrifuged at 10,000 g for 10 minutes at -15°. The acetone was decanted and the sediment was allowed to dry in the air. The solid was dissolved in 0.01 M potassium bicarbonate (5 ml) and freeze dried. The lyophilized material was dissolved in water (5 ml) and again freeze dried. The final product (sp. act. = 4.77 μc/μmole) was 80 per cent pure when measured spectrophotometrically (E_{260}/E_{340} = 3.1).

Comments.--The time taken between adding the sodium dithionite to the NADP and the precipitation of the NADPH from acetone must be kept to a minimum to prevent decomposition of the NADPH.

The freeze drying of the NADPH from 0.1 M potassium bicarbonate results in the NADPH having good keeping qualities when stored as the solid at -15°. No decomposition could be detected after 3 months.

Preparation of [4-^{3}H]-NADP.--The synthesis of [4-^{3}H]-NADP involves the oxidation of [4-$^3H_2$]-NADPH by glutathione reductase via the following reaction:

$$\text{NADPH} + \text{GS-SG} \longrightarrow 2\text{GSH} + \text{NADP}$$

Glutathione reductase (0.12 mg), GS-SG (17.3 mg) and [4-$^3H_2$]-NADPH (90.6 mg, sp. act. = 4.79 μc/μmole) were dissolved in 62 ml of 0.001 M phosphate buffer, pH = 7.5 in a 250 ml Erlenmeyer flask. The absorbance at 340 mμ at

time zero was 2.218, and the reaction was terminated when the absorbance at 340 mμ remained constant (0.119; 95 per cent completion). The [4-^{3}H]-NADP was not isolated but was cooled and used directly for the synthesis of NADP^3H_A after boiling the reaction mixture.

Enzymatic Synthesis of NADP^3H_A.--To the above glutathione reductase mixture was added 50 μmoles of $MgCl_2$; 50 μmoles of glucose-6-phosphate and 0.3 mg of glucose-6-phosphate dehydrogenase. The absorbance at 340 mμ at the beginning of the reaction was 0.121. When the absorbance at 340 mμ reached 2.03 (94 per cent completion, 19.65 μmoles NADPH), the reaction mixture was diluted with 64 ml of 0.01 M glycylglycine buffer, pH = 7.5 and passed over a DEAE-cellulose column (2.6 x 8 cm), equilibrated with the same buffer. The column was eluted with a gradient system consisting of 500 ml of 0.01 M glycylglycine, pH = 7.5 containing 0.6 M NaCl in the reservoir flask and 500 ml of the same buffer without NaCl in the mixing chamber and 10 ml fractions were collected.

Figure 2 shows the chromatographic pattern. NADP^3H_A resided in fractions 34-42; these were combined, diluted with six volumes of 0.01 M glycylglycine buffer, pH = 7.5 and poured onto a small DEAE-cellulose column (2.2 x 11 cm). NADP^3H_A was eluted from the column with 0.3 M NaCl in 0.01 M glycylglycine buffer, pH = 7.5. A total of 16.9 μmoles of NADP^3H_A with a specific activity of 4.45×10^5 cpm/μmole were obtained.

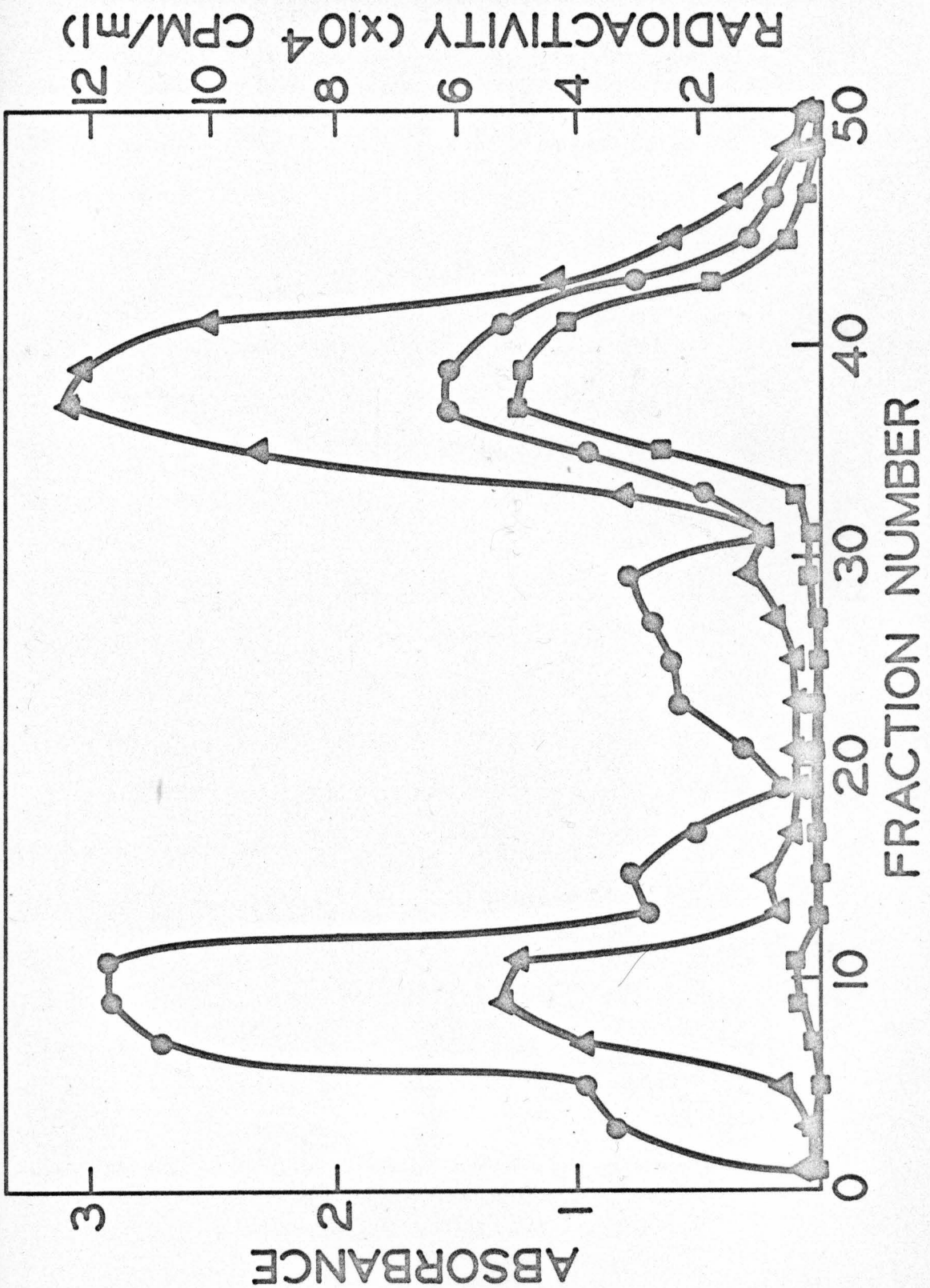
Figure 2. Chromatographic separation of NADP$^{3}H_{A}$ from reaction mixture.

The details are described in the text.

-O-O- = radioactivity cpm/ml;

-△-△- = absorbancy at 260 mμ;

-□-□- = absorbancy at 340 mμ.


ABSORBANCE
0
1
2
3
FRACTION NUMBER
0
10
20
30
40
50
RADIOACTIVITY (x10⁴ CPM/ml)
2
4
6
8
10
12

Assay for Flavoprotein (FP).--The reaction mixture contained 0.1 ml of a 2,6-dichlorophenol indophenol (DCPIP) solution (3 mg/ml), 0.02 ml of a 10 mM solution of NADPH and flavoprotein in a total volume of 1 ml of 0.1 M phosphate buffer, pH = 7.5. The decrease in optical density at 590 mμ during the 30 to 90-second interval was taken as a measure of the initial velocity (this portion of the curve obeyed zero-order kinetics). One flavoprotein unit is that amount of enzyme which catalyzes the reduction of 1 μmole per minute of DCPIP at 590 mμ, using a millimolar extinction coefficient of 19 (64).

NADPH-Cytochrome c Assay for Flavoprotein (FP) or Adrenodoxin (NHIP).--The system consisted of 0.8 mg of Cytochrome c, 0.3 mg of NADPH and aliquots of FP and NHIP in a total volume of 1 ml. When assay of FP is desired, excess NHIP is added or vice versa. The rate of Cytochrome c reduction was measured at 550 mμ on a Gilford spectrophotometer. The initial velocity of reduction of Cytochrome c is expressed as μmoles of Cytochrome c reduced per minute using a millimolar extinction coefficient of 19.1 (65).

Assay for 11β-hydroxylase Activity.--Unless otherwise stated the assay mixture contained the following: 20 μg of DOC containing 0.12 μc of 4-C^{14}-DOC in 0.02 ml of dimethylformamide; 5 μmoles of $MgCl_2$; 2.4 μmoles of NADPH; 0.2 mg of flavoprotein (Y_2 fraction); 0.2 mg of NHIP (R_1 fraction);

and 5 mg of P_{450} (P_2) in a total volume of 3 ml of 0.1 M phosphate buffer, pH = 7.5. The reaction mixture was incubated at 37°C with constant stirring. After 30 minutes, the reaction mixture was extracted with three 2.5 ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness, spotted on a strip of Whatman No. 1 paper, and developed for 3 hours in the toluene propylene glycol system (59). The radioactive chromatographic strips were quantitatively assayed using the Vanguard Automatic Scanner, equipped with an automatic data system. Figure 3 shows the hydroxylase activity vs time while Figure 4 illustrates the hydroxylation activity as a linear function of P_{450} concentration.

Purification of the 11β-Hydroxylase Components.--The Y_2-fraction of adrenodoxin reductase (FP), R_1-fraction of adrenodoxin (NHIP) and P_2-fraction of P_{450}, were isolated according to the method of Omura, et al. (18).

(1) Further purification of adrenodoxin-reductase (Y_2).--Lyophilized Y_2FP (23 mg, specific activity = 0.42 DCPIP units/mg protein) was dissolved in 20 ml of 0.005 M phosphate buffer and applied onto a DEAE-cellulose column (2.2 x 10 cm). The column was eluted with a linear gradient consisting of 350 ml of 0.01 M phosphate buffer, pH = 7.5 in the mixing chamber and 350 ml of 0.05 M phosphate buffer, pH = 7.5 in the reservoir and 9 ml fractions were collected. FP was assayed by the DCPIP reduction method. Figure 5

Figure 3. Hydroxylation as a function of time.

The system contained 20 μg of DOC (0.12 μc) in 0.02 ml of DMF; 5 μmole of $MgCl_2$; 2.4 μmole of NADPH; 2 mg of P_{450} (P_2); 0.2 mg of NHIP (R_1) and 0.2 mg FP (Y_2) in a total volume of 3 ml of 0.1 M phosphate buffer, pH = 7.5. The incubation period was varied as indicated.

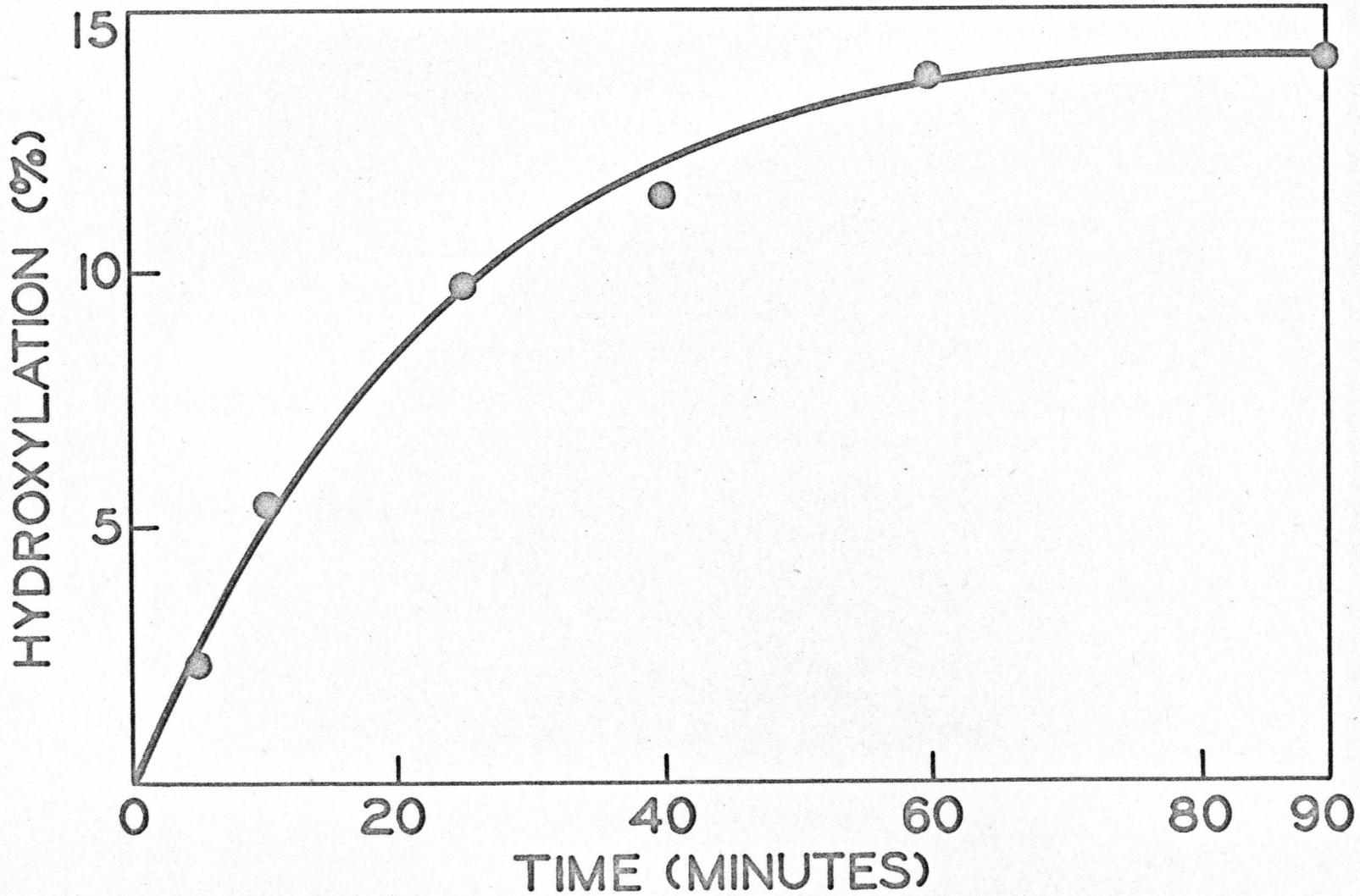

HYDROXYLATION (%)
15
10
5
0
20
40
60
80
90
TIME (MINUTES)

Figure 4. Linear relationship between hydroxylation and P_{450} concentration.

The incubation mixture contained 20 µg of DOC (0.12 µc); 5 µmole of $MgCl_2$; 2.4 µmoles of NADPH; 0.2 mg of FP (Y_2); 0.2 mg of NHIP (R_1) and varying amounts of P_{450} (P_2) as indicated in a total volume of 3 ml. After 30 minutes of incubation at 37°C, the mixture was assayed by the radiochromatographic method as described in the text.

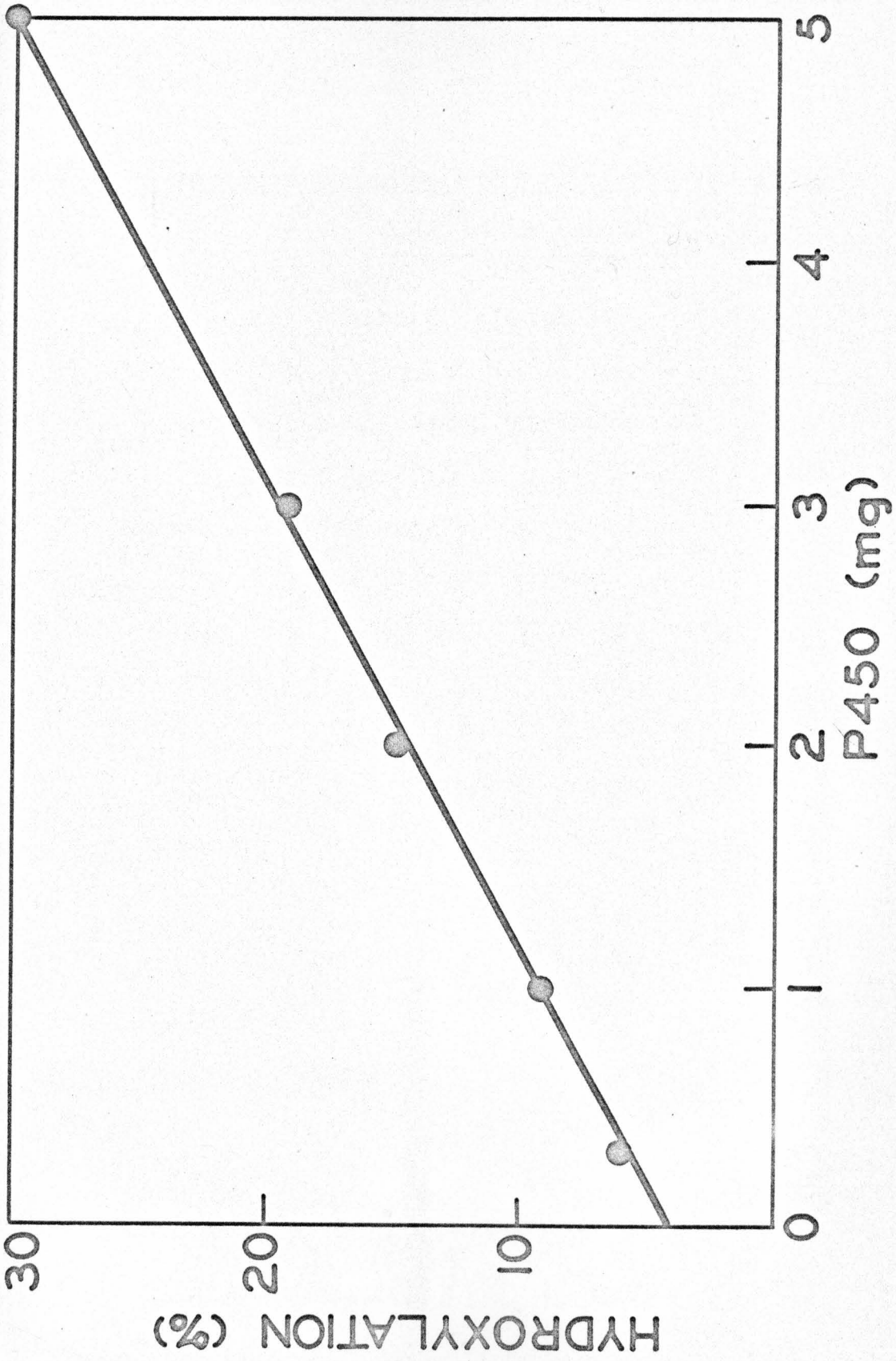
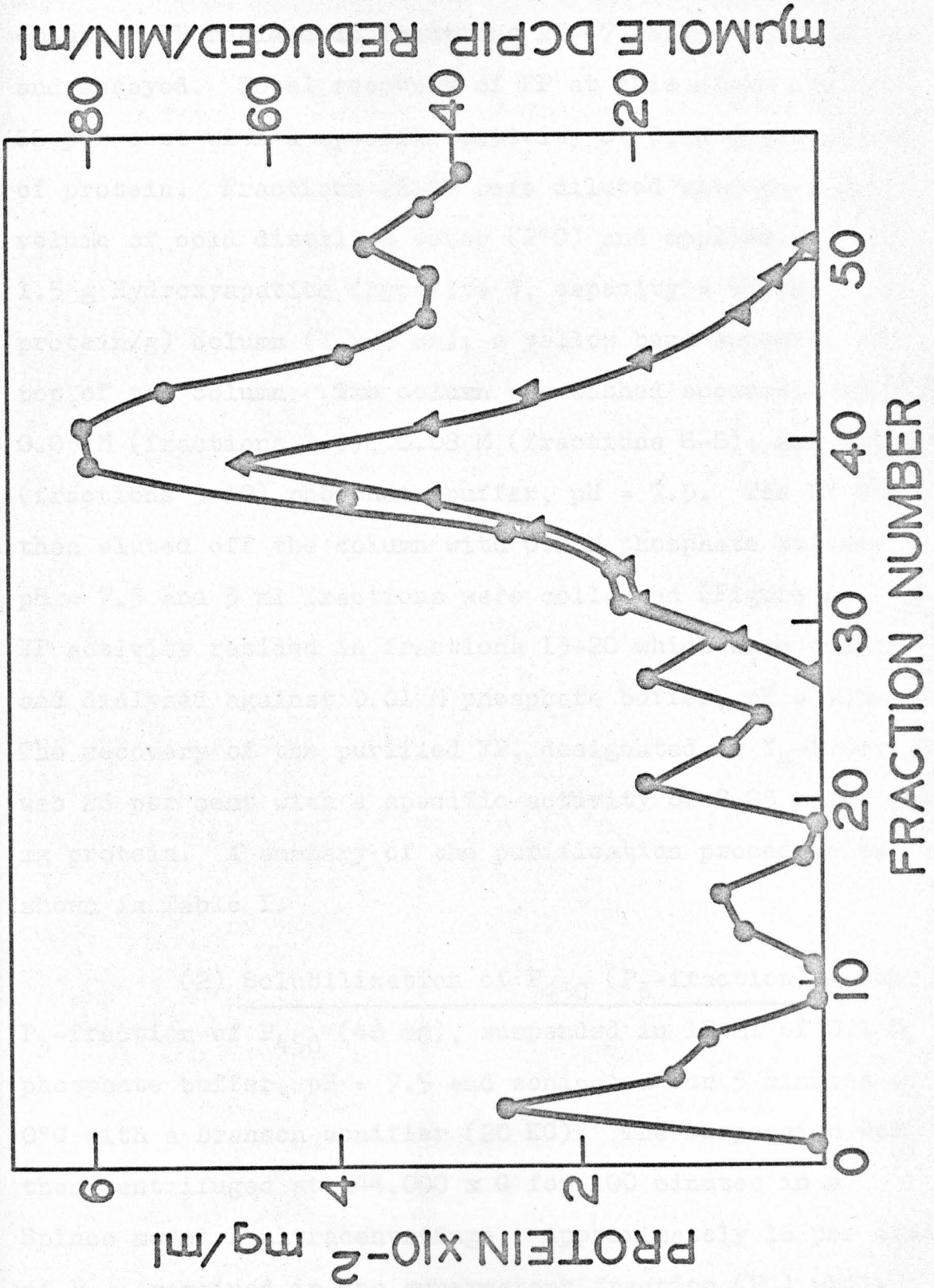

HYDROXYLATION (%)
30
20
10
0
1
2
3
4
5
P450 (mg)

Figure 5. DEAE-cellulose chromatography of the Y_2-fraction of FP.

Protein was estimated by the ratio of absorbancies at 280 and 260 mμ. Adrenodoxin reductase activity was measured via DCPIP reduction. -O-O- = protein; -Δ-Δ- = adrenodoxin reductase (FP) activity.


PROTEIN x10-2 mg/ml
0
2
4
6
FRACTION NUMBER
10
20
30
40
50
mμMOLE DCPIP REDUCED/MIN/ml
20
40
60
80

shows the elution profile of FP from the DEAE-cellulose column. FP resided in fractions 28-47 which were combined and assayed. Total recovery of FP at this stage (Y_3) was 66 per cent with a specific activity of 0.79 units per mg of protein. Fractions 28-47 were diluted with an equal volume of cold distilled water (2°C) and applied onto a 1.5 g Hydroxyapatite (hypatite C, capacity = 93 mg protein/g) column (1 x 3 cm); a yellow band appeared on top of the column. The column was washed successively with 0.05 M (fractions 1-5); 0.08 M (fractions 6-8); and 0.1 M (fractions 9-12) phosphate buffer, pH = 7.5. The FP was then eluted off the column with 0.2 M phosphate buffer, pH = 7.5 and 3 ml fractions were collected (Figure 6). The FP activity resided in fractions 13-20 which were combined and dialyzed against 0.01 M phosphate buffer, pH = 7.5. The recovery of the purified FP, designated as Y_4-fraction was 26 per cent with a specific activity of 2.95 units per mg protein. A summary of the purification procedure is shown in Table I.

(2) Solubilization of P_{450} (P_3-fraction).--The P_3-fraction of P_{450} (48 mg), suspended in 12 ml of 0.1 M phosphate buffer, pH = 7.5 and sonicated for 5 minutes at 0°C with a Branson sonifier (20 KC). The suspension was then centrifuged at 144,000 x G for 100 minutes in a Spinco model L ultracentrifuge. Approximately 16 per cent of P_{450} remained in the supernatant fraction (P_4) while

Figure 6. Chromatography of FP (Y_3-fraction) on hydroxyapatite.

Protein and adrenodoxin-reductase activity were assayed as in Figure 5. The column was successively eluted with 0.05 M (fractions 1-5); 0.08 M (fractions 6-8); 0.1 M (fractions 9-12); and 0.2 M (fractions 13-20) phosphate buffer, pH = 7.5. -O-O- = protein; -Δ-Δ- = adrenodoxin reductase activity (FP).

PROTEIN x10-2 mg/ml
12
10
8
6
4
2
1
3
5
7
9
11
13
15
17
19
FRACTION NUMBER
1
2
3
x10-2 mμMOLE DCPIP REDUCED/MIN/ml

TABLE I

Further Purification of Adrenodoxin Reductase

(Y_2-Fraction)

	Total protein (mg)	Specific activity units/mg protein	Total activity (units)	Recovery to activity (%)
Lyophilized FP (Y_2)	23.6	0.42	9.96	100
DEAE-cellulose column (Y_3)	8.2	0.79	6.48	66
Hydroxyapatite (Y_4)	0.85	2.95	2.51	26

71 per cent of P_{450} appeared in the precipitate. A summary of the procedure is shown in Table II.

Procedure for the Determination of the Stereochemistry of NADPH.--In all cases, the following method was followed: The reaction mixture (3 ml) was diluted with 32 ml of 0.005 M glycylglycine buffer, pH = 7.5 and applied onto a DEAE-cellulose column (2.2 x 8.5 cm) which had been equilibrated with 0.01 M glycylglycine buffer, pH = 7.5. The column was eluted with a gradient system comprising 200 ml of 0.01 M glycylglycine buffer, pH = 7.5, in the mixing chamber and 200 ml of 0.6 M NaCl in the same buffer in the reservoir flask. The flow rate was 1 ml per minute and 4 ml fractions were collected. The combined fractions of NADP and NADPH were diluted again with 6 volumes of 0.01 M glycylglycine buffer, pH = 7.5 and chromatographed over a second DEAE-cellulose column (2.2 x 6.5 cm). The column was eluted with a gradient system consisting of 120 ml of 0.01 M glycylglycine buffer, pH = 7.5 and 120 ml of the same buffer containing 0.6 M NaCl. Fractions containing NADP or NADPH were pooled and the radioactive specific activities were determined.

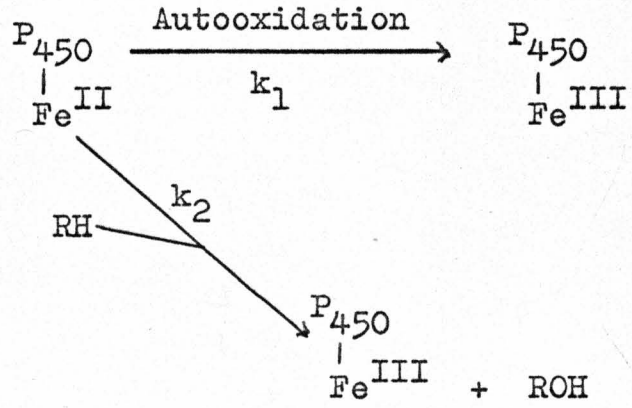
TABLE II

Solubilization of P_{450} by Sonication

	Total protein (mg)	Total activity umoles of DOC hydroxylated	Specific activity umoles of DOC hydroxylated per mg of P_{450} protein	Recovery of activity (%)
P_{450}, P_3-fraction	39	1.078	0.028	--
P_{450}, (precipitate P_4-fraction) After P_3 sonicated for 5 min.	30	0.760	0.025	71
P_{450}, (supernatant P_4-fraction) After P_3 sonicated for 5 min.	5.0	0.168	0.033	16

RESULTS AND DISCUSSION

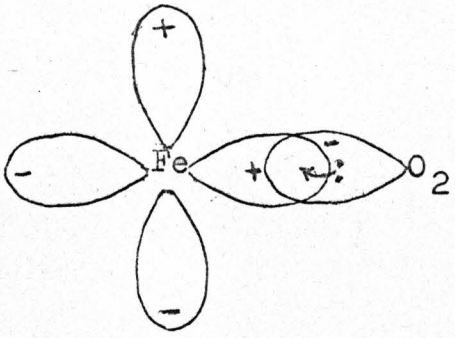
The stoichiometry of oxygen consumption, NADPH utilization and product formation has been shown to be 1:1:1 in some P_{450}-containing mixed function oxidases (66,67). As considerable endogenous NADPH oxidation occurs in the absence of substrates, the stoichiometry for NADPH was obtained by a difference in the amount of NADPH utilized in the presence and absence of substrates. On addition of substrate, a rapid disappearance of NADPH occurs, indicating that there should be little autooxidation of P_{450}-Fe^{II} in the presence of substrate (RH) or $k_2 \gg k_1$.



Therefore, if the model as illustrated in Scheme I is operational, the stoichiometry of the reaction should be proportional to the total amount of NADPH consumed from the time of substrate addition rather than due to a difference

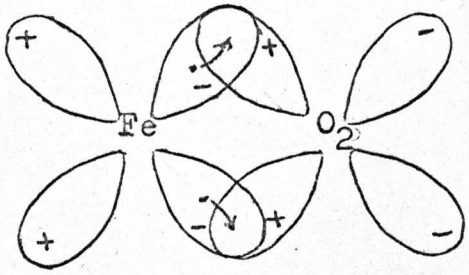
in the amount of NADPH utilized at various times, t, as proposed.

Another cogent objection to Scheme I is the postulation of the porferryl ion, $Fe^{II}-O_2$ as the so-called "active oxygen" for it is known that in general oxygen complexes of d_6 transition metals are kinetically stable towards oxidation of unactivated carbon-hydrogen bonds. This is because in $Fe^{II}-O_2$, both σ and π bonding occur. In the σ bond the ligand acts as a Lewis base and shares a pair of electrons with an empty e_g ($dx^2 - y^2$) orbital while in the π bond, O_2 acts as a Lewis acid and accepts electrons from the filled t_{2g} orbital of the iron (d_{xy} orbital). This bonding and "back-bonding" strengthens the metal-ligand and contributes to the unusual stability of the $Fe^{II}-O_2$ complex.



σ bond

$Fe-O_2$



π bond

FeO_2

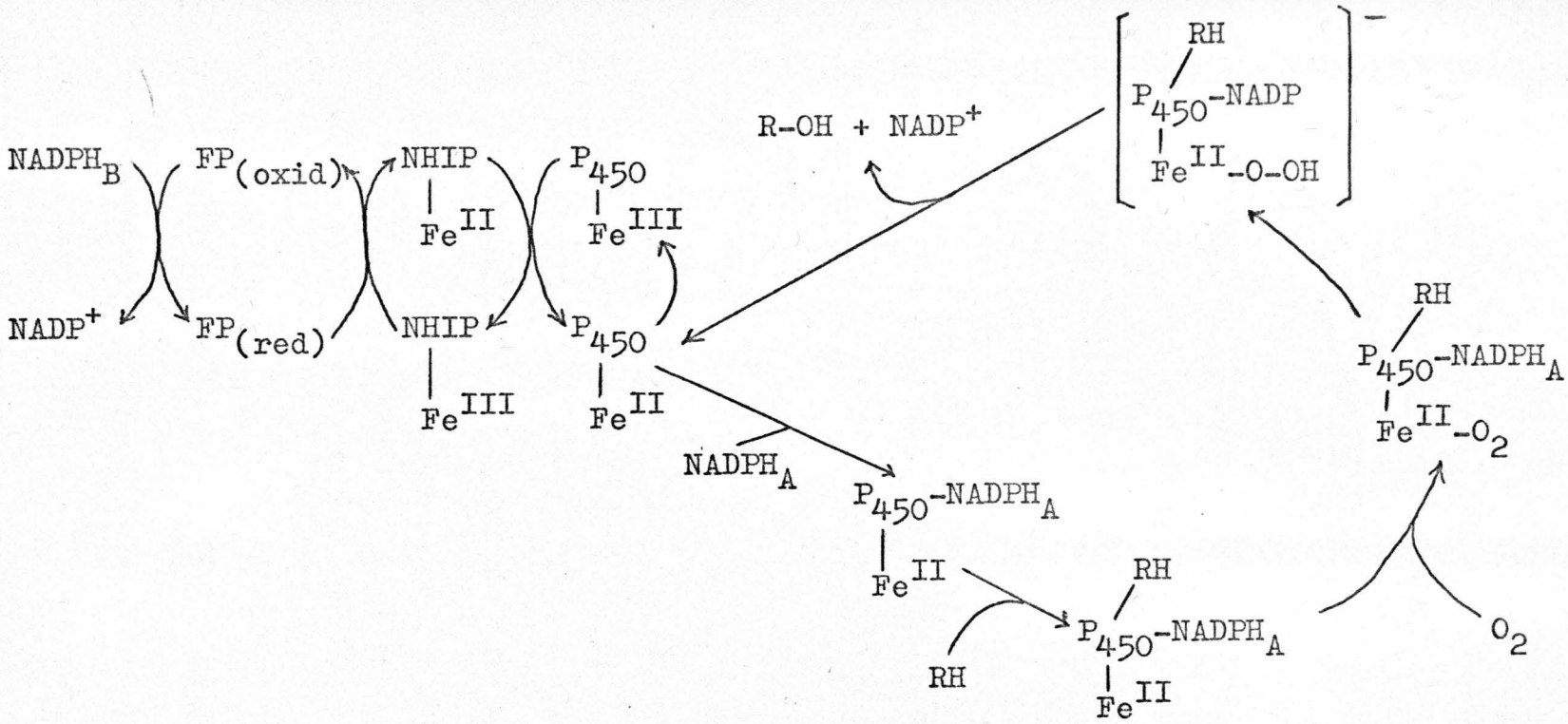
On the other hand, a much more reactive species would be the hydroperoxo complex of ferrous ion, $[Fe^{II}-O-OH]^-$.

Within this complex, all the p-orbitals of oxygen are occupied and "back bonding" by metal is not possible, thus facilitating the rupture of the oxygen-oxygen bond, generating the ferryl ion complex. Fe^{II}-$\ddot{O}$, which is a highly reactive species (68).

The direct involvement of NADPH for the formation of $[Fe^{II}$-O-OH$]^-$ in steroid hydroxylation had been proposed by Hayano (50), which is not only conceptually simpler but also satisfies all the experimental data to date (Scheme III).

The following investigations were undertaken in an attempt to deduce the correct mechanism for steroid hydroxylation.

(a) Analog Studies.--The reduction of adrenodoxin by NADH can also be catalyzed by adrenodoxin reductase (FP) but at a rate 400 times slower than NADPH (19). According to the mechanism as outlined in Scheme I, the inability of NADH to support 11β-hydroxylation must be due to the slow rate of electron transfer from NADH to adrenodoxin thereby unable to keep Cytochrome-P_{450} in the ferrous level of oxidation, required for hydroxylation. If this supposition is correct, one should be able to raise the concentration of FP, so that the rate of reduction of NHIP by NADH is identical to that of NADPH. Under the adjusted conditions, one would expect the rate of 11β-hydroxylation in the presence of NADH or NADPH to be the same. Since oxidized



Scheme III. Proposed mechanism of steroid hydroxylation

NHIP shows two prominent absorption peaks at 415 mμ and 455 mμ (Figure 7), which become bleached upon reduction, NADPH-adrenodoxin reductase (FP) activity could be readily assayed by measuring the disappearance in absorbancy at 415 mμ. As shown in Figure 8, the rate of reduction of NHIP by NADH was identical to that of NADPH, if the FP concentration was increased 20-fold. However, the results in Table 3 clearly show that NADH still failed to support 11β-hydroxylation to any significant extent, a finding incompatible with the mechanism depicted in Scheme I.

As a number of pyridine nucleotide analogs of NADPH could be readily prepared, it appeared desirable to evaluate these compounds for their abilities to substitute for NADPH in DOC-hydroxylation and as substrates for adrenodoxin reductase (FP). Unfortunately, none of the analogs were capable to serve as substrates for FP nor supported DOC-hydroxylation. These included: tetrahydro-NADP; hexahydro-NADP and thio-NADPH. Since the chemical structures of these analogs bear close resemblances to NADPH, one would expect them to occupy the same enzyme sites as those occupied by NADPH or to act as competitive inhibitors. Therefore, the effects of these three analogs on DOC-hydroxylation and adrenodoxin-reductase (FP) activity were examined (Figures 9-11). In each instance, DOC-hydroxylation was inhibited to a greater degree by these analogs than adrenodoxin-reductase, as evidenced by the differences in the slopes of the lines. The difference of

Figure 7. Absorption spectrum of oxidized adrenodoxin (NHIP).

The solution contained 0.9 mg of NHIP per ml. The spectrum was recorded on a Cary 14 recording spectrophotometer.

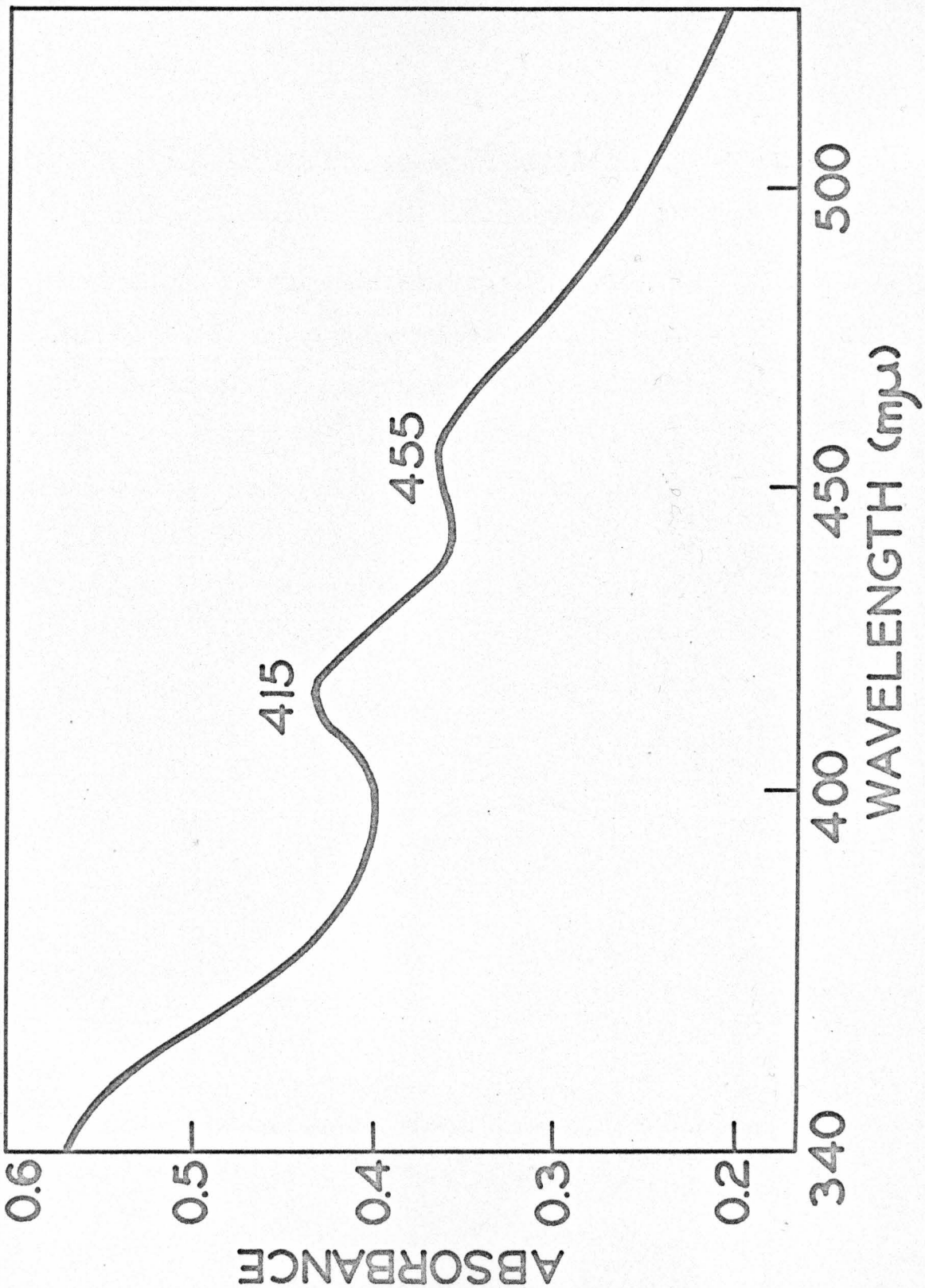

ABSORBANCE
0.6
0.5
0.4
0.3
0.2
340
400
450
500
WAVELENGTH (mμ)
415
455

Figure 8. Kinetics of NHIP reduction by NADH or NADPH catalyzed by different levels of FP.

The reactions were carried out in Thunberg cuvettes under anaerobic conditions. The reaction mixture contained 1.5 mg of NHIP; 3 μmoles of NADPH; 0.2 mg of FP in a total volume of 2.7 ml of 0.1 M phosphate buffer, pH = 7.5. For NADH, the system contained 1.5 mg of NHIP; 3 μmoles of NADH; and 4 mg of FP in a final volume of 2.7 ml.

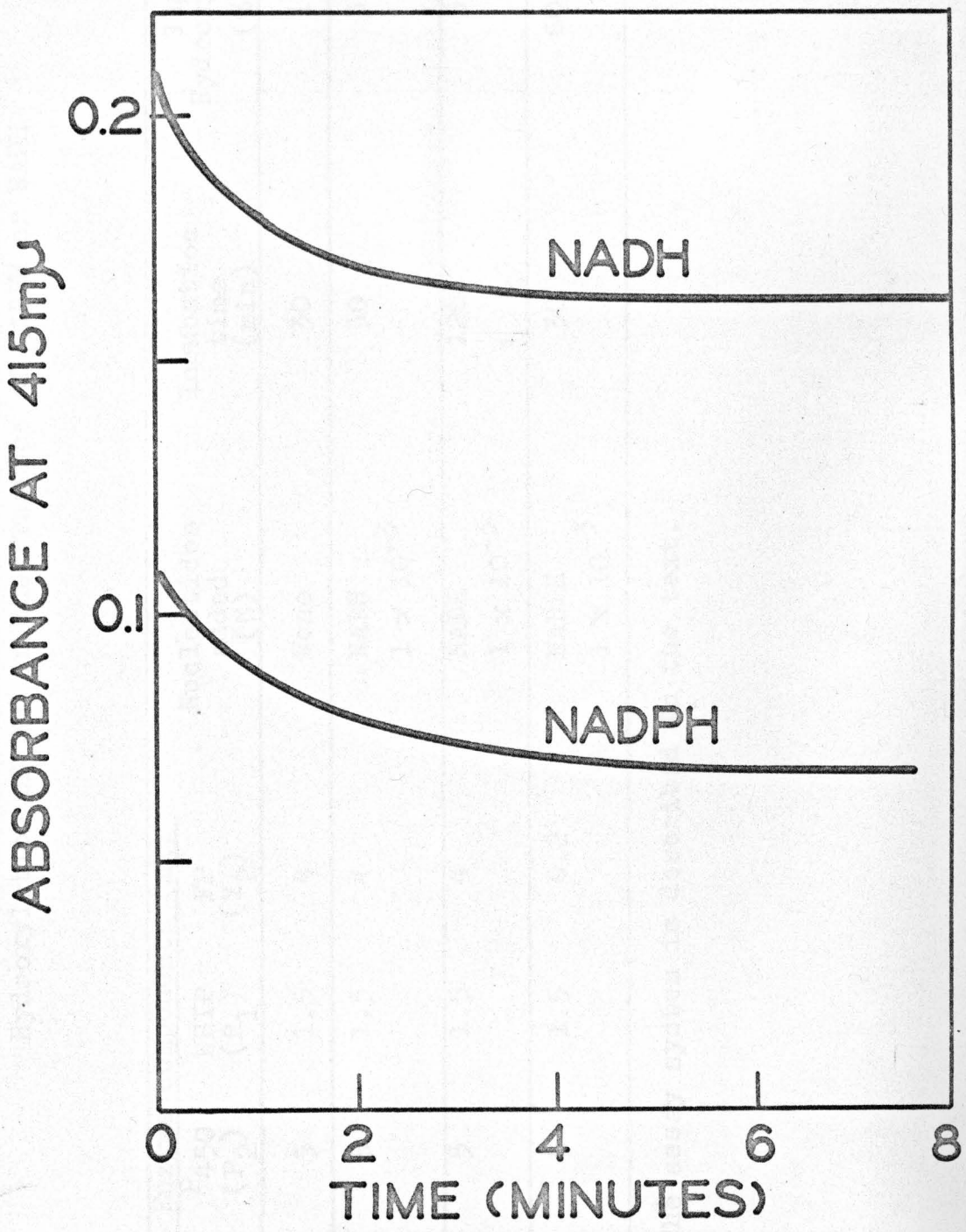

ABSORBANCE AT 415mμ
0.2
0.1
NADH
NADPH
0
2
4
6
8
TIME (MINUTES)

TABLE III

Hydroxylation of DOC in the Presence of NADPH or NADH

Enzyme components (mg)			Nucleotides added (M)	Incubation time (min)	11β-Hydroxylation (%)
P_{450} (P_2)	NHIP (R_1)	FP (Y_2)			
5	1.5	4	None	30	1
5	1.5	4	NADH 1×10^{-3}	30	3
5	1.5	4	NADH 1×10^{-3}	120	5
5	1.5	0.2	NADPH 1×10^{-3}	30	60

The assay system is described in the text.

Figure 9. Differential inhibition of adrenodoxin reductase (FP) activity and DOC-hydroxylation by tetrahydro-NADPH.

The NADPH-Cytochrome c method was used for the assay of adrenodoxin reductase (FP). The system consisted of 0.067 mg FP (Y_2); 0.067 mg NHIP (R_1); 0.8 μmole NADPH; 0.8 mg Cytochrome c; and varying amounts of tetrahydro-NADP in a total volume of 1 ml of 0.1 M phosphate buffer, pH = 7.5. The rate of reduction of Cytochrome c was measured at 550 mμ. 11β-Hydroxylase activity was assayed conventionally as described in the presence of various concentrations of tetrahydro-NADP as indicated.
-O-O- = FP activity; -Δ-Δ- = 11β-hydroxylase activity.

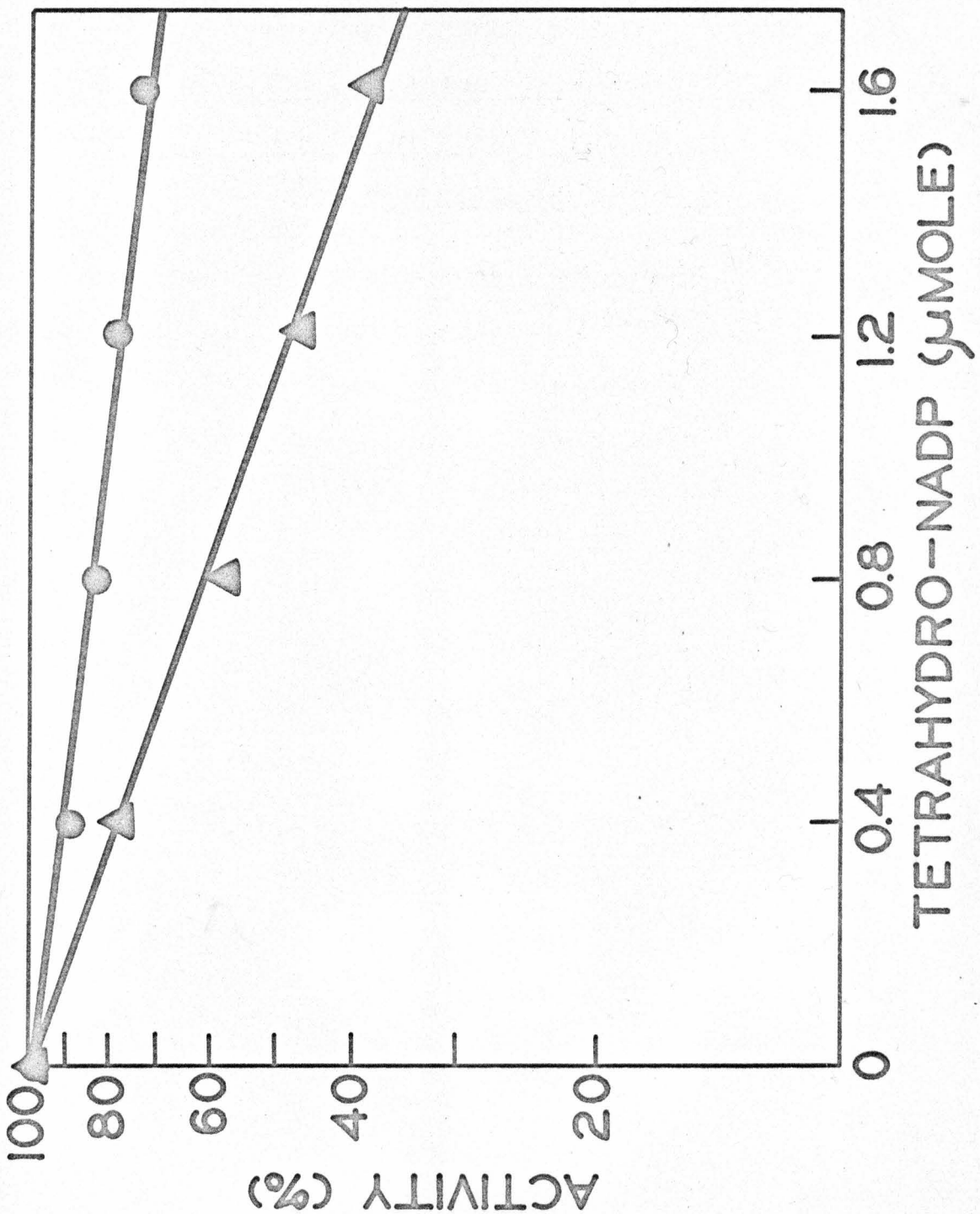

ACTIVITY (%)
100
80
60
40
20
0
0.4
0.8
1.2
1.6
TETRAHYDRO-NADP (μMOLE)

Figure 10. Differential inhibition of adrenodoxin reductase (FP) activity and DOC hydroxylation by hexahydro-NADPH.

The assay procedures were identical to those of Figure 8 except hexahydro-NADP was used as the inhibitor. -O-O- = FP activity; -Δ-Δ- = 11β-hydroxylase activity.

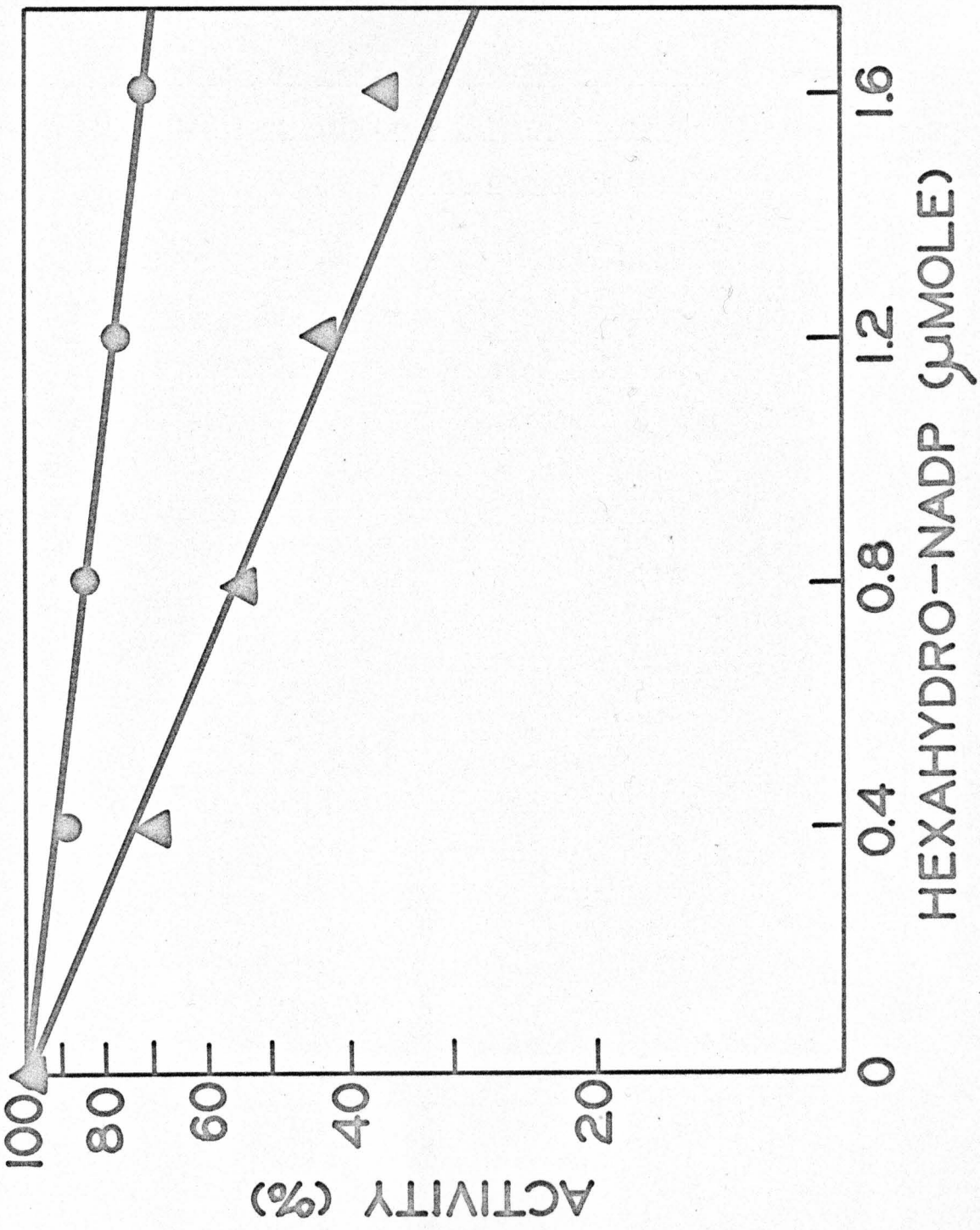
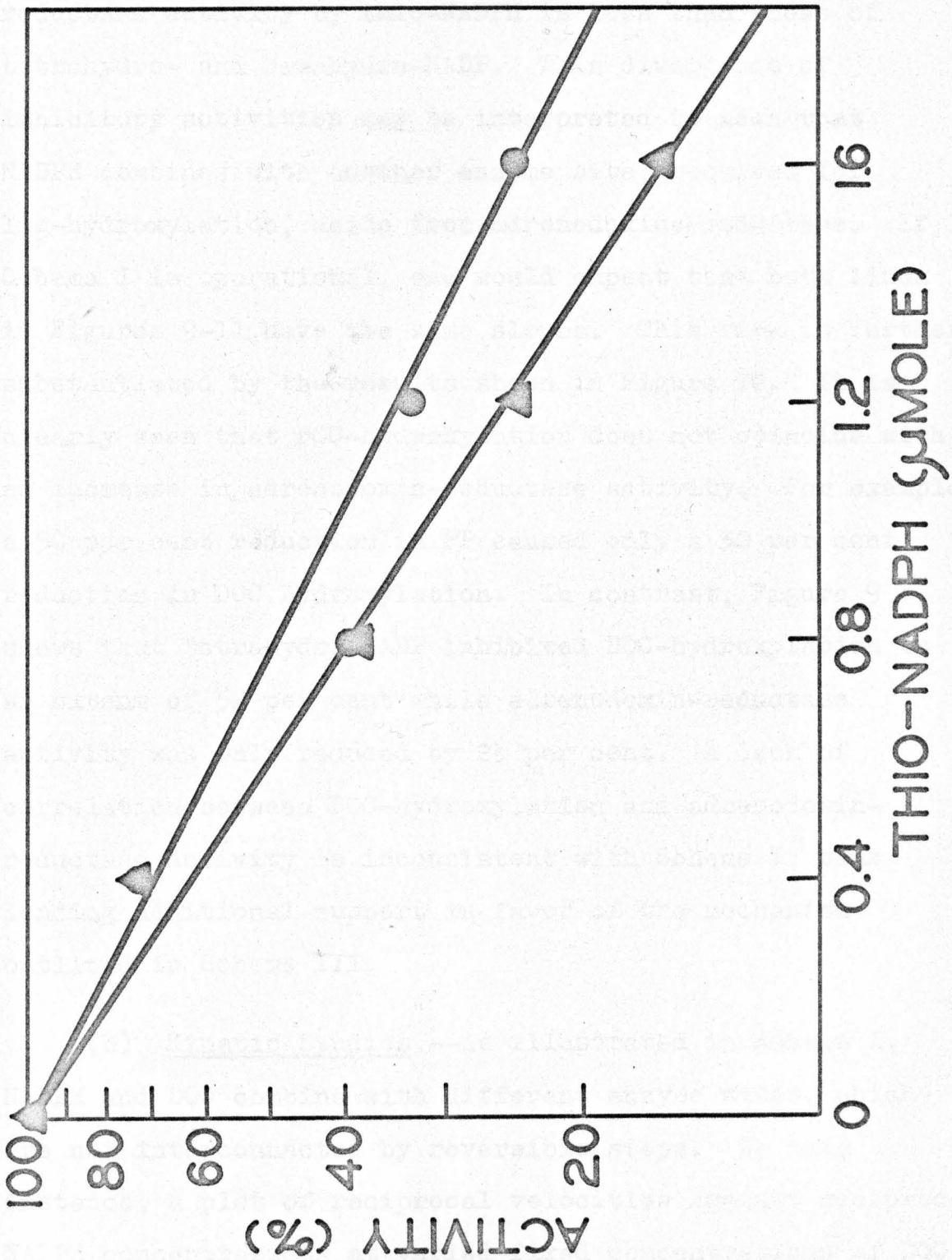

ACTIVITY (%)
100
80
60
40
20
0
0.4
0.8
1.2
1.6
HEXAHYDRO-NADP (μMOLE)

Figure 11. Comparative inhibition of adrenodoxin reductase (FP) activity and DOC hydroxylation by thio-NADPH.

Thio-NADPH was used as the inhibitor; otherwise, the assay methods used were identical to those of Figure 8. -O-O- = FP activity; -Δ-Δ- = 11β-hydroxylation activity.

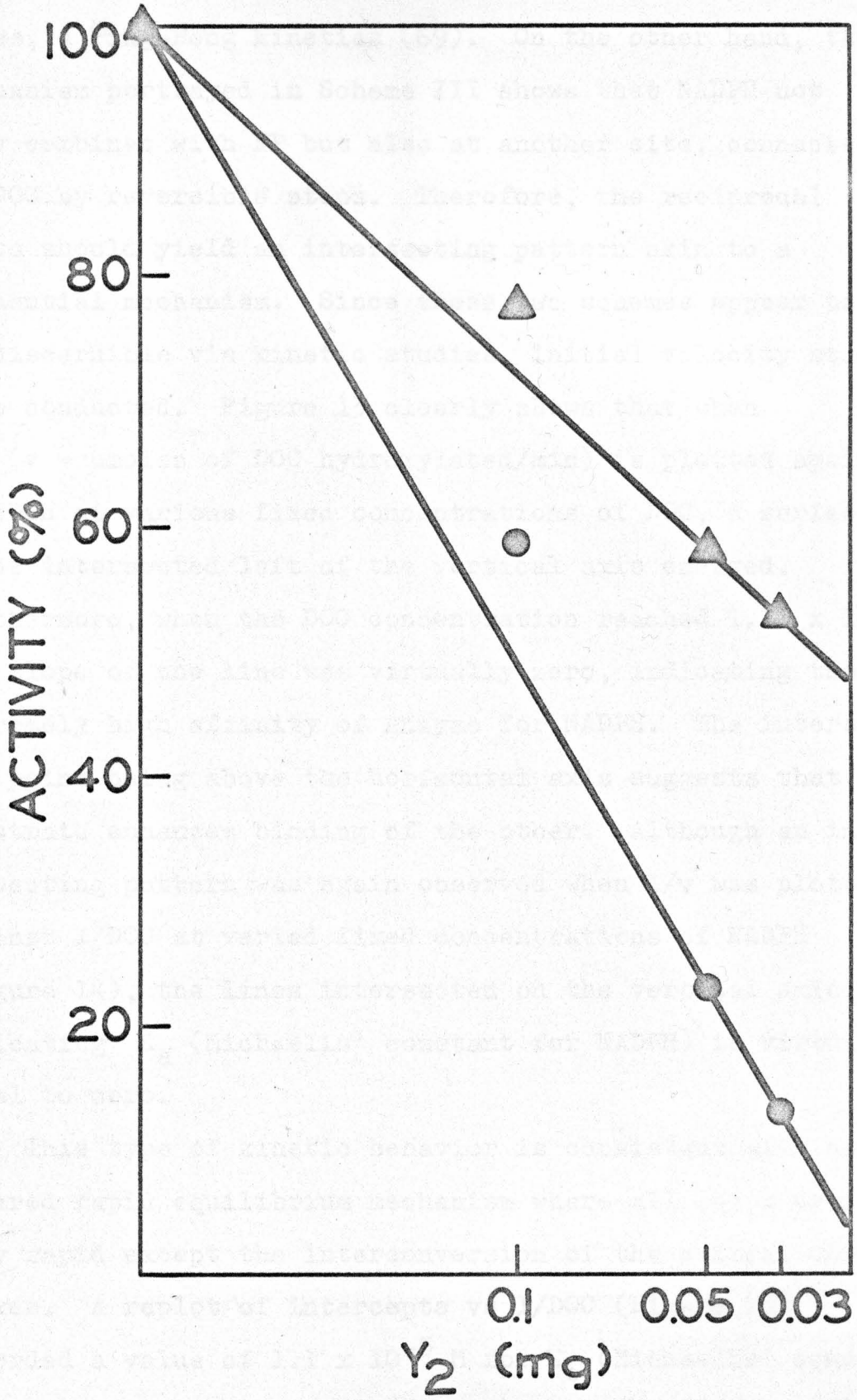

ACTIVITY (%)
100
80
60
40
20
0
0.4
0.8
1.2
1.6
THIO-NADPH (μMOLE)

inhibitory activities in DOC-hydroxylation and adrenodoxin-reductase activity by thio-NADPH is less than those of tetrahydro- and hexahydro-NADP. This divergence of inhibitory activities may be interpreted to mean that NADPH combines with another enzyme site, required for 11β-hydroxylation, aside from adrenodoxine-reductase. If Scheme I is operational, one would expect that both lines in Figures 9-11 have the same slopes. This view is further substantiated by the results shown in Figure 12. It is clearly seen that DOC-hydroxylation does not coincide with an increase in adrenodoxin-reductase activity. For example, a 50 per cent reduction in FP caused only a 30 per cent reduction in DOC hydroxylation. In contrast, Figure 9 shows that tetrahydro-NADP inhibited DOC-hydroxylation to an extent of 52 per cent while adrenodoxin-reductase activity was only reduced by 23 per cent. A lack of correlation between DOC-hydroxylation and adrenodoxin-reductase activity is inconsistent with Scheme I, thus lending additional support in favor of the mechanism outlined in Scheme III.

(b) Kinetic Studies.--As illustrated in Scheme I, NADPH and DOC combine with different enzyme sites, which are not interconnected by reversible steps. In this instance, a plot of reciprocal velocities against reciprocal NADPH concentrations at varied fixed concentrations of DOC while O_2 remained constant should yield a family of parallel

Figure 12. Effect of adrenodoxin-reductase (FP) concentration on DOC hydroxylation and NHIP reduction.

The conventional assays were used as described in Figure 8 except no pyridine nucleotide analog was added and the amount of FP (Y_2) was varied as indicated.
-Δ-Δ- = 11β-hydroxylase activity;
-O-O- = NHIP reduction.


100
80
60
40
20
ACTIVITY (%)
0.1
0.05
0.03
Y2 (mg)

lines, a Ping-Pong kinetics (69). On the other hand, the mechanism portrayed in Scheme III shows that NADPH not only combines with FP but also at another site, connected to DOC by reversible steps. Therefore, the reciprocal plots should yield an intersecting pattern akin to a sequential mechanism. Since these two schemes appear to be discernible via kinetic studies, initial velocity studies were conducted. Figure 13 clearly shows that when 1/v (v = μmoles of DOC hydroxylated/min) is plotted against 1/NADPH at various fixed concentrations of DOC, a series of lines intersected left of the vertical axis emerged. Furthermore, when the DOC concentration reached 1.11×10^{-4} M, the slope of the line was virtually zero, indicating the extremely high affinity of enzyme for NADPH. The intersecting point being above the horizontal axis suggests that the substrate enhances binding of the other. Although an intersecting pattern was again observed when 1/v was plotted against 1/DOC at varied fixed concentrations of NADPH (Figure 14), the lines intersected on the vertical axis, indicating K_a (Michaelis' constant for NADPH) is virtually equal to zero.

This type of kinetic behavior is consistent with an ordered rapid equilibrium mechanism where all steps are very rapid except the interconversion of the central complexes. A replot of intercepts vs 1/DOC (Figure 15) afforded a value of 1.1×10^{-5} M for K_b (Michaelis' constant for DOC) while a replot of slopes vs 1/NADPH (Figure 16)

Figure 13. Double reciprocal plots of 1/v vs 1/NADPH at various fixed concentrations of DOC.

The assay system contained 5 mg of P_{450} (P_2); 0.2 mg of NHIP (R_1); 0.2 mg of FP (Y_2); 5 μmole of $MgCl_2$ and indicated concentration of NADPH and DOC in a total volume of 3 ml of 0.1 M phosphate buffer, pH = 7.5. After incubation at 37°C for 10 min, C^{14}-corticosterone was assayed by the radiochromatographic method.

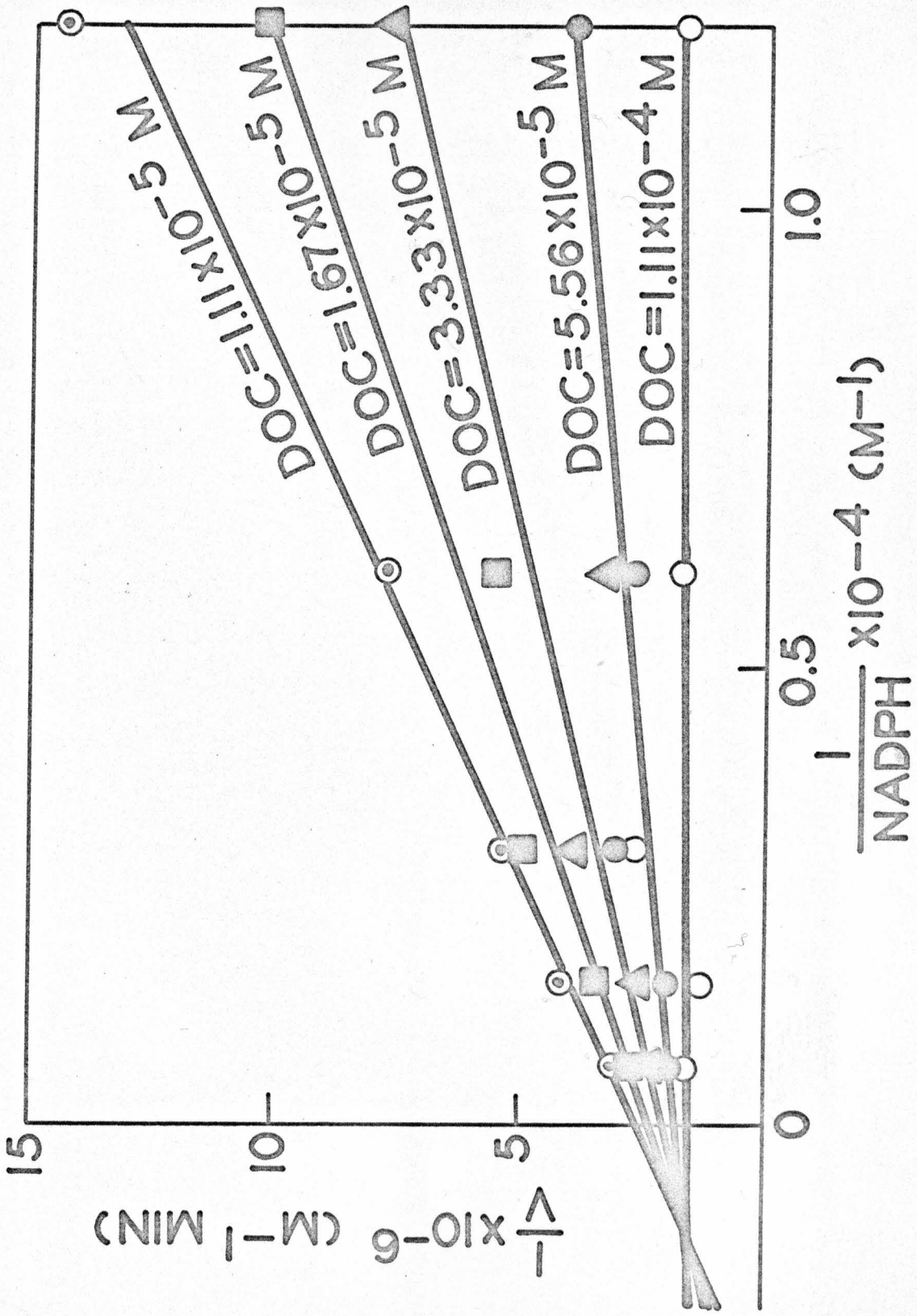

DOC=1.11x10-5 M
DOC=1.67x10-5 M
DOC=3.33x10-5 M
DOC=5.56x10-5 M
DOC=1.11x10-4 M
15
10
5
0
0.5
1.0
1/v x10-6 (M-1 MIN)
1/NADPH x10-4 (M-1)

Figure 14. Double reciprocal plots of 1/v vs 1/DOC at various fixed concentrations of NADPH.

The incubation and assay were the same as those of Figure 13.

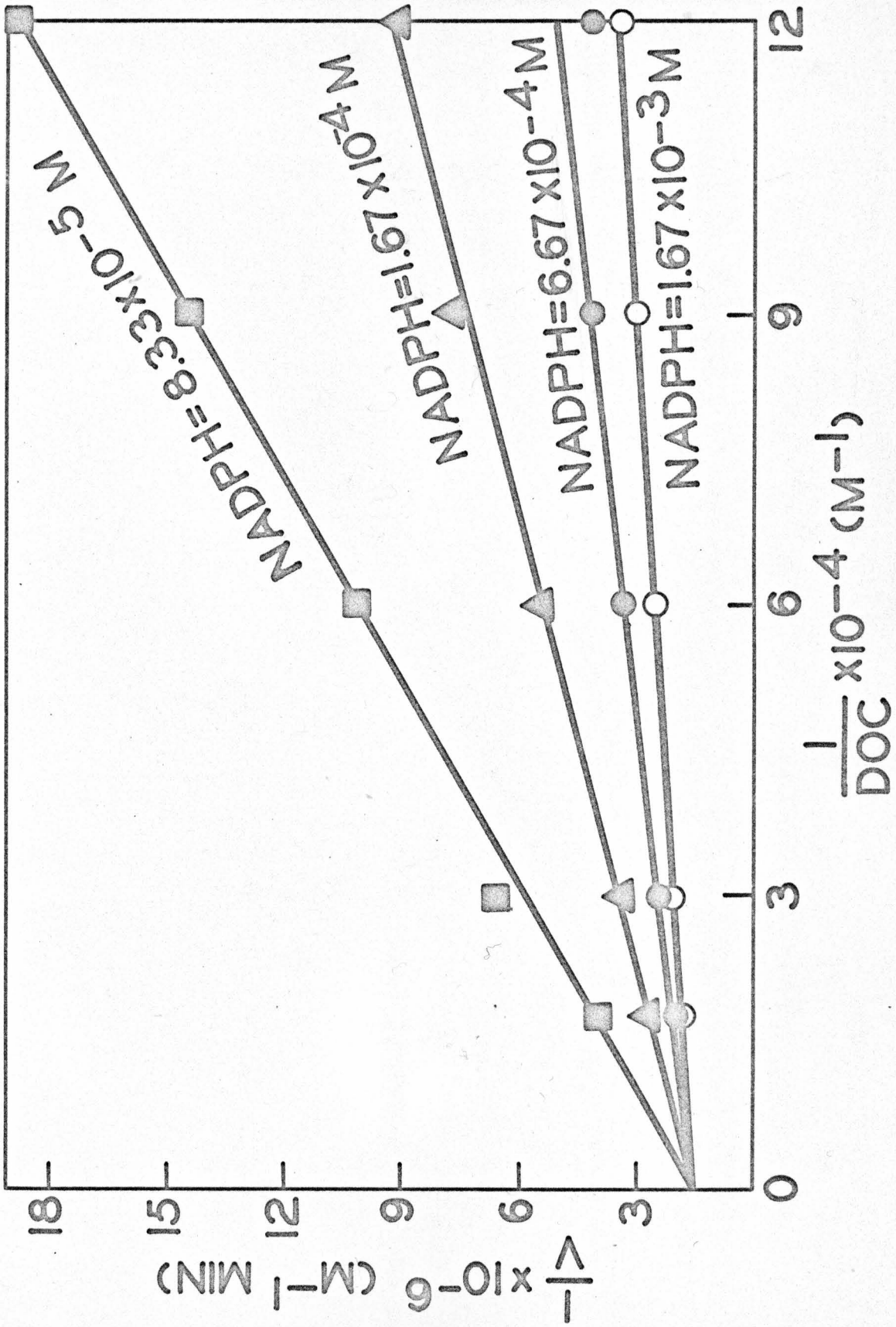

NADPH=8.33x10-5 M
NADPH=1.67x10-4 M
NADPH=6.67x10-4 M
NADPH=1.67x10-3 M
1/V x10-6 (M-1 MIN)
1/DOC x10-4 (M-1)
0
3
6
9
12
15
18

Figure 15. Replot of intercept vs 1/DOC.

The intercept values are those of Figure 13.

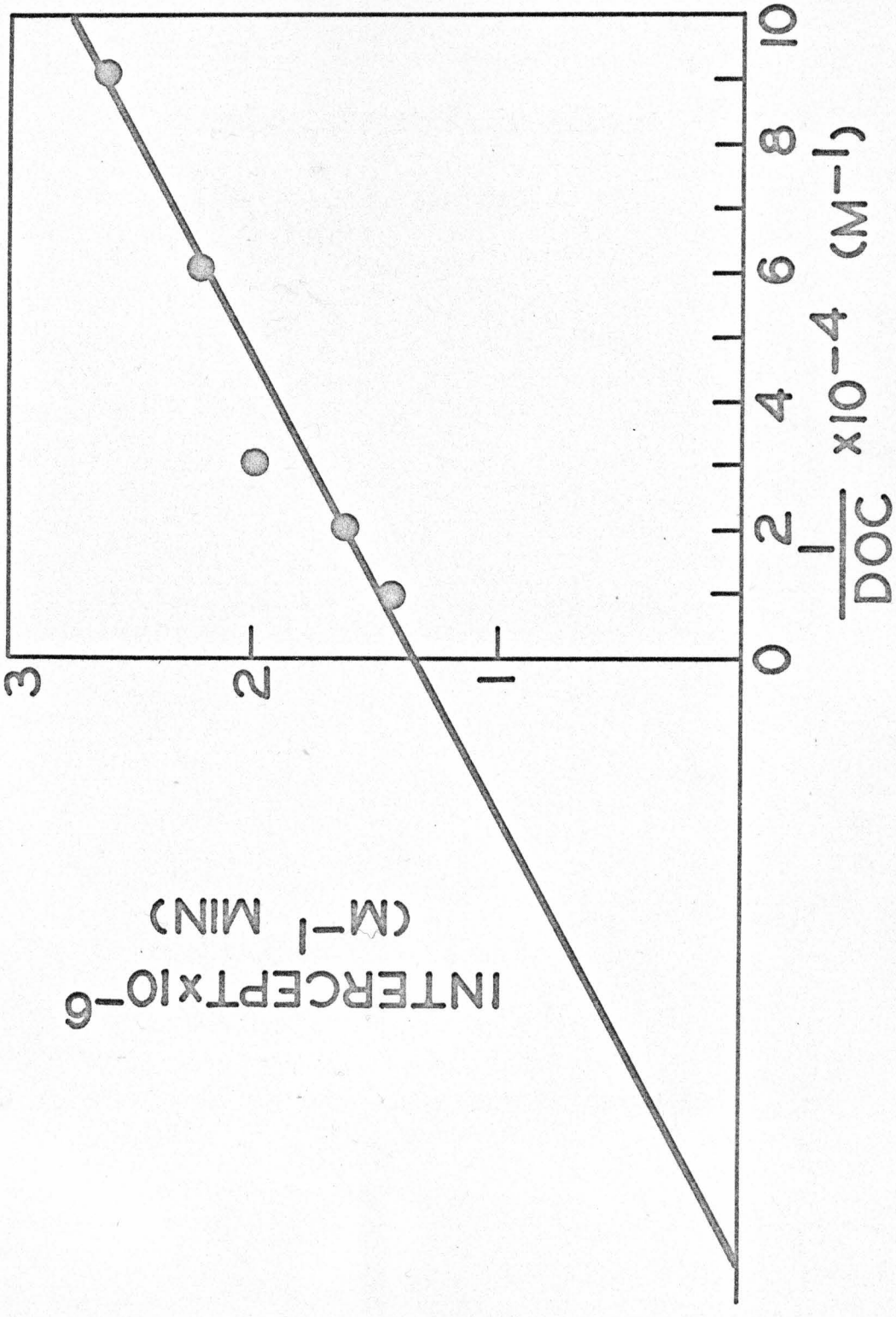
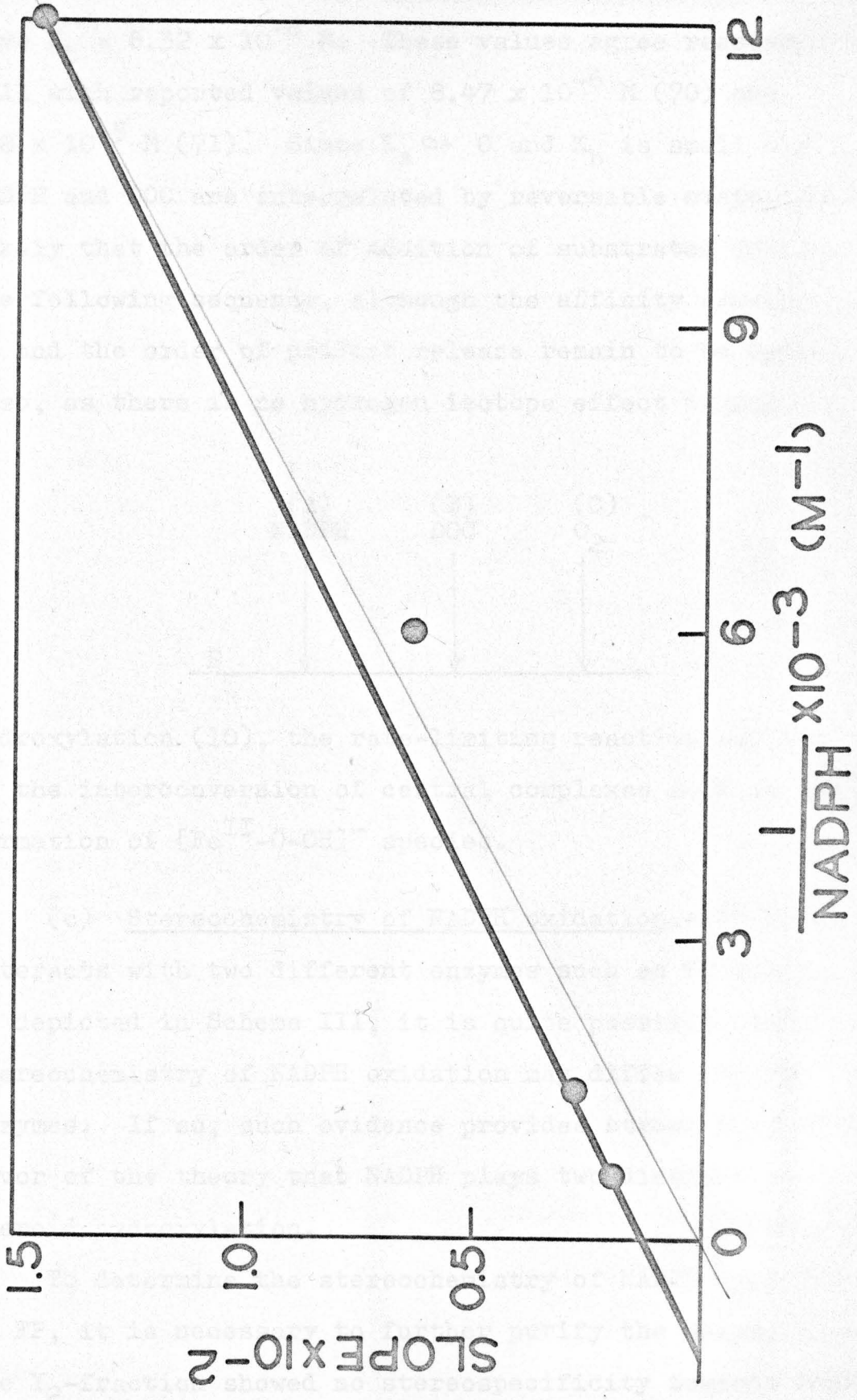
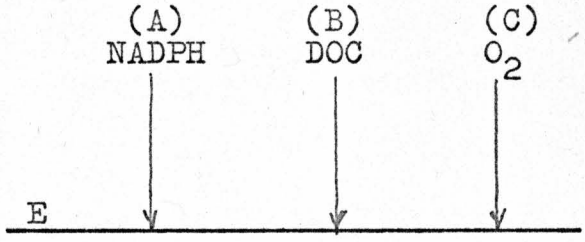

INTERCEPT x10⁻⁶
(M⁻¹ MIN)
3
2
1
0
2
4
6
8
10
1/DOC x10⁻⁴ (M⁻¹)

Figure 16. Replot of slopes vs 1/NADPH.

The values are those of Figure 14.


SLOPE x10-2
1.5
1.0
0.5
0
3
6
9
12
1/NADPH x10-3 (M-1)

gave K_b = 8.32 x 10^{-6} M. These values agree reasonably well with reported values of 8.47 x 10^{-6} M (70) and 1.8 x 10^{-5} M (71). Since $K_a \simeq 0$ and K_b is small and NADPH and DOC are interrelated by reversible steps, it is likely that the order of addition of substrates follows the following sequence, although the affinity constant for O_2 and the order of product release remain to be determined. Also, as there is no hydrogen isotope effect during



hydroxylation (10), the rate-limiting reaction may indeed be the interconversion of central complexes such as the formation of $[Fe^{II}\text{-}O\text{-}OH]^-$ species.

(c) Stereochemistry of NADPH oxidation.--If NADPH interacts with two different enzymes such as FP and P_{450} as depicted in Scheme III, it is quite possible that the stereochemistry of NADPH oxidation may differ for the two enzymes. If so, such evidence provides strong support in favor of the theory that NADPH plays two distinct roles in steroid hydroxylation.

To determine the stereochemistry of NADPH oxidation by FP, it is necessary to further purify the enzyme since the Y_2-fraction showed no stereospecificity towards $NADPH_B$

oxidation. The difficulty has been the extremely low concentration of FP in adrenal cortex mitochondria. Generally, one kilogram of adrenal cortex yields 4-5 mg of FP. Chromatography of the Y_2-fraction on a DEAE-cellulose column gave Y_3, whose specific activity about doubled. Rechromatography of Y_3 on a hydroxyapatite column afforded Y_4, which has a specific activity of 2.95 DCPIP units, representing a seven-fold purification of Y_2 with a recovery of 26 per cent. Thus, from 1 kilogram of adrenal cortex, one obtains about 15-20 μg of Y_4. When $NADPT_B$ was incubated with FP (Y_4) in the presence of NHIP, it was oxidized with greater than 88 per cent stereospecificity (Table IV). Since adrenodoxin appeared to catalyze considerable amount of exchange, as indicated by the radioactivity as T_2O, the stereospecificity of FP (Y_4) oxidation of $NADPT_B$ was calculated from the ratio of the specific activities of recovered NADP and NADPH from DEAE-cellulose column chromatography (Figure 17).

Apparently, Y_4 by itself does not catalyze significant quantities of exchange between $NADPT_B$ and H_2O. Conversely, when $NADPT_A$ was exposed to FP (Y_4), the recovered NADP and NADPH had specific activities of 3.46×10^5 and 3.8×10^5, indicating 90 per cent stereospecificity. It is apparent that the H_A hydrogen of NADPH undergoes little exchange. It is quite evident from these results that <u>adrenodoxin-reductase (FP) preferentially removes the H_B hydrogen of NADPH</u>.

TABLE IV

The Stereochemistry of NADPT Oxidation Catalyzed by FP (Y_4)

	First radioactive peak ($T_2$0) (cpm)	Amount of nucleotides recovered (μmole)		Radioactivity recovered (cpm)		Specific activity recovered (cpm/μmole)	
		NADP	NADPH	NADP	NADPH	NADP	NADPH
FP + $NADPT_B$	5.0×10^3	0.080	1.55	1.44×10^4	9.3×10^5	1.8×10^5	6.0×10^5
NHIP + $NADPT_B$	1.16×10^5	0.127	1.34	5.64×10^4	7.46×10^5	4.44×10^5	5.57×10^5
FP + NHIP + $NADPT_B$	7.41×10^5	1.30	0.23	7.72×10^4	1.13×10^5	5.94×10^4	4.91×10^5
FP + NHIP + $NADPT_A$	2.79×10^4	1.28	0.23	4.43×10^5	8.74×10^4	3.46×10^5	3.8×10^5

The reaction mixture contained: 1.65 μmole of $NADPT_B$ (specific activity = 9.15×10^5 cpm/μmole), or $NADPT_A$ (specific activity = 4.45×10^5 cpm/μmole), and where indicated 29 μg of FP (Y_4), 2 mg of NHIP (R_1) in a total volume of 3 ml of 0.05 M glycylglycine buffer, pH = 7.5. After incubation at 37°C for 30 minutes, the reaction was terminated by freezing the contents in dry-ice-acetone mixture.

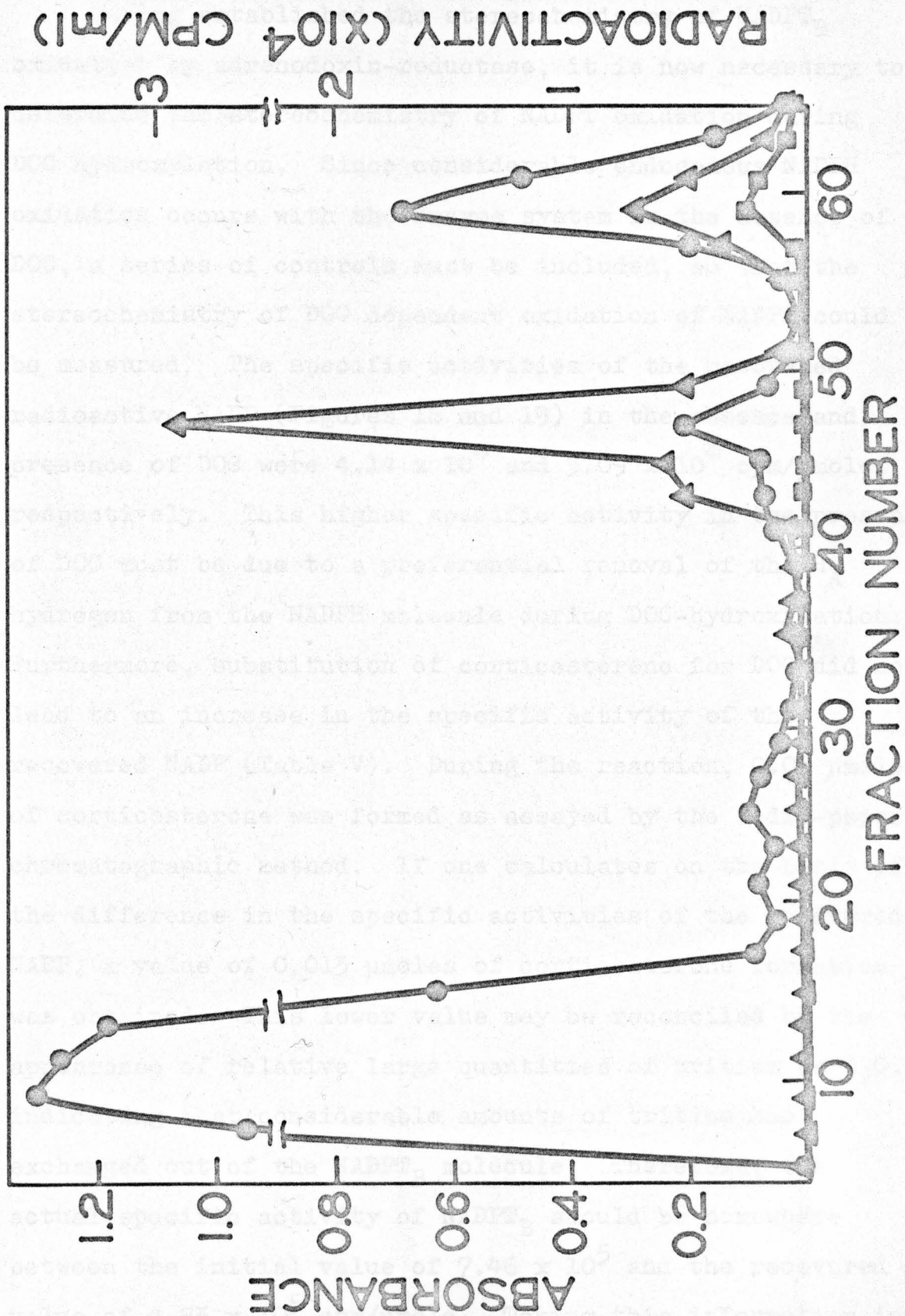
Figure 17. Isolation of radioactive NADP and NADPH from the reaction mixture - FP + NHIP + $NADPT_B$ on DEAE-cellulose column.

T_2O (fractions 5-17); NADP (fractions 41-51); NADPH (fractions 53-65).

-O-O- = radioactivity (cpm/ml);

-Δ-Δ- = absorbancy at 260 mμ;

-□-□- = absorbancy at 340 mμ.


RADIOACTIVITY (x10⁴ CPM/ml)
3
2
1
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
FRACTION NUMBER

Having established the stereochemistry of $NADPT_B$ oxidation by adrenodoxin-reductase, it is now necessary to determine the stereochemistry of NADPT oxidation during DOC hydroxylation. Since considerable endogenous NADPH oxidation occurs with the enzyme system in the absence of DOC, a series of controls must be included, so that the stereochemistry of DOC dependent oxidation of NADPT could be measured. The specific activities of the recovered radioactive NADP (Figures 18 and 19) in the absence and presence of DOC were 4.14×10^4 and 5.09×10^4 cpm/μmole, respectively. This higher specific activity in the presence of DOC must be due to a preferential removal of the H_A hydrogen from the NADPH molecule during DOC-hydroxylation. Furthermore, substitution of corticosterone for DOC did not lead to an increase in the specific activity of the recovered NADP (Table V). During the reaction, 0.03 μmole of corticosterone was formed as assayed by the radio-paper chromatographic method. If one calculates on the basis of the difference in the specific activities of the recovered NADP, a value of 0.013 μmoles of corticosterone formation was obtained. This lower value may be reconciled by the appearance of relative large quantities of tritium as T_2O, indicating that considerable amounts of tritium had exchanged out of the $NADPT_B$ molecule. Therefore, the actual specific activity of $NADPT_B$ should be somewhere between the initial value of 7.46×10^5 and the recovered value of 4.83×10^5 cpm/μmole. Taking this information into

Figure 18. Chromatographic resolution of tritiated NADP and NADPH from the reaction mixture - FP + NHIP + P_{450} + $NADPT_B$ on DEAE-cellulose Column.

Fractions (5-19) - T_2O; (39-45) - NADP; (47-59) - NADPH. -O-O- = radioactivity cpm/ml; -Δ-Δ- = absorbancy at 260 mμ; -□-□- = absorbancy at 340 mμ.

ABSORBANCE
0.5
1.0
1.5
FRACTION NUMBER
0
10
20
30
40
50
60
RADIOACTIVITY (x10⁴ CPM/ml)
1
2
3
4

Figure 19. Separation of tritiated NADP and NADPH from the reaction mixture - FP + NHIP + DOC + P_{450} + $NADPT_B$ on DEAE-cellulose column.

Fractions (5-19) - T_2O; (33-41) - NADP; (43-55) - NADPH. -O-O- = radioactivity cpm/ml; -Δ-Δ- = absorbancy at 260 mμ; -□-□- = absorbancy at 340 mμ.

ABSORBANCE
1.5
1.0
0.5
0
10
20
30
40
50
FRACTION NUMBER
RADIOACTIVITY (x10⁴ CPM/ml)
4
3
2
1

TABLE V

Stereochemistry of $NADPT_B$ Oxidation During DOC-Hydroxylation

	First radioactive peak T_2^0 (cpm)	Amounts of nucleotides recovered (μmole)		Radioactivity recovered (cpm)		Specific activity (cpm/μmole)		Hydroxylation %
		NADP	NADPH	NADP	NADPH	NADP	NADPH	
FP + NHIP + P_{450} + $NADPT_B$	8.82 x 10^5	0.81	1.13	3.35 x 10^4	5.54 x 10^5	4.14 x 10^4	4.90 x 10^5	--
FP + NHIP + P_{450} + $NADPT_B$ + DOC	8.40 x 10^5	0.86	1.11	4.38 x 10^4	5.36 x 10^5	5.09 x 10^4	4.83 x 10^5	49
FP + NHIP + P_{450} + $NADPT_B$ +corticosterone	8.36 x 10^5	0.76	1.10	3.11 x 10^4	5.45 x 10^5	4.09 x 10^4	4.95 x 10^5	--

The system contained 2.48 μmole of $NADPT_B$ (specific activity = 7.46 x 10^5 cpm/μmole); 29 μg of FP (Y_4); 0.2 mg of NHIP (R_1); 2 mg of P_{450} (P_4); and where indicated 20 μg of DOC or corticosterone in a total volume of 3 ml of 0.05 M glycylglycine buffer, pH = 7.5. Incubation conditions were the same as those of Table IV.

consideration, the two values for corticosterone formation are in reasonable agreement. The same experiment was repeated on a larger scale and similar results emerged (Table VI). However, in this experiment the amount of corticosterone formed as assayed by the radiochromatographic method was 0.065 μmoles as compared to 0.060 μmoles, calculated by the difference in the specific activities of recovered NADP. The closer agreement here is probably attributed to a more active hydroxylation preparation since the P_{450} (P_4) fraction was increased three-fold over that of Table V.

To further confirm the stereochemistry of NADPH oxidation during DOC-hydroxylation, $NADPT_A$ was prepared and incubated with the 11β-hydroxylase system. As should be the case, the specific activities of the recovered NADP in the presence of DOC was 2.92×10^5, which is lower than 3.21×10^5 cpm/μmole obtained in the absence of DOC or with corticosterone. This difference in specific activities of the recovered NADP should account for the formation of 0.065 μmoles of corticosterone, but by radiochromatographic assay only 0.029 μmoles of corticosterone was obtained. It is worth noting that relatively small amounts of tritium exchanged out of $NADPT_A$ molecule as T_2O, as compared to $NADPT_B$. This discrepancy can be readily explained however if the following reactions are taken into consideration. During DOC hydroxylation, $NADPT_A$ undergoes three reactions simultaneously at different rates. These are: (a) exchange

TABLE VI

A Larger-scale Experiment Confirming the Stereochemistry of $NADPT_B$ Oxidation

	First radioactive peak (T_2O) (cpm)	Amounts of nucleotides recovered (μmole)		Radioactivity recovered (cpm)		Specific activity recovered cpm/μmole		Hydroxylation %
		NADP	NADPH	NADP	NADPH	NADP	NADPH	
FP + NHIP + P_{450} + $NADPT_B$	4.33 x 10^6	1.19	2.11	2.48 x 10^5	2.98 x 10^6	2.08 x 10^5	1.41 x 10^6	--
FP + NHIP + P_{450} +$NADPT_B$ + DOC	4.39 x 10^6	1.32	2.07	4.50 x 10^5	3.79 (2.79?) x 10^6	3.41 x 10^5	1.35 x 10^6	21.38
FP + NHIP + P_{450} + $NADPT_B$ + corticosterone	4.21 x 10^6	1.26	1.99	2.75 x 10^5	2.73 x 10^6	2.18 x 10^5	1.37 x 10^6	--

The reaction mixture contained: 29 μg of FP (Y_4); 0.2 mg of NHIP (R_1); 6 mg of P_{450} (P_4); 4 μmoles of $NADPT_B$ (specific activity = 2.24 x 10^6 cpm/μmole) and where indicated 100 μg of either DOC or corticosterone in a total volume of 3 ml of 0.05 M glycylglycine buffer, pH = 7.5. Incubation conditions were the same as those of Table V.

reaction, (b) non-DOC dependent removal of H_B from $NADPT_A$, (c) DOC-dependent removal of H_A from $NADPT_A$. If the rates of these reactions follow the order b > a > c, one can then envisage the low value of the recovered NADP specific activity in the presence of DOC. In any event, these results coincide with those obtained from $NADPT_B$ studies verifying that DOC-dependent oxidation of NADPH involves the removal of the H_A-hydrogens.

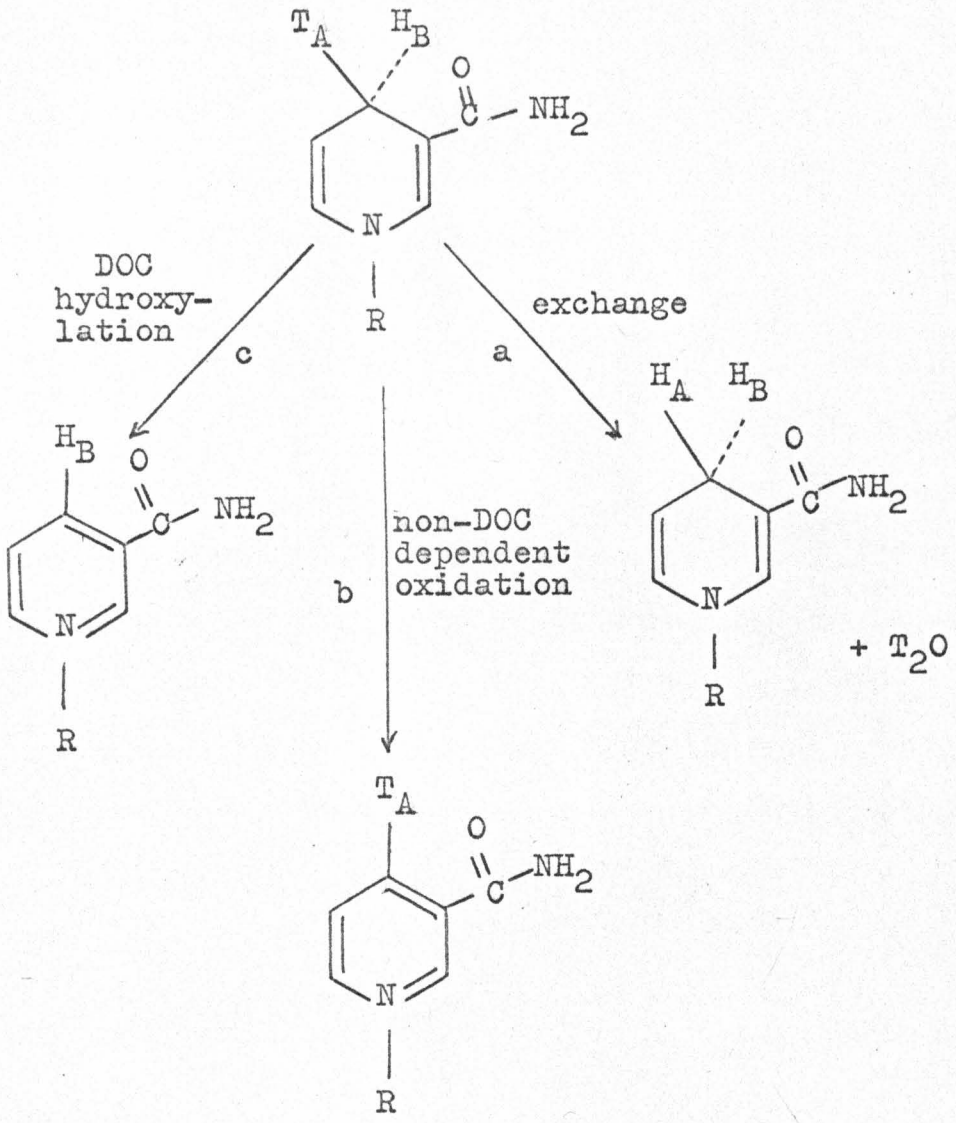
From the analog, kinetic and stereochemical studies it is quite obvious that the results are incompatible with Scheme I but are completely consistent with our proposed mechanism as outlined in Scheme III. Inherent in this mechanism is the dual function of NADPH. NADPH serves in an accessory capacity keeping the autooxidizable P_{450} in the reduced state via the NADPH-Cytochrome-P_{450} reductase system, a sequence which is steroid independent. Secondly, NADPH is directly involved in the steroid hydroxylation reaction to generate the hydroperoxo complex, $[Fe^{II}\text{-}O\text{-}OH]^-$, as shown. It should be noted that there is no overall valence change of the P_{450} on completion of one stoichiometric cycle of the reaction. Since the 11 -hydroxylation reaction proceeds with retention of configuration and obeys the rule of Bloom and Shull, it is represented as an electrophilic displacement reaction where, the "oxenoid" species (68), OH^+, stereospecifically displaces the H on the steroid. The order of addition of substrates appears to follow the sequence: NADPH; DOC and O_2 with the reduction

TABLE VII

Stereochemistry of $NADPT_A$ Oxidation During DOC-Hydroxylation

	First radioactive peak (T_2O) (cpm)	Amounts of nucleotides recovered (μmole)		Radioactivity recovered (cpm)		Specific activity recovered (cpm/μmole)		Hydroxylation %
		NADP	NADPH	NADP	NADPH	NADP	NADPH	
FP + NHIP + P_{450} + $NADPT_A$	3.42 x 10^4	0.73	1.16	2.37 x 10^5	4.46 x 10^5	3.21 x 10^5	3.84 x 10^5	--
FP + NHIP + P_{450} + $NADPT_A$ + DOC	3.28 x 10^4	0.79	1.12	2.29 x 10^5	4.24 x 10^5	2.92 x 10^5	3.79 x 10^5	47
FP + NHIP + P_{450} + $NADPT_A$ + corticosterone	3.85 x 10^4	0.74	1.18	2.38 x 10^5	4.51 x 10^5	3.21 x 10^5	3.82 x 10^5	--

The system contained: 29 μg of FP (Y_4); 0.2 mg NHIP (R_1); 2 mg P_{450} (P_4); 2.48 μmole $NADPT_A$ (specific activity = 4.45 x 10^5 cpm/μmole) and where indicated 20 μg of either DOC or corticosterone in a final volume of 3 ml of 0.05 M glycylglycine buffer, pH = 7.5. Incubation conditions were the same as those in Table VI.


DOC
hydroxy-
lation
c
exchange
a
b
non-DOC
dependent
oxidation
+ T2O

b >> a > c

of $Fe^{II}-O_2$ by NADPH as the rate-limiting step. Postulation of reduction of an iron-oxygen complex with formation of a reactive species is consistent with studies of the autooxidation of iron (II), whose second order dependence on ferrous ion concentration suggests rate-determining reduction of ferrous ion-oxygen complex (72,73).

Although the existence of the proposed reactive, transient species, $[Fe^{II}-O-OH]^-$, has yet to be demonstrated, in the absence of a better explanation for the DOC-dependent NADPH oxidation, a course which removes the H_A hydrogen, the suggestion that NADPH is involved in the reduction of $Fe^{II}-O_2$ either directly or indirectly is most likely to be correct. It should be interesting to apply similar experimental approaches to examine other mixed function oxidase systems, to establish whether the proposed mechanism where NADPH is involved in the generation of the so-called "active oxygen" or oxygen atom with 6 electrons (68), is common to all external mixed function oxidases (2).

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