THE PEROXIDASE PROPERTIES OF CYTOCHROME P-450

EUGENE G. HRYCAY

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A thesis submitted to the

Faculty of Graduate Studies

of Memorial University of Newfoundland

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

in the

Department of Biochemistry

by

Eugene G. Hrycay

May, 1973

ACKNOWLEDGEMENTS

The major portion of this work was carried out in the Department of Biochemistry, Memorial University of Newfoundland. The author expresses gratitude to Dr. Peter J. O'Brien for his overall supervision of the work. Thanks are also due to Dr. Johan E. van Lier, Biochemistry Laboratories, Centre Hospitalier Universitaire, Sherbrooke, Quebec, for supplying the steroid hydroperoxides and for use of his facilities in carrying out part of this work.

The author would like to express his gratitude to Professor George S. Boyd, Department of Biochemistry, Edinburgh University, Scotland, for his many stimulating discussions, for use of his research facilities, and for providing moral support in times of stress.

Finally, thanks go out to Ms. Barbara Hennessey and Mrs. Kay Cherian for assistance in certain enzyme preparations and to Mrs. Helen Kennedy for typing the manuscript.

ABSTRACT

The decomposition of steroid and other organic hydroperoxides by microsomes from rat liver and bovine adrenal cortex has been examined using TMPD as an electron donor. A comparison of the hydroperoxide specificity of microsomal peroxidase revealed the 17α-hydroperoxide derivatives of progesterone and pregnenolone to be very effective substrates. Treatment of rats with phenobarbital or 3-methylcholanthrene enhanced the specific activity of microsomal peroxidase and the cytochrome P-450 content to a similar extent. Microsomal "P-450 particles" devoid of cytochrome $\underline{b}_5$ showed the same peroxidase activity per mole of P-450 as did original microsomes. Further evidence for P-450 being a microsomal peroxidase was the inhibition of peroxidase activity by type I, type II, and modified type II compounds and by reagents converting P-450 to P-420.

The rate of NADPH or NADH oxidation by microsomal fractions was markedly enhanced by the hydroperoxides. Evidence for the involvement of NADPH-cytochrome $\underline{c}$ reductase in NADPH-peroxidase activity indluded a similar K_m for NADPH; inhibition by $\underline{p}$-mercuribenzoate with partial protection by NADPH; inhibition by $NADP^+$; and inactivation by antiserum to the flavoenzyme. Evidence of a role for NADH-cytochrome $\underline{b}_5$ reductase in NADH-peroxidase activity included a similar K_m for NADH; inhibition by $\underline{p}$-mercuribenzoate with partial protection by NADH; and inactivation by antibody to the flavoprotein.

Evidence for the involvement of P-450 in the NAD(P)H-peroxidase

reaction included inhibition by type I, type II, and modified type II compounds; inhibition by reagents converting P-450 to P-420; and marked stimulation by *in vivo* phenobarbital treatment. The NADPH- and NADH-reduced forms of P-450 were oxidized very rapidly by cumene hydroperoxide under a CO atmosphere. Incubation of the hydroperoxides with microsomal fractions gave rise to the corresponding hydroxy derivatives as the major products.

Mechanisms for the P-450-dependent reduction of hydroperoxides are proposed and the role of hydroperoxides as possible intermediates in biological hydroxylation reactions discussed.

ABBREVIATIONS

NADPH	- reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	- nicotinamide adenine dinucleotide phosphate
NADH	- reduced nicotinamide adenine dinucleotide
NAD^+	- nicotinamide adenine dinucleotide
GSH	- reduced glutathione
TMPD	- N,N,N',N'-tetramethyl-p-phenylenediamine
gc	- gas chromatography
ms	- mass spectrometry
tlc	- thin layer chromatography
EDTA	- ethylenediaminetetraacetate
P-450	- cytochrome P-450

Systematic nomenclature for compounds given trivial names in the text include:

pregn-4-ene-3,20-dione —— progesterone

3β-hydroxypregn-5-en-20-one —— pregnenolone

17α,21-dihydroxypregn-4-ene-3,20-dione —— 17α,21-dihydroxyprogesterone

17α-hydroxypregn-4-ene-3,20-dione —— 17α-hydroxyprogesterone

3β,17α-dihydroxypregn-5-en-20-one —— 17α-hydroxypregnenolone

androst-4-ene-3,17-dione —— androstenedione

3β-hydroxyandrost-5-en-17-one —— dehydroepiandrosterone

3β-hydroxy-5α-pregnan-20-one —— allopregnanolone

17β-hydroxyandrost-4-en-3-one —— testosterone

cholest-5-en-3β-ol —— cholesterol

4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol —— lanosterol

2-phenyl-2-hydroperoxypropane —— cumene hydroperoxide

p-ethoxyacetanilide —— phenacetin

o-biphenylenemethane —— fluorene

1,2,3,4-tetrahydronaphthalene —— tetralin

1,2,3,4-tetrahydro-2-naphthol —— tetralol

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INTRODUCTION

Enzymes that incorporate one atom of molecular oxygen into substrate while concomitantly reducing the other atom to water are termed mixed-function oxidases or mono-oxygenases (1). The stoichiometry of the reaction may be represented as

$$RH + NAD(P)H + H^+ + O_2 \longrightarrow ROH + NAD(P)^+ + H_2O$$

where RH is the substrate and NAD(P)H the electron donor. The reaction results in the oxidation of one mole of NAD(P)H and consumption of one mole of oxygen during the formation of one mole of hydroxylated product. The importance of mixed-function oxidases in biological systems is evident from the wide diversity of substances that are metabolized by these enzymes (2-5).

I. Mixed-function oxidase reactions in liver microsomes

1. Hydroxylation of drugs and foreign compounds

Studies *in vitro* have revealed that the endoplasmic reticulum of liver is the major site for the metabolism of a large variety of drugs, carcinogens, and other foreign compounds by nonspecific enzymes. These oxidative enzymes fit the category of mixed-function oxidases and require the participation of NADPH and molecular oxygen. In some instances, NADH may substitute for NADPH but the rate of the reaction is markedly reduced. The versatility of these enzyme systems is virtually unique in biochemistry as they catalyze such diverse reactions as the oxidation of aliphatic and aromatic hydrocarbons, the deamination of various substances, the dealkylation of secondary and tertiary amines, the oxidative cleavage

of ethers, the sulfoxidation of thio-ethers, and the epoxidation of aromatic hydrocarbons and alkenes (2,3).

Shortly after the discovery of these reactions, Brodie and colleagues (4) pointed out that many of these reactions could be visualized as hydroxylation reactions in which unstable hydroxylated intermediates were formed. These investigators also suggested that NADPH participates in these reactions by reducing a component in liver microsomes that reacts with oxygen to form an "active oxygen" complex which in turn hydroxylates the various substrates. The component reduced by NADPH was found to be a carbon monoxide-binding pigment named cytochrome P-450. It was later established that the flavoenzyme NADPH-cytochrome c reductase (EC 1.6.2.3) mediates the reduction of cytochrome P-450 by NADPH (3).

2. *Hydroxylation of physiological substrates*

A large number of lipid-soluble drugs and other foreign compounds are known to undergo hydroxylation in liver microsomes in the presence of NADPH and oxygen. However, the hepatic microsomal NADPH-dependent enzymes metabolize not only drugs but also a host of normal body substrates which include steroids such as testosterone, progesterone, 17β-estradiol, deoxycorticosterone, corticosterone, and androstenedione; fatty acids; tyramine and other amines; thyroxin; anthranilic acid; and various indoles such as tryptamine and indoleacetic acid. Similarly, the biosynthesis of cholesterol requires the participation of NADPH-dependent enzymes in liver microsomes (5) as does the conversion of cholesterol to bile acids (6). Various steroid substrates can be hydroxylated by the liver microsomal enzyme system at the 2β, 6β, 7α, 7β, 12α, and 16α positions (5-7).

The similarities between drug and steroid hydroxylases in liver microsomes led investigators to suspect that drugs and steroids are substrates for the same hydroxylating enzymes. This concept was strengthened by the finding that steroid hormones could act as competitive inhibitors of the drug-metabolizing enzymes (5). The inhibitory effect of carbon monoxide on liver microsomal steroid hydroxylations suggested that cytochrome P-450 was involved in these reactions. Further evidence for the involvement of the hemoprotein in steroid hydroxylation came from the studies of Conney and coworkers (7) who demonstrated that the inhibitory effect of carbon monoxide on the 6β-, 7α-, and 16α-hydroxylation of testosterone by liver microsomes is prevented by monochromatic light at 450 nm. In addition, the low Michaelis-Menten constants found for the hydroxylation of testosterone, progesterone, and 17β-estradiol by liver microsomes provided additional support for the concept that steroid hormones are normal physiological substrates for the oxidative drug-metabolizing enzymes (8).

II. Mixed-function oxidase reactions in non-hepatic tissues

1. Hydroxylation of drugs and carcinogens

Although oxidative drug-metabolizing enzymes are localized primarily in liver microsomes, low levels of these enzymes are also present in various non-hepatic tissues (5). For example, Wattenberg and Leong (9) found NADPH-dependent 3,4-benzpyrene hydroxylase activity in the kidney, adrenal, and small intestine of normal rats. Benzpyrene and 3-methylcholanthrene hydroxylase activity has also been detected in the lung, thyroid, testis, placenta, skin, and leukocyte (5, 10). Ichikawa and colleagues (11) observed the hydroxylation of aniline, aminopyrine, and p-nitroanisole with microsomes of lung and kidney.

2. Hydroxylation of steroids

The endocrine glands are the main site for the biosynthesis of the steroid hormones from cholesterol. In the adrenal, hydroxylases for the following positions of the steroid molecule have been partially purified: 2α, 6α, 6β, 11β, 17α, 18, 19, 20, 21 and 22. The 2α-, 17α-, and 21-hydroxylases are located in the microsomes whereas the remaining enzymes are localized in the mitochondria (12). In the ovary, testis and placenta, the 17α-hydroxylases appear to be associated with the endoplasmic reticulum (13, 14). A mixed-function oxidase enzyme system that utilizes NADPH and molecular oxygen to catalyze the side-chain cleavage of cholesterol to pregnenolone appears to be an integral part of the mitochondrial structure of steroid-producing organs (14-16).

III. Cytochrome P-450 and its role in hydroxylation reactions

1. Activities catalyzed by cytochrome P-450-dependent enzyme systems

Cytochrome P-450 is distributed in the microsomes of a large variety of tissues and in the microsomes and mitochondria of various steroid-producing organs (11-18). Some of the well-known activities that are catalyzed by the cytochrome P-450-dependent enzyme systems in mammalian tissues are outlined in Table 1. All the reactions require the presence of NADPH and oxygen.

2. The role of NADPH-cytochrome c reductase in cytochrome P-450-catalyzed hydroxylation reactions

Cytochrome P-450 is the terminal oxidase which catalyzes the hydroxylation of a large number of drugs, steroids, carcinogens, alkanes, and fatty acids in liver microsomes (5, 22) and the hydroxylation of steroids in various steroidogenic organs (12-16). In these reactions, the necessary reducing equivalents for the reduction of cytochrome P-450 are supplied by NADPH via an electron transport system which includes the flavoenzyme

TABLE 1

SOME REACTIONS CATALYZED BY CYTOCHROME P-450-DEPENDENT ENZYME SYSTEMS

Number	Activity	Substrate(s)	Source	Species	References
1.	Hydroxylation of drugs and foreign compounds	codeine; acetanilide; 4-methyl-aminoantipyrine	liver microsomes	rat	(19, 20)
		cyclohexane; hexane; octane	liver microsomes	rat, rabbit	(21, 22)
		benzphetamine; ethylmorphine; hexobarbital; aminopyrine; norcodeine	liver microsomes	rat, rabbit	(21)
		3,4-benzpyrene; chlorcyclizine	liver microsomes	rat	(23)
2.	ω-Hydroxylation of fatty acids	laurate; octanoate; palmitoleate	liver microsomes	rat, rabbit	(21, 22)
		laurate	kidney cortex microsomes	rat	(24)
3.	Oxygenation of heme	heme	liver microsomes	rat, pig	(25)
		heme	spleen microsomes	rat, pig	(25)
4.	Lanosterol biosynthesis	squalene	liver microsomes	rat	(26)
5.	Hydroxylation of steroids -				
	1) 2β-hydroxylation	testosterone	liver microsomes	rat	(26)
	2) 6β-hydroxylation	testosterone; androstenedione; 17β-estradiol	liver microsomes	rat	(7, 26)
	3) 7α-hydroxylation	cholesterol; testosterone	liver microsomes	rat	(6, 7)
	4) 16α-hydroxylation	testosterone; 17β-estradiol; pregnenolone	liver microsomes	rat	(26, 27)
	5) 11β-hydroxylation	deoxycorticosterone	adrenal cortex mitochondria	steer	(12)
	6) 18-hydroxylation	corticosterone	adrenal cortex mitochondria	steer	(12)
	7) 17α-hydroxylation	progesterone	adrenal cortex microsomes	steer	(12)
			testis microsomes	rat	(13)
	8) 21-hydroxylation	17α-hydroxyprogesterone	adrenal cortex microsomes	steer	(12)
6.	Side-chain cleavage	cholesterol	mitochondria from adrenal cortex, testis, ovary, placenta	steer, rat	(14-16)

NADPH-cytochrome c reductase as the acceptor of electrons from NADPH.

The reduction of cytochrome P-450 by NADPH is normally measured at 450 nm under a carbon monoxide atmosphere in the absence of oxygen since the reduced hemoprotein in the membrane-bound state is highly autoxidizable. Carbon monoxide binds only the reduced form of cytochrome P-450, However, actual rates of cytochrome P-450 reduction are difficult to obtain since the initial phase of the reaction is very rapid. Therefore, exogenous cytochrome c is usually employed in the assay medium as the *in vitro* acceptor of electrons from NADPH.

The K_m for NADPH during aminopyrine demethylation in rabbit liver microsomes was found by Cohen and Estabrook (28) to be approximately 1 μM. This value correlates very well with the K_m values reported for NADPH (1-4 μM) by other investigators for purified NADPH-cytochrome c reductase (11, 29, 30).

The activity of microsomal NADPH-cytochrome c reductase is inhibited by $NADP^+$, by low concentrations of sulfhydryl reagents, and by antibody prepared against the flavoprotein (29-31). These compounds are known to inhibit to a similar extent various microsomal hydroxylation reactions (28, 32, 33).

3. Type I, Type II, and modified Type II spectral changes

Interaction of various compounds with oxidized cytochrome P-450 in liver microsomes is known to cause three types of alterations of the difference spectrum termed type I, type II, and modified type II spectral changes (34, 35). The type I spectral change is characterized by the formation of a peak at about 390 nm and a trough at about 420 nm in the difference spectrum. This type of spectral change was reported to be caused by a

modification of an existing ligand of the P-450 heme. A large number of compounds ranging from drugs and carcinogens to fatty acids and steroids are known to cause the type I spectral change and most of these substances appear to be substrates for the cytochrome P-450-dependent enzyme system (35).

Compounds causing the type II spectral change produce an absorption maximum at 425-435 nm and an absorption minimum at 390-395 nm in the difference spectrum. The type II ligands are believed to bind directly to the heme iron of oxidized cytochrome P-450 to form a hemichrome. At present, only a few type II compounds are known to act as substrates for cytochrome P-450 (35).

A third class of compounds cause spectral changes of the modified type II category. This spectral change was suggested as being due to the displacement of some pre-existing substrate, bound *in vivo*, from cytochrome P-450. Interaction of the hemoprotein with modified type II compounds appears to be at a site different from the type I-binding site and distinct from the type II (heme iron) site (35).

The type I, type II, and modified type II compounds are known to act as effective inhibitors of various cytochrome P-450-dependent hydroxylation reactions (11, 21, 36).

4. Conversion of cytochrome P-450 to cytochrome P-420

Cytochrome P-450 in liver microsomes acts as a site of both oxygen and substrate activation for hydroxylations of lipid-soluble compounds. The reactive area of cytochrome P-450, i.e. the vicinity of the heme, appears to be embedded in a highly hydrophobic portion of P-450 protein or phospholipid of the microsomal membrane and this hydrophobic environment seems to be essential for substrate hydroxylation. Reagents such as proteases, sulfhydryl

reagents, detergents, alcohols, protein denaturants, strong oxidizing agents, acidic or alkaline solutions alter the hydrophobic environment of the heme and change the conformation of cytochrome P-450 and this process results in the conversion of cytochrome P-450 into a modified form called cytochrome P-420, termed in this manner because the carbon monoxide complex of the reduced hemoprotein shows an absorption maximum at 420 nm. The conversion of cytochrome P-450 to cytochrome P-420 is usually accompanied by an inactivation of hydroxylating activity (11, 26, 37, 38).

5. Effect of trypsin on microsomal hydroxylation activity

Treatment of liver microsomes with low concentrations of trypsin (10 μg/mg protein) is known to solubilize NADPH-cytochrome c reductase completely but to leave the major portion of cytochrome P-450 still attached to the microsomal membrane. The trypsin treatment results in an inactivation of NADPH-cytochrome P-450 reductase activity parallel to the release of NADPH-cytochrome c reductase from the microsomal membrane. Associated with these two phenomena is a concomitant inhibition of the NADPH-dependent drug hydroxylation activity. This concentration of trypsin has no effect on cytochrome P-450 as such, indicating that the inhibitions observed are not due to the conversion of cytochrome P-450 into its inactive P-420 form. On the other hand, NADPH-cytochrome c reductase activity is not inhibited by the trypsin treatment and is in fact slightly enhanced. Orrenius and collaborators (38) concluded that the release of NADPH-cytochrome c reductase from the microsomal membrane is responsible for the decreased rate of cytochrome P-450 reduction by NADPH and the concomitant inhibition of drug hydroxylation activity.

6. Cytochrome b_5 and NADH in microsomal hydroxylation reactions

In addition to the NADPH-linked electron transport pathway, liver microsomes contain a second electron transport system, composed of NADH-cytochrome b_5 reductase (EC 1.6.2.2) and cytochrome b_5, which utilizes NADH as the source of reducing equivalents. NADH is capable of reducing cytochrome P-450 but the electron transport components involved in this reaction sequence have not been identified with certainty (32, 39). Ichikawa and associates (11) found that purified NADPH-cytochrome c reductase can accept NADH as an electron donor. However, the K_m of NADH (1.3 mM) for the isolated flavoenzyme was found to be about 400 times larger than the K_m of NADPH (3.2 μM) suggesting that the flavoprotein does not normally accept reducing equivalents from NADH. Later, Ichikawa and Loehr (39) discovered that cytochrome P-450 in hepatic submicrosomal particles devoid of cytochrome b_5 could be reduced by physiological concentrations of NADH (10 μM) presumably by way of the flavoenzyme NADH-cytochrome b_5 reductase. The K_m of NADH for the purified flavoprotein has been reported to be 2.7 μM (40).

The process of substrate hydroxylation by cytochrome P-450-dependent enzymes requires two reducing equivalents whereas the reduction of ferric cytochrome P-450 to ferrous cytochrome P-450 by NADPH is a one-electron process. Both NADPH and NADH can reduce cytochrome b_5 as well as cytochrome P-450 in liver microsomes. The first electron required for NADPH-dependent hydroxylation reactions is believed to be transferred to cytochrome P-450 from NADPH via the flavoenzyme NADPH-cytochrome c reductase. The pathway of the second electron transfer from NADPH to

cytochrome P-450 (see scheme 1) is less understood. Hildebrandt and Estabrook (32) suggested that reduced cytochrome $\underline{b}_5$ mediates the transfer of the second electron into the cytochrome P-450 system. On the other hand, Omura* (41) found that antibody against cytochrome $\underline{b}_5$ does not inhibit the NADPH-dependent hydroxylation of either aniline or aminopyrine indicating that cytochrome $\underline{b}_5$ may not be involved in the microsomal hydroxylation mechanism.

7. Different forms of cytochrome P-450

Cytochrome P-450 in microsomes exists both in a free state and in a form bound to endogenous substrate (42-44). Spectrophotometric and ESR studies have demonstrated the presence in liver microsomes of at least three forms of oxidized cytochrome P-450: (1) the low spin form of the hemoprotein observed in the absence of substrate; (2) a low spin form of cytochrome P-450 obtained when type II substances interact with the hemoprotein; (3) a high spin form of the ferric hemoprotein observed in the presence of type I compounds (44).

In addition to these three forms, two other species of cytochrome P-450 can be obtained in liver microsomes by pretreatment of animals with various barbiturates such as phenobarbital or with polycyclic carcinogens such as 3-methylcholanthrene. The phenobarbital-induced form of cytochrome P-450 is detectable by ESR spectroscopy in a low spin state whereas the 3-methylcholanthrene-induced form is detected as a high spin hemoprotein. These two forms of cytochrome P-450 differ from each other and from normal P-450 in structure and function (23, 41, 44-47). A sixth species of cytochrome P-450 that is detectable in a low spin state and exhibits a high

* Work cited in reference 41.

affinity for cyanide has recently been purified from liver microsomes by Comai and Gaylor (48).

In mammals, cytochrome P-450 is present in a large variety of tissues such as liver, lung, spleen, kidney, small intestine, pituitary, and in steroidogenic organs such as the adrenal, ovary, testis, and placenta. Although the hemoprotein is widely distributed, there is a considerable body of evidence indicating that the cytochrome P-450's found in different cell types or within a single cell differ both in structure and in function (5, 11, 12-20, 24, 25, 41, 44).

8. Induction of hepatic microsomal enzymes

Although more than 200 different compounds are known to enhance drug metabolism by inducing liver microsomal enzymes, most studies have been carried out with phenobarbital and the polycyclic hydrocarbons 3-methylcholanthrene and 3,4-benzpyrene. It has been established that phenobarbital and the polycyclic hydrocarbons exert their inducing effects by different mechanisms (5). For example, treatment of rats with phenobarbital accelerates almost all of the reactions catalyzed by cytochrome P-450 including the metabolism of ethylmorphine, hexobarbital, aminopyrine, acetanilide, and 3,4-benzpyrene whereas the administration of 3-methylcholanthrene induces relatively few enzymic reactions such as the metabolism of acetanilide and 3,4-benzpyrene but not that of ethylmorphine, hexobarbital, or aminopyrine.

Phenobarbital-like inducers markedly enhance the activity of NADPH-cytochrome c reductase and the concentration of cytochrome P-450, thus increasing the rate of reduction of the hemoprotein. Polycyclic hydrocarbons

such as 3-methylcholanthrene enhances the concentration of cytochrome P-450 but not the activity of NADPH-cytochrome c reductase or the rate of cytochrome P-450 reduction. It is thought that 3-methylcholanthrene induces the formation of a variant of cytochrome P-450 termed cytochrome P-448 which exhibits different spectral properties and affinities for the various drug substrates than does normal cytochrome P-450 (45).

9. *Reconstitution of the cytochrome P-450-dependent hydroxylating system from liver microsomes.*

Numerous attempts have been made in the past to solubilize and purify the liver microsomal cytochrome P-450 hydroxylating enzyme system in a functional state but investigators have been continually frustrated in thier attempts because of the ready conversion of the hemoprotein into its inactive P-420 form. In recent years, the liver microsomal cytochrome P-450 enzyme system has been solubilized and partially purified but these preparations do not catalyze the hydroxylation of substrates (49-51). Recently, however, Lu and collaborators (21-23, 52-55) have resolved a solubilized preparation of the cytochrome P-450 system from liver microsomes into three components: cytochrome P-450, NADPH-cytochrome c reductase, and a lipid fraction. The active component of the lipid fraction required for efficient reconstitution of hydroxylating activity was identified as phosphatidylcholine (56). This cytochrome P-450 preparation was capable of hydroxylating a large number of compounds such as drugs, steroids, fatty acids, and various hydrocarbons. However, it must be pointed out that the reductase fraction and the cytochrome P-450 preparations were by no means pure, and therefore, more efficient

reconstitution of hydroxylating activity must await further purification of these electron transport components in a functional state.

10. Mechanism suggested for substrate hydroxylation

A mechanism for substrate hydroxylation which has gained considerable support over the years is shown in scheme 1. There are at least six steps which occur in the cyclic reduction and oxidation of cytochrome P-450:

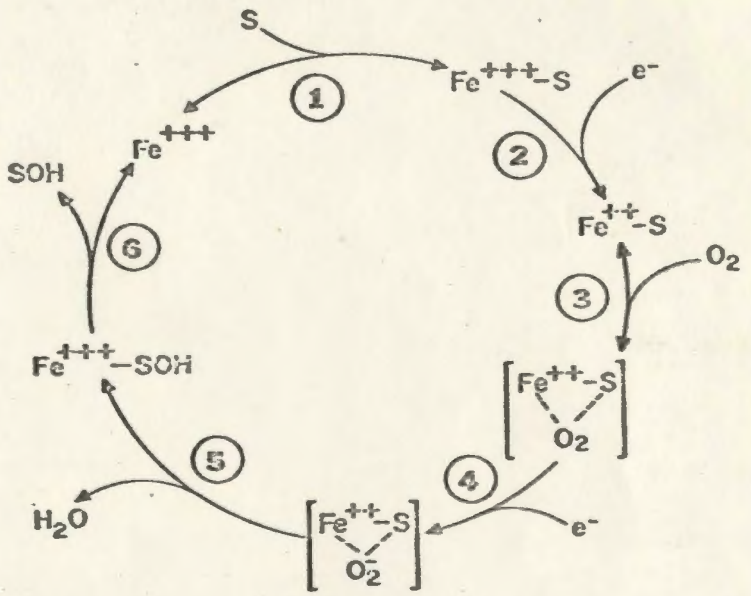
i) the reversible interaction of a substrate molecule with low spin ferric cytochrome P-450 accompanied by the formation of a high spin form of the ferric-substrate complex of the hemoprotein;

ii) the one-electron reduction of the high spin ferric-substrate complex of cytochrome P-450 to a ferrous-substrate complex;

iii) the reversible interaction of oxygen with the ferrous-substrate complex of cytochrome P-450 to form an oxygenated or oxy-ferrous-substrate complex;

iv) a second one-electron reduction step required to generate an intermediate which is as yet undefined. This intermediate may be a superoxide anion-ferrous-substrate complex of cytochrome P-450 or it may be a hydroperoxide derivative of the ferric-substrate complex of cytochrome P-450 or one of a variety of other forms;

v) a proposed rearrangement accompanying internal oxidation and reduction reactions resulting in the introduction of one atom of molecular oxygen into the organic substrate in the form of a hydroxyl group concomitant with the release of the other atom of oxygen as water. The mechanism of transfer of one atom of oxygen to substrate with the



Scheme 1. Mechanism for substrate (S) hydroxylation by cytochrome P-450

resultant formation of water from the remaining atom of oxygen remains to be elucidated;

vi) the dissociation of the hydroxylated product from ferric cytochrome P-450 with the regeneration of a low spin form of the hemoprotein, thus allowing for its participation in another cycle of the reaction (44).

Although not conclusive, the results obtained with the adrenal mitochondrial cytochrome P-450 and the liver and adrenal microsomal cytochrome P-450 enzyme systems (43,44) are consistent with the mechanism proposed in scheme 1.

11. Sterol demethylation activity in liver microsomes

In the conversion of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) to cholesterol, the methyl groups in positions 4α, 4β, and 14α of lanosterol are oxidized to carbon dioxide. The initial reaction of the oxidative demethylation process is an aerobic hydroxylation of each methyl group by a mixed-function oxidase enzyme requiring reduced pyridine nucleotide (either NADPH or NADH) as cofactor. The conversion of the hydroxymethyl groups to acetaldehyde derivatives followed by the formation of the carboxylic acid compounds and subsequent decarboxylation produces carbon dioxide. The first three steps require NAD(P)H and molecular oxygen whereas the decarboxylation process can occur under anaerobic conditions.

Gaylor and associates (48, 57-59) have recently proposed the involvement in the initial NADH-dependent hydroxylation reaction of a cytochrome P-450 species that exhibits a high affinity for cyanide. However, the sterol demethylation reaction is unique in that the activity is insensitive to inhibition by carbon monoxide and ethyl isocyanide, reagents which normally inhibit other microsomal cytochrome P-450-dependent

reactions.

12. Hydroperoxides as possible intermediates in hydroxylation reactions

A number of "activated oxygen" species (e.g. superoxide anions, hydroxyl radicals, oxenoid species, alkoxy free radicals, epoxide intermediates) have been proposed as initial intermediates in the cytochrome P-450-catalyzed hydroxylation mechanism (44,60-62). Hydroperoxides have also been postulated to occur as intermediates in enzymic hydroxylation reactions. For example, Grant (63) studied the 11β-hydroxylation of steroids by adrenal tissue and suggested that 11β-hydroperoxide was an intermediate. Hydroperoxides have been identified as intermediates in the NADPH-dependent hydroxylation of fluorene, tetralin, and other organic compounds by liver microsomal preparations (64-66). Cholesterol 20α-hydroperoxide was found to undergo an enzymic rearrangement to 20α,21-dihydroxycholesterol and 20 ,22-dihydroxycholesterol by a mitochondrial cytochrome P-450 preparation from adrenal cortex and the hydroperoxide has been proposed as an initial intermediate in pregnenolone biosynthesis (67-70). Boyer and associates (71) were the first to report the NADH-dependent reduction of alkyl hydroperoxides to alcohols by an enzyme preparation from Pseudomonas oleovorens.

13. Cytochrome P-450 as a microsomal peroxidase in lipid peroxide decomposition

The unsaturated fatty acids of intracellular membrane phospholipids readily undergo peroxidation in the presence of oxygen. Many deleterious effects of lipid peroxides on biochemical cellular constituents and subcellular fractions have been reported. Hochstein and Ernster (72) demonstrated the inactivation by lipid peroxides of enzymes associated

with the membranes of the endoplasmic reticulum.

The lipid peroxides formed in tissues are rapidly decomposed. O'Brien and Little (73) studied the decomposition of linoleic acid hydroperoxide by subcellular fractions of rat liver and found two types of mechanisms associated with the decomposition: (1) a reduction of the hydroperoxide by glutathione of the cytosol fraction catalyzed by glutathione peroxidase and resulting in the formation of monohydroxy linoleic acid; (2) a cytochrome-catalyzed decomposition of the hydroperoxide by the mitochondrial and microsomal fractions in which a complex range of products was formed presumably as a result of the interaction of free-radical intermediates.

The mechanism of hydroperoxide decomposition by liver microsomes was recently investigated by Hrycay and O'Brien (74) who identified cytochrome P-450 as the microsomal peroxidase responsible for the decomposition of a lipid peroxide substrate. The peroxidase activity of hepatic microsomes was measured using TMPD as the electron donor. Cytochrome P-450 is distributed in a large variety of tissues (17) and could therefore be an important intracellular site for the decomposition of various hydroperoxides.

Aim of the Thesis

The aim of the present work was to investigate the mechanism by which steroid and other organic hydroperoxides are decomposed by the cytochrome P-450-dependent oxidase system of microsomal fractions in order to assess the role of hydroperoxides as possible intermediates in microsomal hydroxylation reactions.

CHAPTER I

CYTOCHROME P-450 AS A MICROSOMAL PEROXIDASE IN STEROID HYDROPEROXIDE DECOMPOSITION

CHAPTER I: CYTOCHROME P-450 AS A MICROSOMAL PEROXIDASE IN STEROID HYDROPEROXIDE DECOMPOSITION

In the following chapter, the decomposition of steroid and other organic hydroperoxides by microsomal fractions will be investigated using TMPD as an electron donor.

MATERIALS AND METHODS

Materials

Steroids, nucleotides, n-octylamine, vitamin E, heme compounds, trypsin, trypsin inhibitor, p-hydroxymercuribenzoate, and GSH were purchased from Sigma. Phenacetin and TMPD were from British Drug Houses, Toronto. Cumene hydroperoxide and aminopyrine were supplied by Matheson, Coleman and Bell. Lubrol WX was kindly donated by Imperial Chemical Industries, Providence, Rhode Island. 2-Phenyl-2-propanol was obtained from Aldrich Chemical Company, Milwaukee. All other reagents were of the highest grade commercially available.

Preparation of steroid hydroperoxides

The 17α-hydroperoxide derivatives of progesterone, pregnenolone, and allopregnenolone were prepared by the method of Bailey et al.(75). Cholesterol 7α-, 7β-, 20α-, 25-, and 26-hydroperoxides as well as cholest-4-en-3-one 6 -hydroperoxide were prepared by autoxidation of cholesterol as previously described (76-78). Their purity was verified by tlc, gc, ms, or chemical analysis (75-78).

Preparation of linoleic acid hydroperoxide

Linoleic acid hydroperoxide was prepared by aerial oxidation of

linoleic acid and purified by silica gel chromatography as described by O'Brien (79). The concentration was determined by measuring the absorbance at 233 nm in ethanol using an extinction coefficient of 25.25 $cm^{-1}mM^{-1}$.

Preparation of microsomes

Liver microsomes were prepared from perfused livers of Sprague-Dawley rats (250-350 g) as previously described (74). The microsomes were suspended in 0.3 M sucrose - 2 mM EDTA (pH 7.4) and used within 24 hours of preparation. No loss in activity occurred during this period when microsomes were stored at 0°. Adrenocortical microsomes were prepared from bovine adrenal glands. The adrenals were defatted, demedullated, and the cortical tissue washed with cold isotonic KCl to remove adsorbed hemoglobin. The cortical tissue was homogenized in 4 volumes of cold sucrose-EDTA solution, the homogenate centrifuged at 14,000 g for 20 minutes and the pellet discarded. The supernatant was centrifuged at 105,000 g for 60 minutes, the microsomal pellet was resuspended in 0.15 M KCl - 2 mM EDTA (pH 7.4) and centrifuged as above. The washed microsomes were finally suspended in 0.3 M sucrose - 2 mM EDTA (pH 7.4) and used within 24 hours of preparation. Microsomes from other tissues of the rat were obtained by a similar procedure as was used for the preparation of adrenocortical microsomes.

Assay of peroxidase activity

Peroxidase activity in microsomal fractions was assayed at 23° C with TMPD as hydrogen donor (74) using a reaction medium normally containing 0.033 M sodium phosphate buffer (pH 7.4), 1.5 mM EDTA, 0.27 M sucrose, 0.2 mM TMPD, 1 mg microsomal protein and 0.05 mM organic hydroperoxide in a final volume of 3 ml. The rate of TMPD oxidation was followed spectrophotometrically at 610 nm in the first minute of reaction by measuring the

formation of Wurster's blue free radical (80). Reaction rates were corrected for TMPD oxidation in the absence of hydroperoxide. An extinction coefficient for TMPD of 11.6 cm^{-1} mM^{-1} (80) was employed in the calculation of the reaction rates. The use of large concentrations of TMPD in the assay medium was to be avoided because of the relatively high rate of autoxidation of TMPD at pH 7.4 (81).

When the peroxidase reaction was measured using adrenocortical microsomes, a substantial control rate of TMPD oxidation due to mitochondrial contamination, as evidenced by the presence of cytochrome oxidase activity, was observed in the absence of hydroperoxide. Therefore, assays were carried out in the presence of 0.1 mM sodium cyanide which reduced the control rate to an almost negligible amount.

Steroid hydroperoxides were dissolved in ethanol prior to use and microliter amounts were added to the assay medium. Because of the poor solubility in aqueous medium of the steroid hydroperoxides, assays were carried out in the presence of 0.27 M sucrose to assist in the solubilization of the hydroperoxides.

Analytical Methods

Cytochromes P-450, P-420, and b_5 were determined as described previously (74). Protein was measured by the method of Lowry et al (82) using bovine serum albumin as standard.

RESULTS

The TMPD-peroxidase assay

The peroxidase activity of microsomal fractions and heme compounds was investigated using TMPD as hydrogen donor and cumene hydroperoxide and a wide variety of steroid hydroperoxides as substrates. The oxidation of TMPD was followed at 610 nm by measuring the rate of formation of Wurster's blue free radical. This is a new peroxidase assay and has the advantage of high sensitivity, a product that is stable over a wide pH range, good stoichiometry, and an almost negligible control rate in the absence of hydroperoxide or catalyst (74), 80). Other hydrogen donors used in peroxidase assays such as guaiacol, pyrogallol, o-dianisidine, hydroquinone, catechol, and resorcinol gave no satisfactory reaction rate for microsomal peroxidase with the concentration of hydroperoxide normally employed in the TMPD assay method.

Kinetic properties of microsomal peroxidase

The kinetic properties of microsomal peroxidase were investigated using liver or adrenocortical microsomes as the enzyme source and cumene hydroperoxide and the 17α-hydroperoxide derivatives of progesterone and pregnenolone as substrates. The reaction rate with cumene hydroperoxide was linear for the first 5 minutes of reaction whereas the rate with steroid hydroperoxides was non-linear with time because of product inhibition. Maintaining the TMPD concentration at 0.2 mM, the reaction rate was found to be first-order with respect to microsomal protein concentration (Fig. 1) and first-order with respect to cumene hydroperoxide concentration (Fig. 2) in the concentration range 0.05 - 1.0 mM*. Using the 17α-

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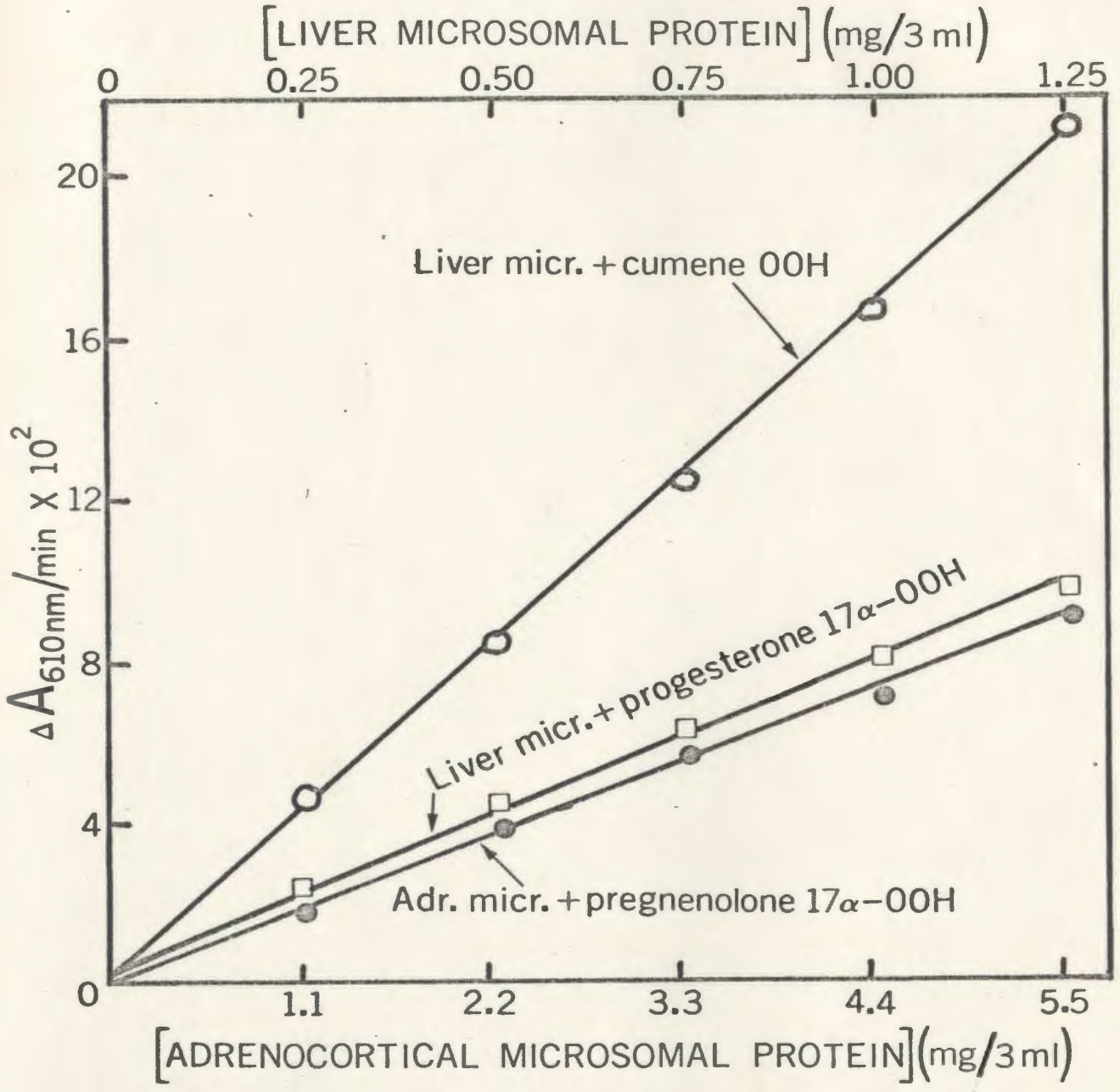
* *Higher concentrations produced very large reaction rates which could not be measured accurately.*

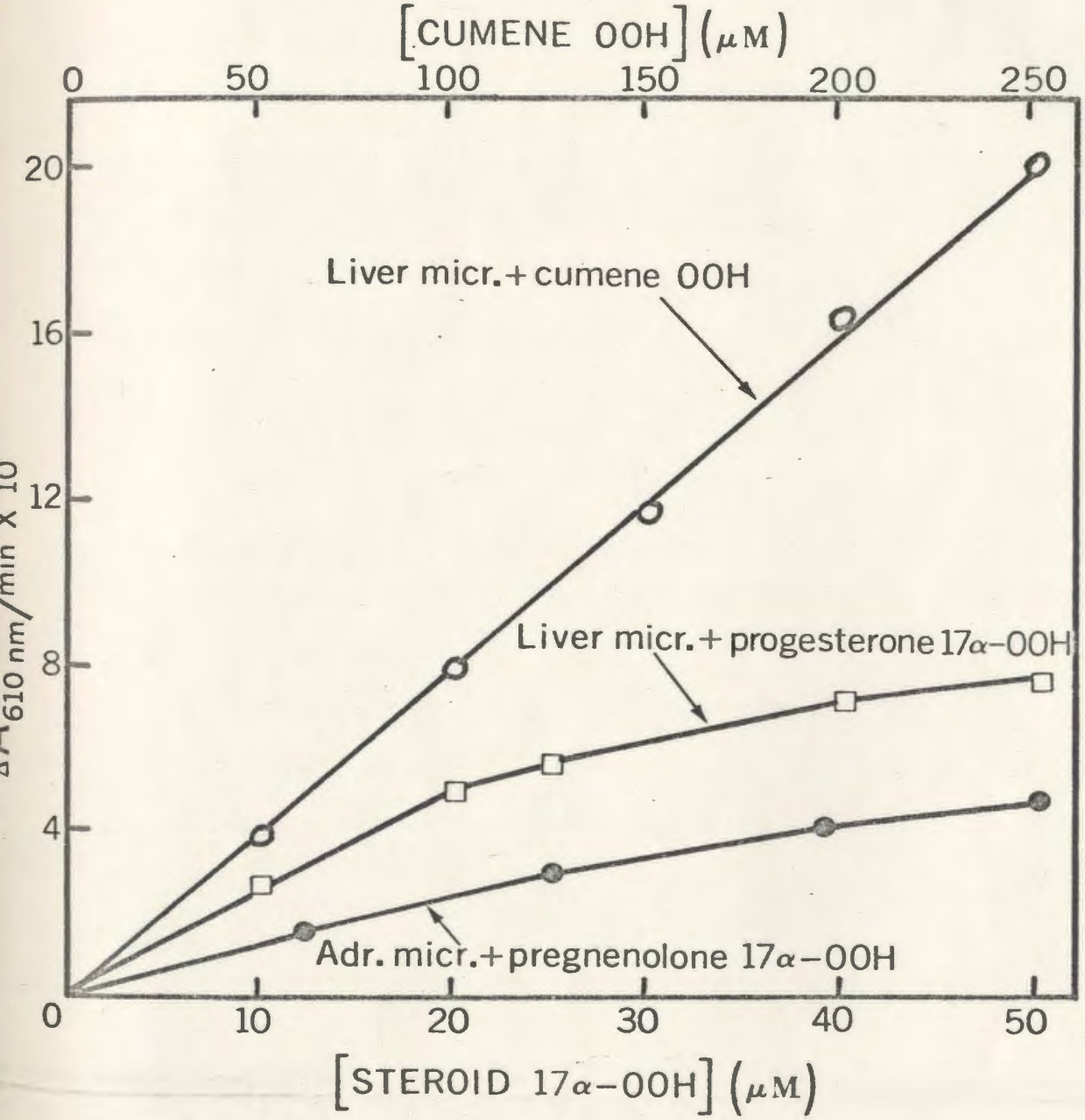
Figure 1. Effect of microsomal protein concentration on the rate of TMPD oxidation by hydroperoxides.



Peroxidase activity was measured at 23° with TMPD as hydrogen donor at 610 nm in a 3 ml system consisting of 0.033 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.27 M sucrose, 0.2 mM TMPD, varying amounts of microsomal protein and 0.2 mM cumene hydroperoxide or 0.05 mM progesterone 17α-hydroperoxide or pregnenolone 17α-hydroperoxide.

Figure 2. Effect of hydroperoxide concentration on the rate of catalyzed TMPD oxidation.

Assay conditions were similar to those in Figure 1 except that 1 mg liver microsomal protein and 2.2 mg adrenocortical microsomal protein were used and the hydroperoxide concentration varied.



hydroperoxide derivatives of progesterone and pregnenolone as substrates, the reaction rate showed saturation kinetics with increasing concentrations of hydroperoxide (Fig. 2). When the concentration of TMPD was varied, the reaction rate showed saturation kinetics with increasing concentrations of TMPD (Fig. 3). The range of steroid hydroperoxide concentrations examined was 0.01 - 0.1 mM. Concentrations exceeding 0.1 mM could not be used as the steroid hydroperoxides came out of aqueous solution under the specified conditions of the peroxidase assay.

The effect of pH on the peroxidase activity of liver microsomes using cumene hydroperoxide and progesterone 17α-hydroperoxide as substrates is illustrated in Fig. 4. The reaction rate with cumene hydroperoxide showed a pH optimum in the range 6.5 - 7.0. A pH optimum of between 6.8 and 7.6 was obtained with progesterone 17α-hydroperoxide.

Hydroperoxide specificity of microsomal peroxidase

The hydroperoxide specificity of microsomal peroxidase is presented in Table 2. It is seen that the 17α-hydroperoxide derivatives of progesterone and pregnenolone were very effective substrates with either liver or adrenocortical microsomes as the enzyme source. The cholesterol hydroperoxides were found to be much less effective substrates for microsomal peroxidase, possibly because the cholesterol side chain sterically hinders the interaction between the hydroperoxide moiety and the heme catalyst.

Figure 3. Effect of TMPD concentration on the rate of catalyzed TMPD oxidation by hydroperoxides.

Assay conditions were similar to those in Figure 1 except that 1 mg liver microsomal protein and 2.2 mg adrenocortical microsomal protein were used and the concentration of TMPD varied. Reaction rates were corrected for TMPD oxidation in the absence of hydroperoxide.

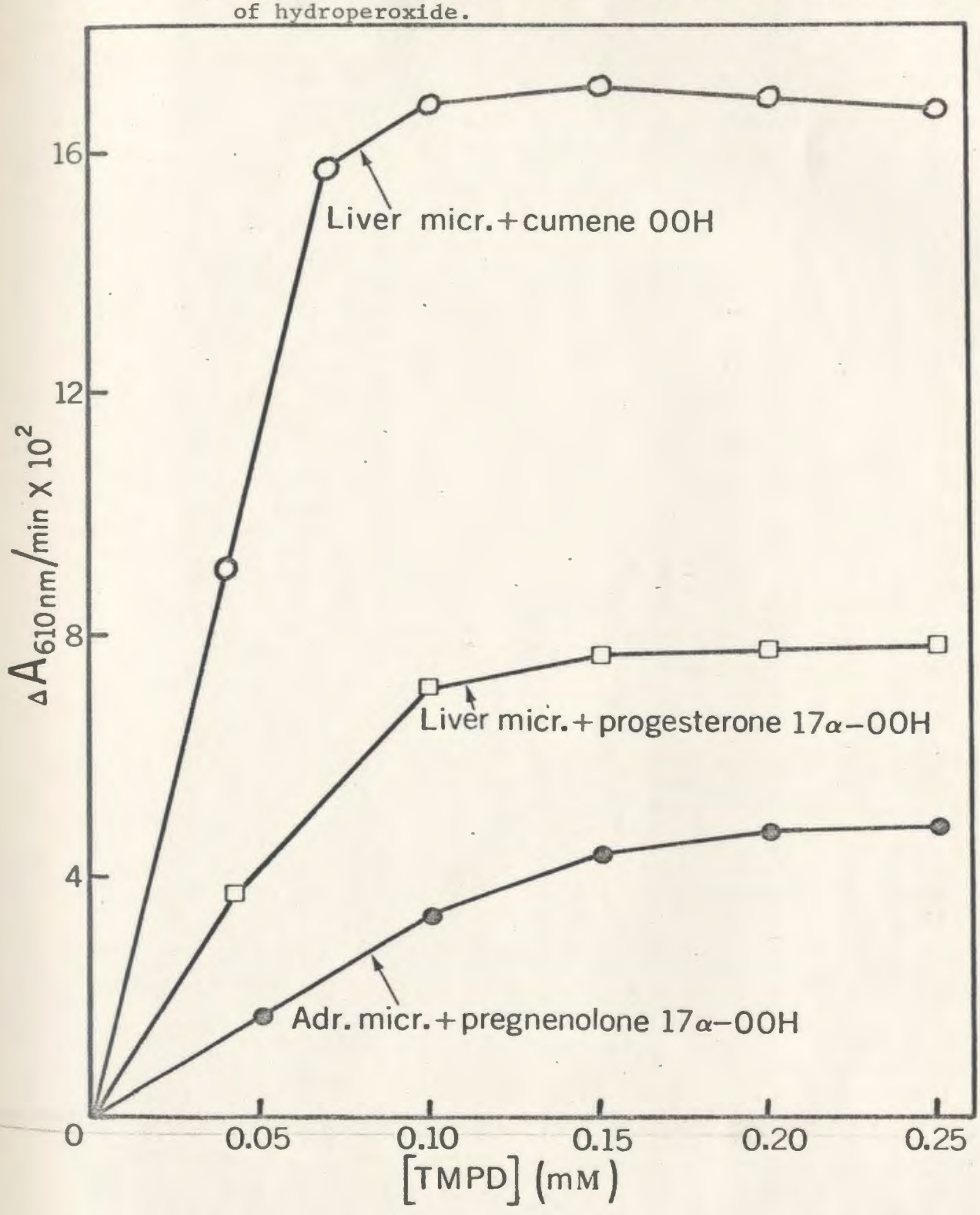


Figure 4. Effect of pH on the peroxidase activity of liver microsomes.

The peroxidase reaction was measured in a 3 ml solution containing 1 mM EDTA, 0.27 M sucrose, 0.2 mM TMPD, 1 mg microsomal protein, and 0.2 mM cumene hydroperoxide or 0.05 mM progesterone 17α-hydroperoxide. The pH was adjusted with HCl or NaOH. At pH values of between 4.2 and 4.8, an increase in turbidity due to protein aggregation was observed prior to addition of hydroperoxide. In these cases, the reaction rate was measured by addition of hydroperoxide after there was no more increase in turbidity.

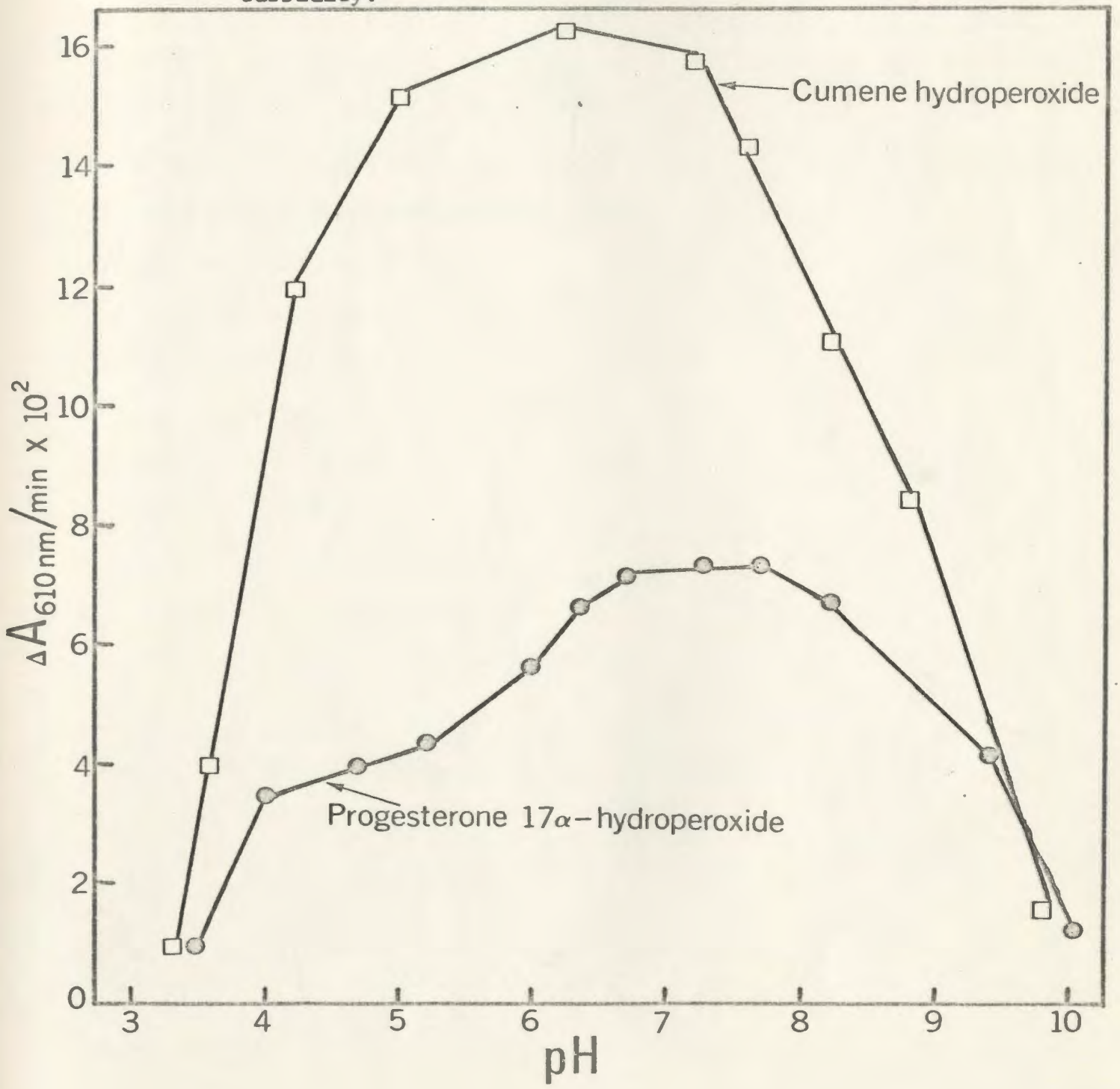


TABLE 2

HYDROPEROXIDE SPECIFICITY OF MICROSOMAL PEROXIDASE[a]

Hydroperoxide	Peroxidase Activity: Liver Microsomes	Peroxidase Activity: Adrenocortical Microsomes
Progesterone 17α-hydroperoxide	92	31
Pregnenolone 17α-hydroperoxide	80	30
Allopregnanolone 17α-hydroperoxide	76	27
Linoleic acid hydroperoxide	75	25
Cumene hydroperoxide	34	12
Cholesterol 7β-hydroperoxide	30	17
Cholest-4-ene-3-one 6β-hydroperoxide	30	10
Cholesterol 7α-hydroperoxide	22	9
Cholesterol 25-hydroperoxide	17	9
Cholesterol 26-hydroperoxide	15	9
Cholesterol 20α-hydroperoxide	12	5

[a] Assay medium contained 0.033 M sodium phosphate (pH 7.4), 1.5 mM EDTA, 0.27 M sucrose, 0.2 mM TMPD, 2 mg microsomal protein, and 0.05 mM hydroperoxide in a 3 ml volume. The peroxidase activity of adrenocortical microsomes was measured in the presence of 0.1 mM sodium cyanide to inhibit the control TMPD oxidase rate. Reaction rates were corrected for TMPD oxidation in the absence of hydroperoxide and are expressed as $\Delta A'_{610\ nm}$/min/μ mole P-450.

Tissue distribution of microsomal peroxidase activity

In Table 3 is shown the tissue distribution of microsomal peroxidase activity. It is seen that microsomal fractions from tissues which contained high amounts of cytochrome P-450 per milligram protein (e.g. liver, adrenal gland) were the most active in catalyzing the peroxidase reaction whereas microsomes from tissues that contained little or no cytochrome P-450 showed either weak activity or no activity at all. Adrenal microsomes were about 4 times less effective than liver microsomes in catalyzing the peroxidase reaction with both fractions containing similar cytochrome P-450 levels.

The properties of microsomal peroxidase from rat liver and bovine adrenal cortex were next examined in detail.

Inhibition of microsomal peroxidase activity by various reagents

The effect of various reagents on the peroxidase activity of liver microsomes using cumene hydroperoxide as substrate is presented in Table 4. Cumene hydroperoxide was chosen as the model substrate for inhibitor studies because it is sufficiently soluble in water (84) to permit study of the peroxidase reaction. It is seen that heat treatment of microsomes completely abolished the activity which indicates the enzymic nature of the peroxidase reaction. Since EDTA did not affect the reaction rate, this suggests that weakly bound inorganic metal ions are unlikely to be involved. Sodium azide (10 mM) produced a 20% inactivation whereas N_2 and sodium fluoride exhibited little effect. CO produced no appreciable inhibition which indicates that the peroxidase activity of microsomes is not due to contaminating hemoglobin. No significant inhibition was

TABLE 3

TISSUE DISTRIBUTION OF MICROSOMAL PEROXIDASE ACTIVITY[a]

Source of Microsomes	P-450 Content	Peroxidase Activity
Rat liver	0.70	0.170
adrenal gland	0.53	0.040
kidney	0.25	0.010
lung	0.05	0.030
testis	0.02	0.008
small intestine	0.02	0.007
heart	0	0
skeletal muscle[b]	0	0
brain	0	0
Bovine adrenal cortex	0.60	0.030

[a] Assay system contained 0.067 M sodium phosphate (pH 7.4), 1 mM EDTA, 0.2 mM TMPD, 1 mg microsomal protein, 0.1 mM sodium cyanide, and 0.2 mM cumene hydroperoxide in a final volume of 3 ml. Peroxidase activity is expressed as $\Delta A_{610\ nm}$/min/ mg protein whereas P-450 content is given as nmoles/mg protein.

[b] The hind legs of the rat were used for the source of skeletal muscle.

TABLE 4

EFFECT OF VARIOUS MODIFIERS ON HEPATIC MICROSOMAL PEROXIDASE ACTIVITY[a]

Modifier	% Inhibition
Heat (80° for 5 min)	95
Sodium azide (10 mM)	20
Carbon monoxide[b]	4
Nitrogen[b]	3
EDTA (10 mM)	7
Sodium fluoride (10 mM)	4
2-phenyl-2-propanol (2 mM)	2

[a] The assay medium contained 0.067 M sodium phosphate (pH 7.5), 1 mM EDTA, 0.2 mM TMPD, 1 mg liver microsomal protein, 0.2 mM cumene hydroperoxide, and specified conc. of various modifiers in a 3 ml reaction volume. The specific activity of microsomal peroxidase before addition of modifier, expressed as $\Delta A_{610\ nm}$/min/mg protein, was 0.17.

[b] An aerobic sample cuvette was gassed for 15 min with nitrogen (99.99% pure) or CO that had been previously passed through a deoxygenating medium (83).

obtained when an excess of 2-phenyl-2-propanol, the hydroxy derivative and main decomposition product of cumene hydroperoxide, was added to the peroxidase assay.

Similar inhibitions by these modifiers were observed when adrenocortical microsomes were employed as the enzyme source.

Inhibition of peroxidase activity by reagents that convert cytochrome P-450 to cytochrome P-420

The conversion of microsomal cytochrome P-450 into its inactive P-420 form is brought about by a wide variety of reagents such as proteases, sulfhydryl reagents, lipophilic substances, surface-active agents, strong oxidizing agents, and acidic or alkaline pH (11, 26, 37, 50). The effects of several of these reagents on microsomal peroxidase activity using cumene hydroperoxide as substrate are illustrated in Table 5. The results strongly suggest that the conversion of microsomal cytochrome P-450 to cytochrome P-420 by various reagents is responsible for the observed inhibition of peroxidase activity.

Similar inhibitions by these P-450 modifiers were obtained when adrenocortical microsomes were substituted for liver microsomes or when progesterone 17α-hydroperoxide was used as substrate.

Inhibition of peroxidase activity by type I and type II compounds

Compounds termed type I, type II, and modified type II substrates interact specifically with cytochrome P-450 to produce characteristic spectral changes (34, 35). The effects of several of these substances on the peroxidase activity of liver microsomes is shown in Table 6. The results indicate that these compounds effectively inhibited the peroxidase

TABLE 5

INHIBITION OF HEPATIC MICROSOMAL PEROXIDASE ACTIVITY BY REAGENTS THAT CONVERT CYTOCHROME P-450 TO CYTOCHROME P-420 [a]

P-450 Modifier	% Inhibition	% P-420 Formed
None	0	0
N-bromosuccinimide (0.4 mM)	50	44
Iodine (0.2 mM)	60	45
p-hydroxymercuribenzoate (0.5 mM)	66	67
Sodium dodecyl sulfate (0.1%)	90	95
Lubrol WX (1%)	62	73
Urea (4 M)	38	44
Trypsin [b]	54	62
n-Propanol	85	100

[a] Liver microsomes at a protein conc. of 5 mg/ml were incubated at 20° for 15 min with specified conc. of various P-450 modifiers in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.05 M sucrose. After incubation samples were diluted with an equal volume of 0.1 M sodium phosphate buffer (pH 7.4), peroxidase activity using 0.2 mM cumene hydroperoxide measured, and the extent of conversion of P-450 to P-420 determined.

[b] Liver microsomes (10 mg protein/ml) were incubated at 23° for 90 minutes with 50 µg trypsin/mg protein in 0.04 M sodium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.3 M sucrose. Peroxidase activity and the extent of conversion of P-450 to P-420 were determined.

TABLE 6

INHIBITION OF HEPATIC MICROSOMAL PEROXIDASE ACTIVITY BY TYPE I AND TYPE II LIGANDS [a]

Type I Ligand	% Inhibition	Type II Ligand	% Inhibition	Modified Type II Ligand	% Inhibition
Androstenedione (0.1 mM)	65	Aniline (10 mM)	83	Sodium cyanide (1 mM)	56
Testosterone (0.1 mM)	65	Imidazole (1 mM)	82	Corticosterone (0.1 mM)	55
17β-estradiol (0.1 mM)	50	Pyridine (10 mM)	82	Phenacetin [b] (1 mM)	55
Aminopyrine (5 mM)	45	n-Octylamine (0.05 mM)	40	Ethanol (7%)	68
Hexobarbital (0.5 mM)	34			Methanol (7%)	66
Linoleic acid (0.1 mM)	34				

[a] Peroxidase activity was measured in the presence of specified amounts of various type I and type II ligands as described in Table 4.

[b] Phenacetin = p-ethoxyacetanilide.

reaction.

In Table 7 is illustrated the effect of various type I and type II ligands on adrenocortical microsomal peroxidase activity using progesterone 17α-hydroperoxide as substrate. The peroxidase reaction was inhibited by 17α-hydroxyprogesterone, the main decomposition product of progesterone 17α-hydroperoxide, suggesting a feedback mechanism of inhibition. Other type I and type II ligands also produced strong inhibitory effects.

Jefcoate et al (85) investigated the binding of the type II ligand, n-octylamine, to the low spin and high spin forms of oxidized cytochrome P-450 present in liver microsomes. Two binding constants for n-octylamine of 0.05 mM and 0.034 mM were obtained for low spin P-450 and a binding constant of 0.3 mM was found for high spin P-450. In contrast to liver microsomes, only the low spin form of oxidized cytochrome P-450 was detected in adrenocortical microsomes and a binding constant for n-octylamine of about 1 mM was observed (85). Our results indicate a 40% inhibition of hepatic microsomal peroxidase activity by 0.05 mM n-octylamine (Table 6) and a 55% inactivation of adrenocortical microsomal peroxidase activity by 1 mM n-octylamine (Table 7), suggesting a possible involvement of low spin cytochrome P-450 in the peroxidase mechanism of hepatic and adrenocortical microsomes.

Inhibition of peroxidase activity by cyanide

Cyanide has been reported to inhibit several cytochrome P-450-dependent oxidation reactions (11, 12, 58, 59). The effect of cyanide on the microsomal TMPD-peroxidase reaction is illustrated in Fig. 5. The

TABLE 7

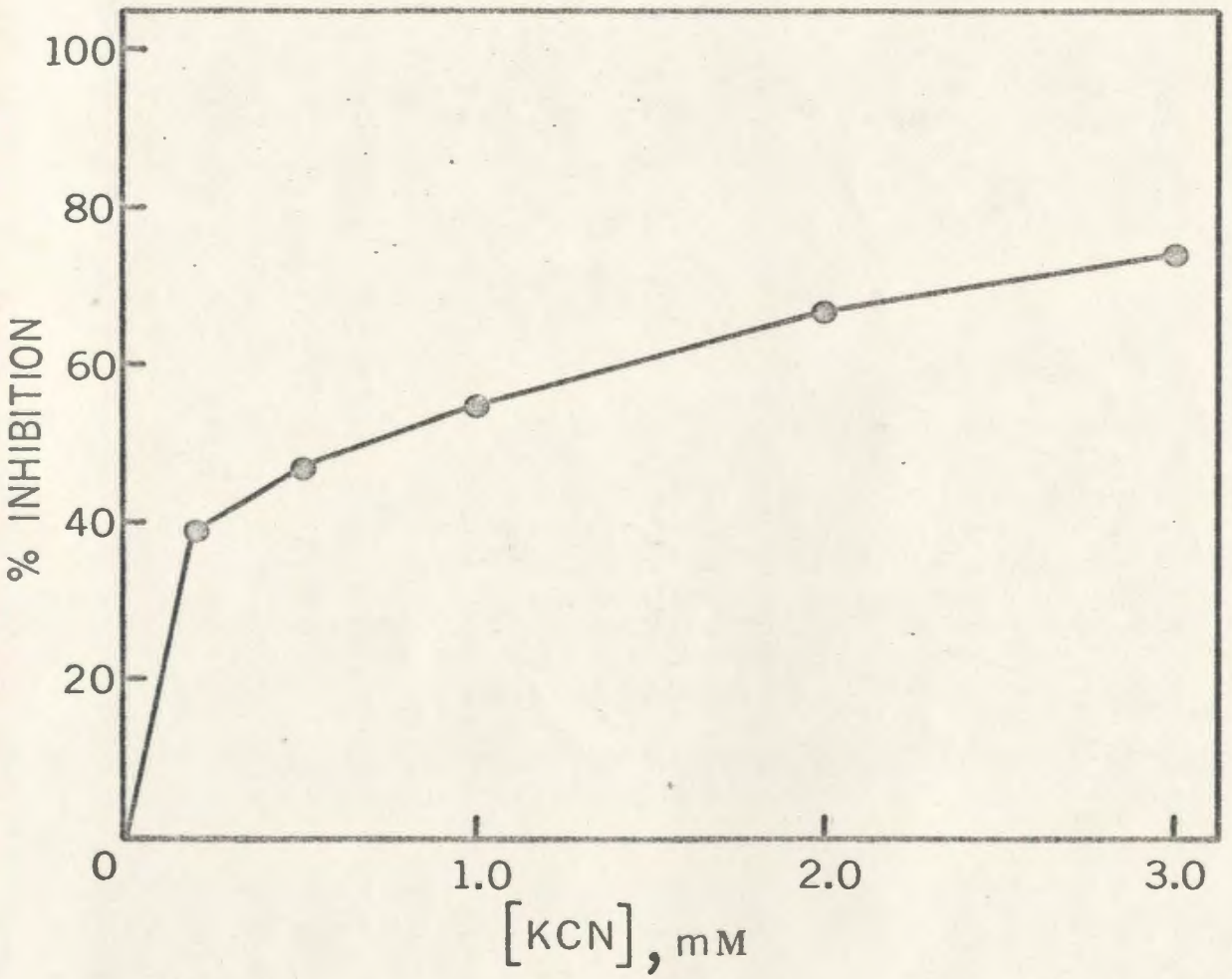
INHIBITION OF ADRENOCORTICAL MICROSOMAL PEROXIDASE ACTIVITY BY TYPE I AND TYPE II LIGANDS [a]

Type I Ligand	% Inhibition
Testosterone (0.1 mM)	44
Progesterone (0.1 mM)	39
17α-Hydroxyprogesterone (0.1 mM)	35
Androstenedione (0.1 mM)	34
Deoxycorticosterone (0.1 mM)	28
Type II Ligand	
n-Octylamine (1 mM)	55
Imidazole (2 mM)	40
Corticosterone (0.1 mM)	28

[a] Assay medium contained 0.033 M sodium phosphate (pH 7.5), 1.5 mM EDTA, 0.27 M sucrose, 0.1 mM sodium cyanide, 0.2 mM TMPD, 2 mg adrenocortical microsomal protein, 0.05 mM progesterone 17α-hydroperoxide, and specified concentrations of modifiers. The specific activity of microsomal peroxidase before addition of ligands, expressed as $\Delta A_{610\ nm}$/min/mg protein, was 0.016.

Figure 5. Cyanide inhibition curve for microsomal TMPD-peroxidase activity.

The reaction was measured in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.2 mM TMPD, 1 mg liver microsomal protein, indicated amounts of potassium cyanide, and 0.2 mM cumene hydroperoxide.



results show that cyanide was a fairly effective inhibitor of the reaction, a 50 percent inhibition occurring at a cyanide concentration of about 0.8 mM.

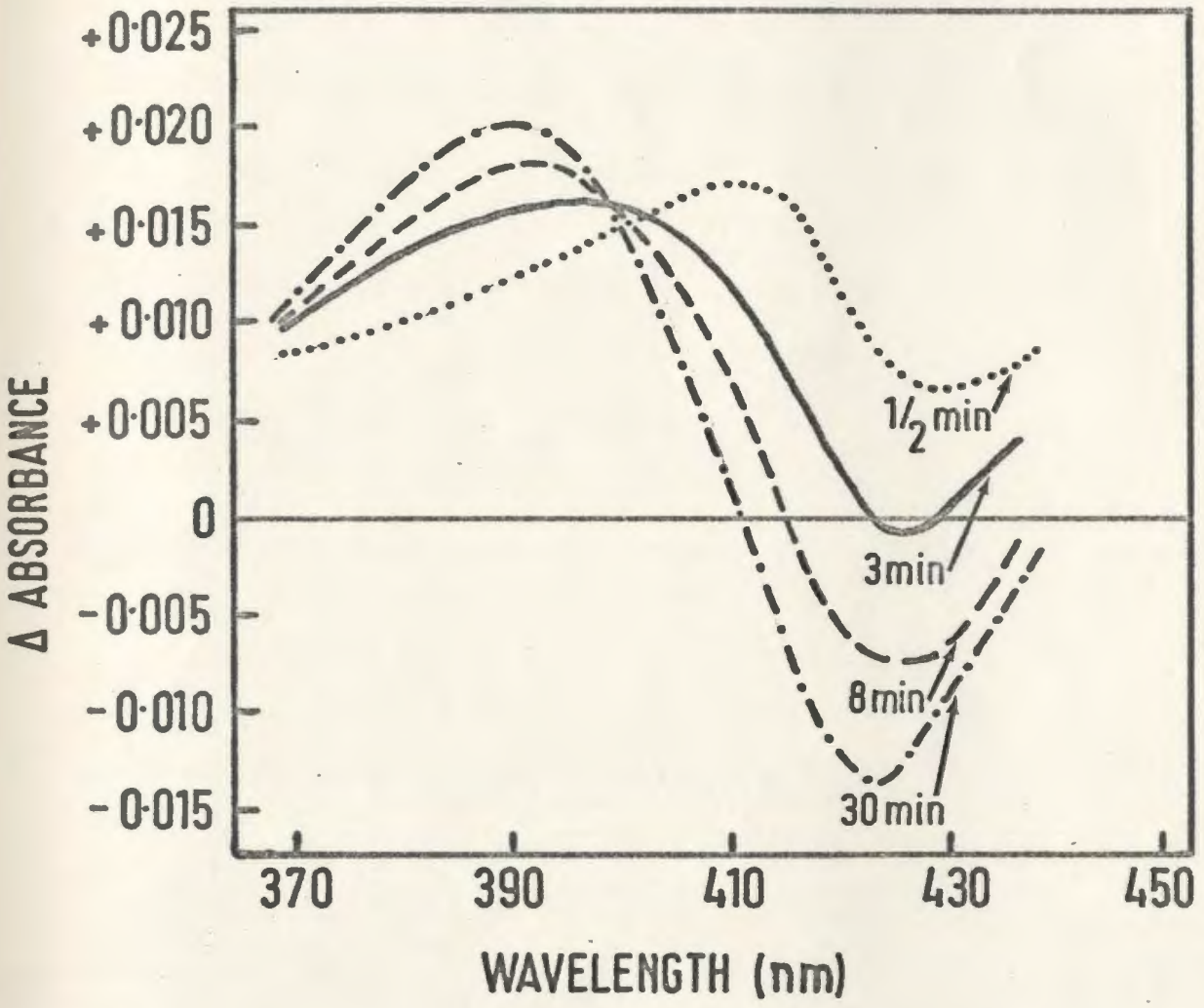
Spectral changes during hydroperoxide interaction with microsomal fractions

Fig. 6 presents the spectral changes induced by progesterone 17α-hydroperoxide interaction with adrenocortical microsomal suspensions. Difference spectra were carried out in the presence of 0.15 M sucrose to assist in the solubilization of the hydroperoxide. However, larger amounts of sucrose were to be avoided since polyols are known to decrease the magnitude of the type I spectral change induced by various steroids (86). The initial difference spectrum of progesterone 17α-hydroperoxide showed a broad peak at about 412 nm and a trough at 430 nm. After 15 minutes a type I difference spectrum with a peak at about 390 nm and a trough at 422 nm was obtained. This spectrum was fully developed after 30 minutes of reaction and was identical to the type I difference spectrum produced by interaction of 0.05 mM 17α-hydroxyprogesterone with a similar concentration of adrenocortical microsomal protein. Extraction of the reaction medium with methylene chloride at 45 minutes and analysis of the products by tlc, gas chromatography, and mass spectrometry (as described in Chapter 2) established that progesterone 17α-hydroperoxide had been converted to 17α-hydroxyprogesterone in 65% yield. A small amount of androstenedione (20%) was also formed and the rest was unconverted hydroperoxide.

Both 17α-hydroxyprogesterone and androstenedione produce type I spectral changes when added to adrenocortical microsomal suspensions but

Figure 6. Spectral changes produced during progesterone 17α-hydroperoxide decomposition by adrenocortical microsomes.

In each of two cuvettes (1 cm light path) was placed 3 ml of a microsomal suspension (2.5 mg protein/ml) containing 0.067 M sodium phosphate buffer (pH 7.5), 1.5 mM EDTA, 0.15 M sucrose and the baseline was recorded using an AMINCO-CHANCE



spectrophotometer. To the sample cuvette was added 20 µl of an ethanolic solution of progesterone 17α-hydroperoxide (final concentration 0.05 mM), the reference cuvette received an equivalent volume of ethanol, and the difference spectrum was recorded at 23° at indicated time intervals. After 30 min, the reaction medium was extracted with methylene chloride and the products analyzed.

the magnitude of the spectral change produced by 17α-hydroxyprogesterone is about 5 times greater than that produced by an equivalent amount of androstenedione (12). The fact that 17α-hydroxyprogesterone was the major reaction product formed during progesterone 17α-hydroperoxide decomposition by adrenocortical microsomes and because the hydroxysteroid produces a much more intense spectral change than does androstenedione suggests that the major portion of the type I-induced difference spectrum produced during the course of progesterone 17α-hydroperoxide decomposition by adrenocortical microsomes is the result of the interaction of newly-formed 17α-hydroxyprogesterone with microsomal cytochrome P-450.

No such spectral changes were observed when progesterone 17α-hydroperoxide or cumene hydroperoxide were incubated with liver microsomal suspensions.

Comparison of adrenocortical microsomal peroxidase activity with the rate of progesterone 17α-hydroperoxide reduction by microsomes

The peroxidase activity of adrenocortical microsomes as measured by TMPD oxidation and using 0.05 mM progesterone 17α-hydroperoxide as substrate gave a rate of approximately 4 nmoles/min/mg protein. The rate of conversion of progesterone 17α-hydroperoxide to 17α-hydroxyprogesterone by adrenocortical microsomes was found to be approximately 1.5 nmoles/min/mg protein. The latter value was calculated using the ΔA (390-420nm) after 3 minutes of reaction (Fig. 5) and employing the relationship 12 μM 17α-hydroxyprogesterone yields a ΔA (390-420 nm) = 0.017/cm/mg protein (12).

Effect of phenobarbital or 3-methylcholanthrene pretreatment of rats on microsomal peroxidase activity

Treatment of rats or rabbits with phenobarbital or 3-methylcholanthrene is known to cause a marked elevation in the specific content of hepatic microsomal cytochrome P-450 without appreciably affecting the concentration of cytochrome b_5 (41). The cytochrome P-450 from liver microsomes of induced animals differs in several of its physical and biochemical properties from normal cytochrome P-450 (45, 46). Whether the new P-450 synthesized in the liver in response to phenobarbital or 3-methylcholanthrene pretreatment possesses similar peroxidase properties to normal P-450 was next examined. The results (Table 8) show that the specific activity of microsomal peroxidase using progesterone 17α-hydroperoxide as substrate was enhanced 2.8-fold by *in vivo* phenobarbital treatment. This activation was accompanied by a 3-fold increase in P-450 content whereas cytochrome b_5 showed only a 1.3-fold increase. Injection of rats with 3-methylcholanthrene increased the specific activity of microsomal peroxidase 1.4-fold, with progesterone 17α-hydroperoxide as substrate, and this stimulation was accompanied by a similar elevation in P-450 content whereas cytochrome b_5 remained virtually unaffected.

In contrast, pronounced differences in the peroxidase activity of microsomal cytochrome P-450 from uninduced and phenobarbital or 3-methylcholanthrene-induced rats were obtained using cumene hydroperoxide as substrate (Table 8). The specific activity of microsomal peroxidase was enhanced 19-fold by phenobarbital treatment whereas the specific content

TABLE 8

EFFECT OF PHENOBARBITAL OR 3-METHYLCHOLANTHRENE PRETREATMENT OF RATS ON MICROSOMAL PEROXIDASE ACTIVITY [a]

Pretreatment	Cytochrome b_5	Cytochrome P-450	Peroxidase Activity	
	nmoles/mg protein	nmoles/mg protein	$\Delta A_{610\ nm}$/min/mg protein	
			progesterone 17α-hydroperoxide	cumene hydroperoxide
Saline	0.40	0.60	0.062	0.021
Phenobarbital	0.51	1.80	0.171	0.411
Olive Oil	0.36	0.58	0.050	0.023
3-Methylcholanthrene	0.40	0.77 [b]	0.070	0.015

[a] Male albino rats (275-325 g) were injected intraperitoneally with sodium phenobarbital (50 mg/kg) in saline twice daily for 5 days or with 3-methylcholanthrene (20 mg/kg) in olive oil once daily for 4 days. Control rats received an equivalent volume of saline or olive oil. The animals were sacrificed 24 hours after the last injection, microsomes were prepared from perfused livers, peroxidase activity and cytochrome b_5 content measured as described in Materials and Methods. The P-450 content of liver microsomes from rats treated with saline, phenobarbital, olive oil was determined from the CO-difference spectrum of dithionite-treated samples using an extinction coefficient of 91 cm^{-1} mM^{-1} (87). Determinations were made on the pooled livers of 3 rats.

[b] The CO-complex of reduced P-450 in liver microsomes from rats treated with 3-methylcholanthrene exhibited an absorption maximum at 448 nm. An extinction coefficient of 103 cm^{-1} mM^{-1} (88) was used to estimate the concentration of the hemoprotein.

of P-450 showed only a 3-fold increase. On the other hand, treatment of rats with 3-methylcholanthrene produced a 35% inactivation of microsomal peroxidase activity whereas the P-450 content was enhanced 1.4-fold. This result suggests that 3-methylcholanthrene-induced P-450 is less active as a peroxidase for cumene hydroperoxide than is normal P-450.

Effectiveness of partially purified cytochrome P-450 and various heme catalysts for peroxidase activity

Table 9 compares the peroxidase activity of hemoprotein P-450 with other heme catalysts using a variety of hydroperoxide substrates. The two hemoproteins, cytochrome $\underline{b}_5$ and hemoprotein P-450, account for virtually all of the protoheme in the microsomal fraction of liver (17, 87). Selective solubilization of cytochrome $\underline{b}_5$ from the microsomal membrane by protease digestion has proven to be a valuable approach in obtaining partially purified cytochrome P-450 preparations. In addition to cytochrome $\underline{b}_5$ release, the flavoprotein NADPH-cytochrome $\underline{c}$ reductase is also completely solubilized by the treatment. The pellet preparations have been termed "P-450 particles" because of their high specific content of P-450 relative to cytochrome $\underline{b}_5$.

In this study, "P-450 particles" were prepared by anaerobic digestion of rat liver microsomes with trypsin essentially by the method of Ichikawa and Yamano (90). The preparation was found to contain about 0.7 nmoles P-450 per milligram protein and a negligible amount of cytochrome $\underline{b}_5$ (i.e., 5% of total protoheme). When tested for peroxidase activity with various hydroperoxide substrates, the partially purified P-450 preparation exhibited a similar peroxidase activity per mole of P-450 as did original

TABLE 9

COMPARATIVE EFFICIENCIES OF HEME CATALYSTS FOR PEROXIDASE ACTIVITY [a]

Heme Catalyst	Peroxidase activity (moles TMPD oxidized/min/mole of heme)			
	Progesterone 17α-hydroperoxide	Pregnenolone 17α-hydroperoxide	Cumene hydroperoxide	Linoleic acid hydroperoxide
Microsomal P-450 [b]	27	23	10	23
Partially purified P-450 [c]	25	19	9	30
Hematin	240	9	5	156
Methemoglobin	6	2	2	122
Cytochrome c	3	2	2	96
High spin P-420 [d]	<3	<2	<2	200
Low spin P-420 [d]	<3	<2	<2	60

[a] The peroxidase activities of various heme catalysts were measured at 23° by following the oxidation of TMPD at 610 nm in a 3 ml solution containing 0.033 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.25 M sucrose, 0.2 mM TMPD, suitable concentrations of heme catalysts, and 0.05 mM hydroperoxide. Reaction rates were first order with respect to heme catalyst and were calculated in terms of moles TMPD oxidized/min/mole of heme using an extinction coefficient for TMPD of 11.6 cm^{-1} mM^{-1} (80).

[b] Liver microsomes containing 0.6 nmoles P-450/mg protein were used in the determinations.

[c] "P-450 particles" containing 0.7 nmoles P-450/mg protein were used in the determinations.

[d] High spin and low spin P-420 was prepared essentially as described previously (89).

microsomes. Hence, cytochrome $\underline{b}_5$ does not appear to be involved in the peroxidase mechanism of liver microsomes.

Hemoprotein P-450 was an effective peroxidase for most hydroperoxides tested (Table 9). Hematin exhibited a high peroxidatic activity towards progesterone 17 α-hydroperoxide. A logical explanation for the effectiveness of hematin in decomposing progesterone 17α-hydroperoxide could be that the heme iron of the hematin molecule, because it is readily exposed and not shielded by a protein residue, can easily interact with the hydroperoxide and catalyze decomposition. On the other hand, hematin showed a weak peroxidatic activity towards pregnenolone 17α-hydroperoxide and cumene hydroperoxide probably because these hydroperoxides do not bind readily to the heme compound and consequently are not decomposed. A similar explanation may hold for the ineffectiveness of methemoglobin and cytochrome $\underline{c}$ in decomposing these hydroperoxides. High spin or low spin cytochrome P-420 was ineffective in decomposing cumene hydroperoxide and the steroid hydroperoxides but acted as an effective peroxidase for linoleic acid hydroperoxide (89).

Destruction of cytochrome P-450 by hydroperoxides

Previous studies (74) reported the destruction of hemoprotein P-450 upon incubation of liver microsomes with linoleic acid hydroperoxide. In this study, the effect of other hydroperoxides on hepatic microsomal P-450 was investigated. The results (Table 10) show that incubation of 30 moles cumene hydroperoxide per mole of P-450 resulted in a 46% destruction of the hemoprotein at pH 7.5. Various hydrogen donors such as GSH, NADH, NADPH, TMPD, and ascorbate protected cytochrome P-450 effectively from damage by the hydroperoxide, possibly by reducing the oxidizing species involved

TABLE 10

EFFECT OF VARIOUS AGENTS ON THE DESTRUCTION OF CYTOCHROME P-450 BY CUMENE HYDROPEROXIDE [a]

Addition	% P-450 remaining
None	100
Cumene hydroperoxide	54
GSH + cumene hydroperoxide	96
TMPD + cumene hydroperoxide	95
Hydroquinone + cumene hydroperoxide	95
Trimethylhydroquinone + cumene hydroperoxide	95
NADH + cumene hydroperoxide	91
NADPH + cumene hydroperoxide	90
Ascorbate + cumene hydroperoxide	83
Vitamin E + cumene hydroperoxide	78
Testosterone [b] + cumene hydroperoxide	78
Progesterone [b] + cumene hydroperoxide	75

[a] A mixture of liver microsomes (4.8 mg protein/ml) containing 0.6 nmoles P-450 per mg protein was incubated for 15 min at 23° with 0.1 mM cumene hydroperoxide in 0.033 M sodium phosphate buffer (pH 7.5) containing 2 mM EDTA and 0.27 M sucrose in a final volume of 3 ml. After incubation the sample was treated with an equal volume of a mixture consisting of 0.1 M sodium phosphate buffer (pH 7.5), 50% glycerol, 2 mM EDTA and 0.4% lubrol WX. The CO-difference spectrum of dithionite-treated samples was measured and the destruction of P-450 determined. The effect of various reagents (2 mM) on P-450 destruction by cumene hydroperoxide was determined by pre-incubation of the P-450 suspension with the agents for 2 min prior to addition of hydroperoxide.

[b] The concentration used was 0.2 mM.

in P-450 destruction. Antioxidants such as hydroquinone and vitamin E also were effective protecting agents. Steroids such as testosterone and progesterone offered some protection to P-450 (Table 10) whereas type II ligands such as imidazole, n-octylamine, and cyanide afforded no appreciable protection. Incubation of 30 moles of progesterone 17α-hydroperoxide per mole of P-450 under identical conditions produced a 25% inactivation of cytochrome P-450.

The susceptibility of adrenocortical microsomal P-450 to destruction by progesterone 17α-hydroperoxide was next determined by incubating a suspension of adrenocortical microsomes (1.9 mg protein/ml) with 0.2 mM hydroperoxide for 30 minutes at 20° in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.03 M sucrose and 0.2 mM EDTA in a final volume of 3 ml. It was found that incubation of 300 moles of progesterone 17α-hydroperoxide per mole of P-450 resulted in only a 15% destruction of the hemoprotein at pH 7.5. GSH (1 mM) protected cytochrome P-450 completely from destruction by the hydroperoxide.

These results illustrate that hepatic microsomal P-450 is much more susceptible to destruction by progesterone 17α-hydroperoxide than is adrenocortical microsomal P-450.

CHAPTER II

PRODUCTS OF DECOMPOSITION OF PREGNENE 17α-HYDROPEROXIDES AND CUMENE HYDROPEROXIDE BY MICROSOMAL FRACTIONS

CHAPTER II: PRODUCTS OF DECOMPOSITION OF PREGNENE 17α-HYDROPEROXIDES AND CUMENE HYDROPEROXIDE BY MICROSOMAL FRACTIONS

In Chapter I, the pregnene 17α-hydroperoxides were found to act as effective substrates for microsomal cytochrome P-450. This chapter presents a detailed analysis of the products formed when these steroid hydroperoxides were exposed to microsomal preparations. In addition the decomposition products of cumene hydroperoxide were also determined.

MATERIALS AND METHODS

Materials

Steroids, nucleotides, and heme compounds were obtained from Sigma. Silica gel HF_{254} was purchased from E. Merck and plastic strips, precoated with silica gel N-HR, were supplied by Brinkmann Instruments. All solvents were predistilled.

Preparation of steroid hydroperoxides

Progesterone 17α-hydroperoxide and pregnenolone 17α-hydroperoxide were prepared by the method of Bailey *et al* (75). Their purity was verified by thin layer chromatography, visualization of the developed spot with N,N-dimethyl-*p*-phenylenediamine reagent (91), and analysis of their characteristic *gc - ms* decomposition patterns (see pages 58 - 60) which were similar to those obtained by Tan and coworkers (92).

Incubation of steroid hydroperoxides with microsomal fractions

Standard incubations were conducted in glass-stoppered 25 ml test tubes. The incubation mixture contained 0.05 M sodium phosphate buffer (pH 7.5), 1.5 mM EDTA, 0.27 M sucrose, and 20 mg microsomal protein in a

final volume of 2 ml. In other incubation mixtures, 3 mM NADPH or NADH was included. Incubations were performed in the presence of sucrose to assist in the solubilization of the steroid hydroperoxides. However, similar products were obtained when sucrose was omitted from the incubation medium although the yield was usually smaller. The reaction was initiated by addition of 0.02 ml of a 10 mg/ml ethanolic solution of hydroperoxide and the mixture was incubated in air or under nitrogen at 23° for 15 minutes. Control incubations without microsomes or steroid substrates were carried out. Products were extracted with 20 ml of methylene chloride and the suspension filtered through Whatman phase separating (silicone-treated) filter paper. The extract was concentrated under vacuum, the residue transferred with ether to a small tube and the solvent removed under nitrogen. The products were redissolved in 0.05 ml methylene chloride and 0.01 ml was used for tlc analysis. The extracts were applied onto a 20 cm x 20 cm chromatoplate of silica gel HF_{254} 0.25 mm thick together with authentic steroids. The chromatoplate was developed three times with benzene-ethyl acetate-acetone (10:1:1) in the case of progesterone 17α-hydroperoxide metabolites or five times with toluene-ethyl acetate-acetone (12:2:1) in the case of pregnenolone 17α-hydroperoxide metabolites. The steroids were visualized under 254 nm ultraviolet light or by their color response towards 50% sulfuric acid at 110° and steroid hydroperoxides were located by spraying with an N,N-dimethyl-p-phenylenediamine solution (91). Mobility data (R_c) are given in terms of progesterone as unity in the two solvent mixtures employed. The recovery of products from the progesterone 17α-hydroperoxide incubations was 70 - 90%.

For analysis of the products by gas chromatography and mass spectrometry, incubations were performed on a larger scale (x 10), products were extracted with 200 ml methylene chloride and the final residue suspended in 0.5 ml methylene chloride. The sample was applied as a fine even line onto a 20 x 20 cm chromatoplate which was developed in the usual manner. The steroids were located under 254 nm ultraviolet light or by spraying a 1 cm strip with 50% sulfuric acid followed by gentle heating for 2 min. Each steroid zone was scraped off the chromatoplate, the steroids were extracted with methylene chloride, each fraction was applied onto a separate chromatoplate 5 cm x 20 cm and again chromatographed and recovered in the usual manner. The products were finally identified by comparison of their chromatographic and spectral properties with those of authentic steroids.

Gas chromatography was conducted on a Hewlett-Packard 420 gas chromatograph using silanized glass U-tubes packed with 3% QF-1 (trifluoropropylmethyl silicone) or 3% SE-30 (methyl silicone) on 80 — 100 mesh Gas-Chrom Q (93). Injection port temperature was 250°, column temperature was 240°, and the carrier gas was nitrogen at a flow rate of 22 ml/min. Gas chromatographic retention times (t_R) are given in terms of progesterone taken as unity. Absolute retention time of progesterone was 10 minutes on 3% QF-1 column and 6 minutes on 3% SE-30 column.

Mass spectral measurements were carried out using an AEI MS-30 double beam instrument, with heptacosafluorobutylamine in the reference beam. Samples were introduced in the sample beam via a membrane separator operated at 215° and connected to a Pye Unicam model 104 gas chromatograph equipped with a 6 ft long, 3 mm internal diameter glass column packed with 3% QF-1 on 80 — 100 mesh Gas Chrom Q, operated at 240° with helium as

carrier gas at a flow rate of 30 ml/min. Spectra of resolved steroids were obtained at 24 eV with a resolution of 1000 and a scanning speed of 10 sec per decade.

Quantitation of progesterone 17α-hydroperoxide metabolites by fluorescence scanning

Progesterone 17α-hydroperoxide was incubated with microsomal fractions and the products were extracted with 20 ml methylene chloride and isolated as described above. Thin layer chromatoplates (20 cm x 20 cm) of silica gel HF_{254} 0.25 mm thick were evenly divided into 20 identical zones 1 cm x 20 cm each. The steroid products from the various incubations and reference steroids were applied onto the chromatoplate which was then developed three times with benzene-ethyl acetate-acetone (10:1:1). Each steroid zone was scanned by reflectance at 254 nm against a blank zone using a Spectrodensitometer Model SD-3000 (Schoeffel Instruments, Westwood, New Jersey) equipped with an SDC 3000 density computer. The percentage of each compound formed was calculated from the area of the peaks obtained on the scanning trace. This method of quantitation appeared to be reliable since the area of the peaks obtained on the scanning trace was directly proportional to the amount of steroid applied onto the chromatoplate.

Incubation of cumene hydroperoxide with microsomal fractions

A mixture containing 0.05 M sodium phosphate buffer (pH 7.5), 1.5 mM EDTA, 20 mg microsomal protein, and 0.25 mM cumene hydroperoxide in a final volume of 2 ml was incubated at 23° for 20 minutes. Other incubations were performed in the presence of 3 mM NADH or NADPH. Control

incubations without microsomal fraction or hydroperoxide were carried out. The reaction mixture was extracted with 20 ml diethyl ether, the water layer removed, and the ether extract concentrated. The residue was applied onto a 20 cm x 20 cm plastic strip coated with silica gel N-HR 0.2 mm thick and chromatographed three times using a toluene-diethyl ether (20:1) solvent system. The products were located by spraying a small portion of the strip with 50% sulfuric acid followed by gentle heating for 2 min. The tlc mobilities and colors were compared with reference samples.

For analysis of the products by gas chromatography, the various product zones were scraped off the tlc strip, eluted with diethyl ether and identified by gas chromatography by comparison with authentic samples. Gas chromatography was conducted on the SE-30 column at an oven temperature of 90° and a carrier gas flow rate of 22 ml/min. Retention times are given in terms of ethanol taken as unity with an absolute retention time for ethanol of 0.7 minutes.

RESULTS

Conversion products of progesterone 17α-hydroperoxide

Progesterone 17α-hydroperoxide was incubated in air or under nitrogen with bovine adrenocortical microsomes in the presence and absence of NADPH or NADH. The products were quantitated by fluorescence scanning and identified by comparison of their chromatographic and spectral properties to those of authentic steroids.

Incubation of progesterone 17α-hydroperoxide in air with adrenocortical microsomes for 15 minutes gave 17α-hydroxyprogesterone (A_1, 58%) and androstenedione (B_1, 17%) as the major metabolites together with some unconverted hydroperoxide (Table 11). A fluorescence scanning trace of the thin layer chromatoplate containing a mixture of these products and a mixture of reference steroids is shown in Fig. 7. Replacing the air phase by a nitrogen atmosphere did not alter the nature or extent of product formation in the incubations (Table 11).

Incubation of progesterone 17α-hydroperoxide in air with adrenocortical microsomal preparations fortified with 3 mM NADPH resulted in the formation of three major products: 17α-hydroxyprogesterone (A_2, 33%), androstenedione (B_2, 11%), and 17α, 21-dihydroxyprogesterone (C_1, 43%) together with a small amount of unconverted hydroperoxide (Table 11). A fluorescence scanning trace of a tlc plate containing a mixture of these products together with a mixture of authentic steroids is shown in Fig. 8. Substitution of NADH for NADPH in the incubation medium also gave rise to similar products although less of the 17α, 21-diol was formed (Table 11). However, when incubations were carried out in the presence

TABLE 11

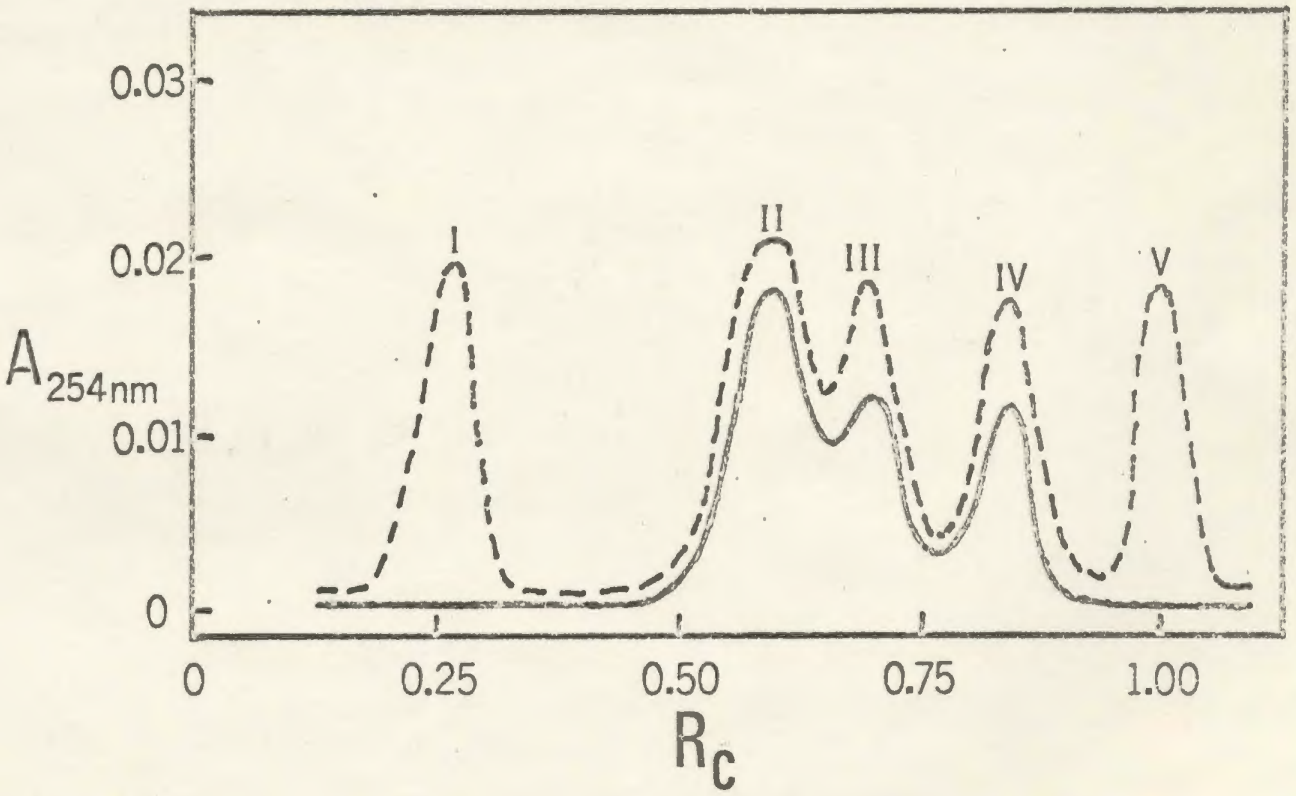
CONVERSION PRODUCTS OF PROGESTERONE 17α-HYDROPEROXIDE INCUBATED WITH ADRENOCORTICAL MICROSOMES UNDER AIR OR NITROGEN [a]

Incubation Conditions	% of Products Recovered							
	17α-hydroxy-progesterone		Androstenedione		17α, 21-dihydroxy-progesterone		Hydroperoxide Remaining	
	Air	N_2	Air	N_2	Air	N_2	Air	N_2
Microsomes	58	58	17	10	0	0	25	32
Microsomes + NADPH	33	60	11	8	43	0	13	32
Microsomes + NADH	34	61	11	9	32	0	23	30

[a] Progesterone 17α-hydroperoxide was incubated with adrenocortical microsomes in the presence of 3 mM NADPH or NADH. Products were identified by tlc, gas chromatography, mass spectrometry and quantitated by fluorescence scanning as described in Materials and Methods.

Figure 7. Fluorescence scanning trace of products obtained upon incubation of progesterone 17α-hydroperoxide with adrenocortical microsomes.

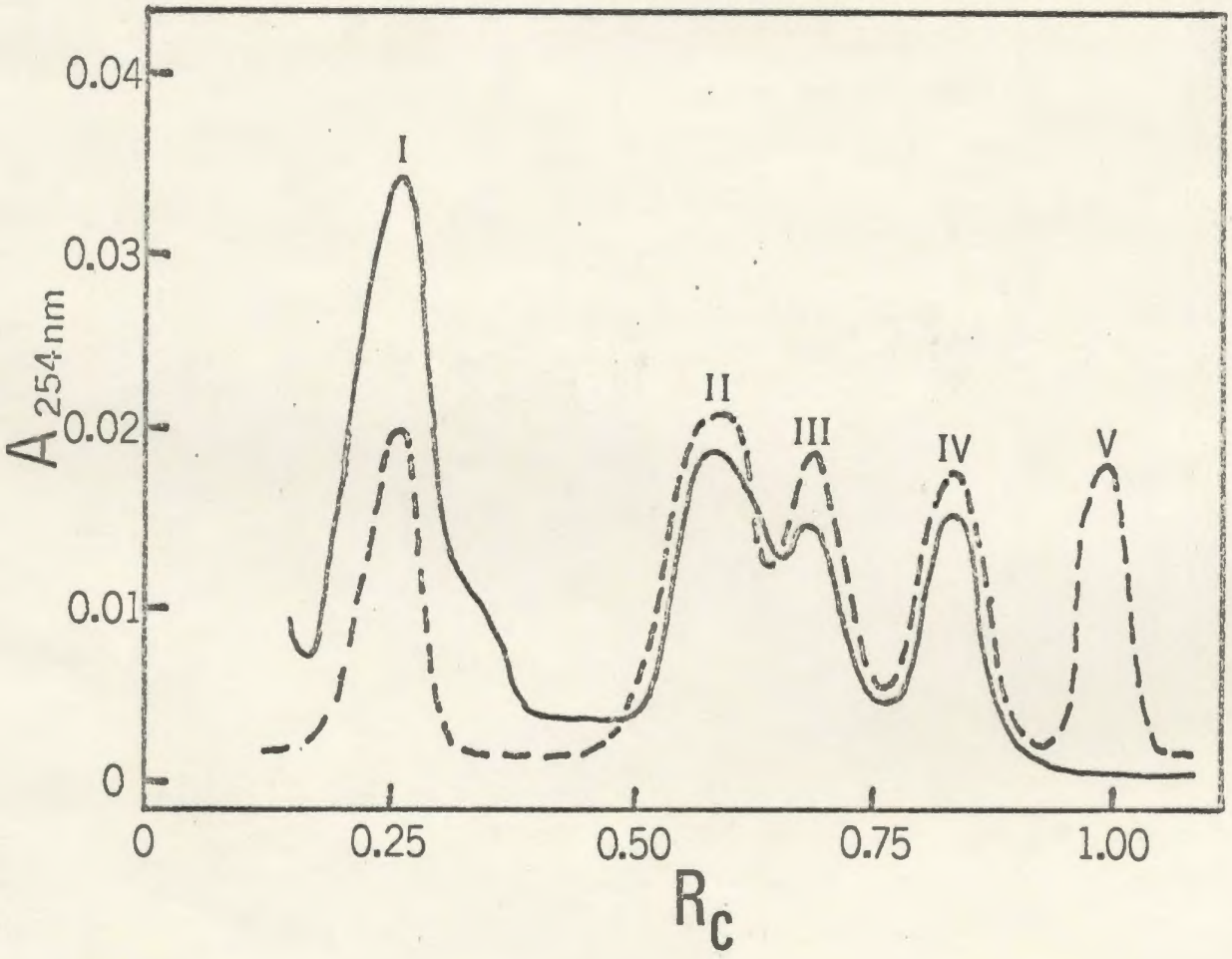
Progesterone 17α-hydroperoxide was incubated with adrenocortical microsomes and the products extracted and chromatographed. The chromatoplate containing products and reference steroids was scanned by reflectance at 254 nm using a 0 to 0.1 absorbance span as described in Materials and Methods. The % of each compound was



calculated from the area of the peaks obtained on the scanning trace. Tracing of the reference steroids is indicated by the broken line. The Roman numerals show the position of the reference compounds as follows: I: 17α,21-dihydroxyprogesterone; II: 17α-hydroxyprogesterone; III: progesterone 17α-hydroperoxide; IV: androstenedione; V: progesterone.

Figure 8. Fluorescence scanning trace of products obtained upon incubation of progesterone 17α-hydroperoxide in air with adrenocortical microsomes in the presence of 3 mM NADPH.

Scanning of metabolites was carried out as described in Figure 7.



of NADPH or NADH under a nitrogen atmosphere, only two products identified as 17α-hydroxyprogesterone and androstenedione were formed and no 17α, 21-diol was detected (Table 11). These results suggest that the oxygen atom at the C-21 position of 17α, 21-dihydroxyprogesterone was supplied by molecular oxygen by way of an active C-21 hydroxylase (94) rather than by a molecular rearrangement of the 17α-hydroperoxy group. This conclusion is strengthened by the fact that incubation of 17α-hydroxyprogesterone in air with adrenocortical microsomal fractions supplemented with NADPH or NADH also resulted in the formation of 17α, 21-dihydroxyprogesterone whereas in the absence of air, diol formation did not occur.

Incubation of progesterone 17α-hydroperoxide in air with rat liver microsomes gave rise to two major products again identified as 17α-hydroxyprogesterone (A_3, 37%) and androstenedione (B_3, 18%) together with at least five more polar and as yet unidentified metabolites. A similar spectrum of products was obtained when incubations were carried out under a nitrogen atmosphere or when the hydroperoxide was exposed to various heme compounds such as cytochrome c or methemoglobin. When incubations were performed in the absence of added microsomal fractions, or with a previously boiled enzyme preparation, progesterone 17α-hydroperoxide remained virtually unchanged.

Progesterone 17α-hydroperoxide gave an R_c of 0.71 (yellow-brown color with sulfuric acid and a positive color reaction with N,N-dimethyl-p-phenylenediamine reagent); gc, characteristic decomposition pattern; ms, solid probe, m/e (%)*: 346 (M^+, 5), 330 (41), 302 (31), 287 (22), 286 (100),

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* *% refers to the relative abundance of the various ions taking the largest peak (in this case at m/e 286) as 100%.*

269 (27), etc. Total decomposition of the hydroperoxide occurred upon injection in the flash heater zone of the gc system, as evidenced by the symmetry of the elution patterns of the various decomposition products on both QF-1 and SE-30 columns. Three major thermal decomposition products were identified as: (1) androstenedione (B_4), (2) progesterone (D_1), and (3) 17α-hydroxyprogesterone (A_4). To a fourth major product, eluting on both QF-1 and SE-30 just before androstenedione and with mass spectral properties similar to androstenedione, we tentatively assign the structure of androst-4-en-3-one 16, 17-epoxide (E_1).

Table 12 (top section) summarizes the tlc, gc, and ms data of the major metabolites of progesterone 17α-hydroperoxide.

Conversion metabolites of pregnenolone 17α-hydroperoxide

Decomposition of pregnenolone 17α-hydroperoxide by adrenocortical microsomes followed a similar pattern as was observed for its progesterone analog. The major product was identified as 17α-hydroxypregnenolone (W_1) and a second product was identified as dehydroepiandrosterone (X_1). When NADPH or NADH was included in the incubation medium again similar products were obtained and no C-21 hydroxy derivative was detected, in contrast to the result obtained when progesterone 17α-hydroperoxide was employed as substrate. It therefore appears that pregnenolone derivatives are not hydroxylated at the C-21 position by the C-21 hydroxylase of adrenocortical microsomes, as was also demonstrated by Ryan and Engel (94).

Incubation of the hydroperoxide with liver microsomes again gave two major products identified as 17α-hydroxypregnenolone (W_2) and dehydroepiandrosterone (X_2) but in addition, several more polar and as yet

unidentified products were recovered. No appreciable formation of these products occurred when incubations were carried out in the absence of microsomal fractions.

Injection of pregnenolone 17α-hydroperoxide (R_c 0.66, purple-brown) in the flash heater zone of gc system resulted in total decomposition. The products gave a characteristic elution pattern on both QF-1 and SE-30 columns. The major products were identified as: 1) dehydroepiandrosterone (X_3), 2) pregnenolone (Y_1), and 3) 17α-hydroxypregnenolone (W_3). To a fourth major product we assign the tentative structure of androst-5-en-3β-ol 16, 17-epoxide (Z_1).

Table 12 (bottom section) summarizes the tlc, gc and ms data of the major decomposition products of pregnenolone 17α-hydroperoxide.

Products of decomposition of cumene hydroperoxide

Cumene hydroperoxide was found to act as a substrate for microsomal cytochrome P-450 (74) and it was therefore desirable to obtain data on the products formed when the hydroperoxide is exposed to microsomal fractions. Cumene hydroperoxide was incubated with liver or adrenocortical microsomes in the presence and absence of NADPH or NADH and the products were identified by tlc and gc. Only one product was detected in significant yield and characterized as 2-phenyl-2-propanol, R_f 0.32 (relative to the solvent front) with toluene-diethyl ether (20:1) as solvent (purple color with sulfuric acid); t_R 5.6 (ethanol as unity) on the SE-30 column, identical in these properties to an authentic sample. No formation of the product was noticed in the absence of microsomal fractions.

TABLE 12

SUMMARY OF THE THIN LAYER CHROMATOGRAPHIC, GAS CHROMATOGRAPHIC AND MASS SPECTRAL ANALYSIS OF PROGESTERONE 17α-HYDROPEROXIDE AND PREGNENOLONE 17α-HYDROPEROXIDE METABOLITES [a]

Products	R_c	Color with 50% H_2SO_4	Retention Time (t_R) QF-1	Retention Time (t_R) SE-30	M^+	Mass Spectra Other Ions m/e (%)[e]	Structure Assigned
A_1, A_2, A_3, A_4[f]	0.61[b]	yellow-brown	1.31	1.39	330(83)	312(24), 302(54) 287(100), 269(39)	17α-hydroxyprogesterone
B_1, B_2, B_3, B_4[f]	0.86[b]	dark green	0.78	0.64	286(100)	271(10, 245(13)	androstenedione
C_1	0.29[b]	magenta	0.78[d] 1.04 1.40	0.64[d] 1.25	346(30)	316(64), 299(31) 287(100), 269(44) 253(20), 244(87)	17α,21-dihydroxypro-gesterone
D_1	1.00[b]	yellow	1.00	1.00	314(100)	272(96), 244(37)	progesterone
E_1			0.57	0.29	286(100)	287(21), 244(44)	androst-4-en-3-one 16, 17-epoxide
W_1, W_2, W_3[f]	0.60[c]	magenta	0.39	0.91	352(57)	314(34), 287(29) 271(57), 253(100)	17α-hydroxypregnenolone
X_1, X_2, X_3[f]	0.78[c]	purple	0.22	0.41	288(93)	273(12), 270(92) 255(100)	dehydroepiandrosterone
Y_1		magenta	0.32	0.78	316(83)	301(21), 298(100) 283(75)	pregnenolone
Z_1			0.16	0.36	288(54)	270(100), 255(80)	androst-5-en-3β-ol 16, 17-epoxide

[a] Metabolites of the pregnene 17α-hydroperoxides were analyzed as described in text.

[b] Benzene-ethyl acetate-acetone (10:1:1)

[c] Toluene-ethyl acetate-acetone (12:2:1)

[d] Decomposition on gc columns to yield a characteristic fragmentation pattern

[e] % refers to the relative abundance of the various ions taking the largest peak as 100%

[f] Each letter refers to a particular product obtained under different incubation conditions as described in the Results section.

Cumene hydroperoxide (R_f 0.61, orange-brown) decomposed in the flash heater zone of the gc system (90°), giving a characteristic elution pattern of decomposition products on the SE-30 column, t_R 14.0 (major), 2.5 (minor), and 5.6 (minor).

CHAPTER III

THE MICROSOMAL NADPH-PEROXIDASE ELECTRON TRANSPORT SYSTEM

CHAPTER III: THE MICROSOMAL NADPH-PEROXIDASE ELECTRON TRANSPORT SYSTEM

This chapter will investigate the role of various electron transport components in the microsomal NADPH-peroxidase enzyme system.

MATERIALS AND METHODS

Materials

Trypsin, soybean trypsin inhibitor, nucleotides, p-hydroxymercuribenzoate, phosphatidylcholine, and lysophosphatidylcholine* were purchased from Sigma. p-Chloro-N-methylaniline was obtained from Calbiochem. Phenacetin was a product of British Drug Houses, Toronto.

Assay of NADPH-peroxidase activity

Microsomal NADPH-peroxidase activity was measured at 23° by following the rate of oxidation of NADPH by hydroperoxides in the first min of reaction at 340 nm in the presence of microsomal fraction. The assay medium contained, unless otherwise stated, 0.067 M sodium phosphate buffer (pH 7.5), 0.27 M sucrose, 2 mg microsomal protein, 0.15 mM NADPH, and 0.05 mM hydroperoxide in a final vol of 3 ml. Reaction rates were routinely corrected for NADPH oxidation in the absence of hydroperoxide and were calculated using an extinction coefficient for NADPH of 6.22 cm^{-1} mM^{-1}. Measurements were normally carried out with a Beckman DB-G double beam spectrophotometer. Turbidity in the sample solutions was balanced by using a reference cuvette containing 0.067 M sodium phosphate buffer and the same

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* *Phosphatidylcholine prepared from egg yolk contained primarily a mixture of stearic and palmitic acids; lysophosphatidylcholine prepared by the action of phospholipase A on phosphatidylcholine also contained palmitic and stearic acids.*

concentration of microsomal protein as employed in the sample cuvette. Reactions which produced low velocities or those which utilized large quantities of protein were measured using a Perkin-Elmer 356 dual wavelength spectrophotometer in the split beam mode.

Steroid hydroperoxides were dissolved in ethanol just before use and 10 microliter amounts were added to the assay medium. Sucrose was included in the reaction medium to assist in the solubilization of the steroid hydroperoxides. Concentrations of steroid hydroperoxides above 0.1 mM could not be used since these hydroperoxides came out of aqueous solution under the specified assay conditions.

Determination of the K_m for NADPH in the NADPH-peroxidase reaction

An estimate of the K_m for NADPH in the NADPH-peroxidase reaction was made by following the rate of oxidation of NADPH at 340 nm on a Perkin-Elmer 356 recording spectrophotometer, with a full-scale deflection of 0.1 absorbance units. The assay medium contained 0.1 M sodium phosphate buffer (pH 7.5), 0.17 mg liver microsomal protein, varying amounts of NADPH, and 0.8 mM cumene hydroperoxide in a final vol of 3 ml. Reaction rates were corrected for NADPH oxidation in the absence of cumene hydroperoxide.

Enzyme assays

The microsomal N-demethylation of p-chloro-N-methylaniline was determined essentially by the method of Kupfer and Bruggeman (95). NADPH- or NADH-cytochrome c reductase activity was measured at 23° by following the reduction of cytochrome c (50 μM) at 550 nm in a 3 ml solution containing 0.033 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH or NADH, and a

suitable amount of microsomal protein. An extinction coefficient between reduced and oxidized cytochrome c of 21 cm^{-1} mM^{-1} (96) was employed in the calculation of the reaction rates.

Preparation of antibodies

Antiserum to NADPH-cytochrome c reductase prepared from rabbits by the method of Glazer et al (31) was kindly supplied by Professor J.B. Schenkman, Yale University. Antibody to cytochrome b_5 prepared by the procedure of Fukushima et al (97) was kindly donated by Professor T. Omura, Kyushu University.

Preparation of microsomal lipid

Lipid was prepared from rat liver microsomes by DEAE-cellulose chromatography of a deoxycholate-solubilized extract followed by extraction with chloroform-methanol according to Strobel et al (56) up to the silicic acid chromatography step. The organic solvent mixture was removed under reduced pressure and the microsomal lipid stored anaerobically at -5°.

Solubilization of lipids

Microsomal lipid was solubilized by sonic oscillation at 4° under a nitrogen atmosphere in 0.01 M Tris-acetate buffer (pH 8.0) containing 0.1 mM EDTA. The lipid was suspended to a concentration of 5 mg/ml and sonicated at 20 sec intervals for 2 min or until the solution turned clear. Phosphatidylcholine, lysophosphatidylcholine, and phosphatidylcholine-lysophosphatidylcholine micelles were prepared by a similar procedure.

Preparation of various microsomal constituents for reconstitution of NADPH-peroxidase activity

Purified NADPH-cytochrome c reductase was prepared from rat liver microsomes by trypsin solubilization following the procedure of Omura and Takesue (98) and by deoxycholate solubilization according to Lu et al (23). Cytochrome b_5 was prepared from rat liver microsomes by trypsin digestion according to Omura and Takesue (98) and by detergent solubilization as described by Spatz and Strittmatter (99). Partially purified cytochrome P-450 was prepared by cholate solubilization of liver microsomes according to Lu et al (23).

RESULTS

The NADPH-peroxidase assay

The NADPH-peroxidase reaction was measured by following the rate of oxidation of NADPH by hydroperoxides at 340 nm in the presence of microsomal fractions. It was found that hydroperoxides can markedly enhance the rate of NADPH oxidation by microsomes. Using cumene hydroperoxide as substrate and rat liver microsomes as the enzyme source, the reaction rate was found to be first-order with respect to microsomal protein concentration (Fig. 9). Using cumene hydroperoxide as substrate, the reaction rate showed saturation kinetics with increasing concentrations of hydroperoxide and an apparent K_m of about 0.4 mM was obtained*(Fig. 10). Under these assay conditions, the rate of NADPH oxidation was calculated to be approximately 28 nmoles/min/mg protein/μmole cumene hydroperoxide.**

An estimate of the K_m for NADPH in the NADPH-dependent peroxidase reaction was made by following the course of oxidation of the reduced nucleotide on a recording spectrophotometer with a full-scale deflection of 0.1 absorbance units as described in Methods. It was found that an NADPH concentration of 6 μM gave an identical initial reaction rate (27 nmoles of NADPH oxidized/min/mg protein/ μmole cumene hydroperoxide) to that obtained when higher amounts of NADPH were employed. It appears, therefore, that the K_m for NADPH in the reaction is smaller than 3 μM. A more precise measurement of the K_m using this technique could not be obtained.

Hydroperoxide specificity of microsomal NADPH-peroxidase

A comparison of the effectiveness of various hydroperoxides in oxidizing NADPH in the presence of liver or adrenocortical microsomes is

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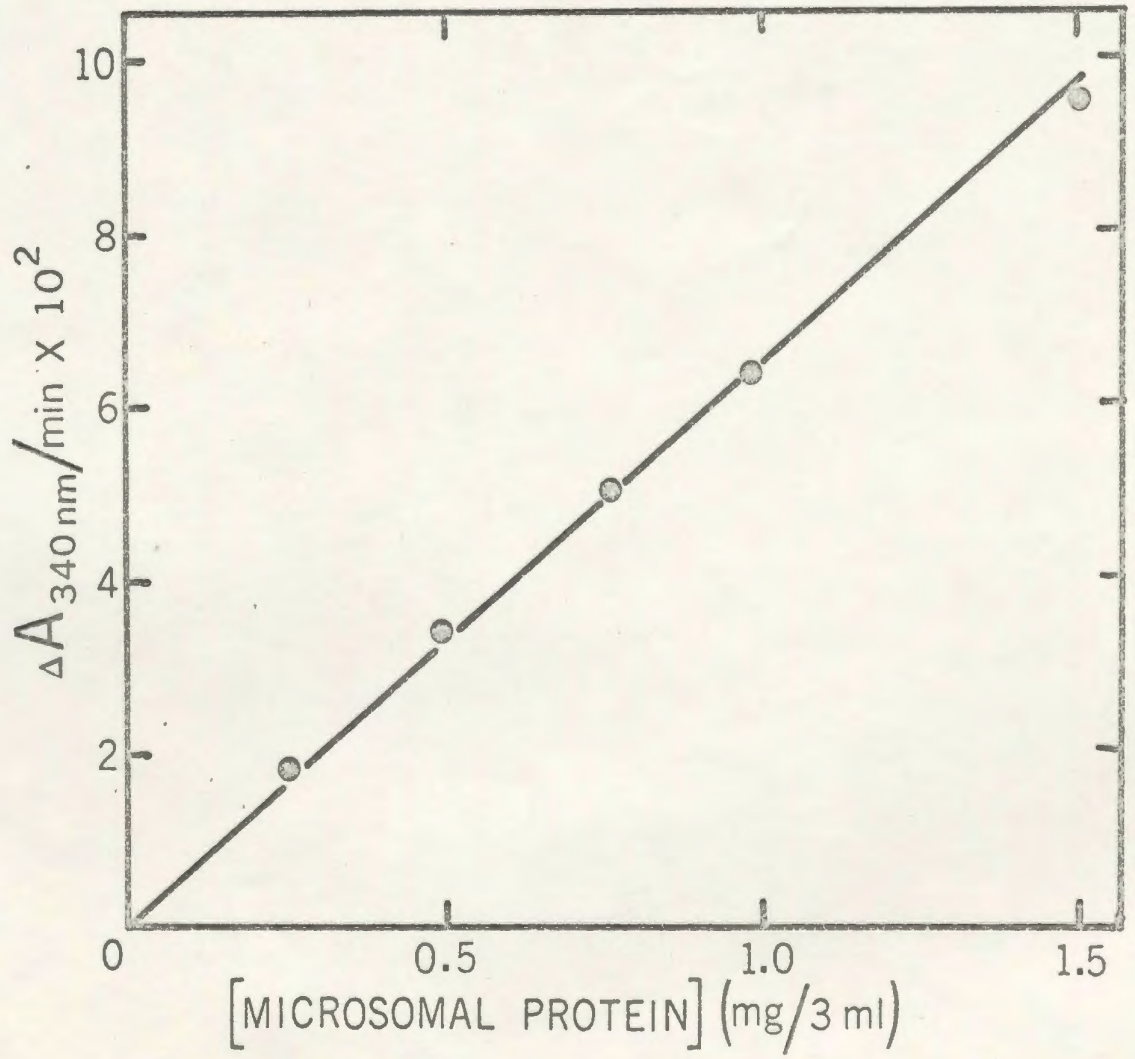
* *A similar K_m value was also obtained using a Lineweaver-Burke plot.*

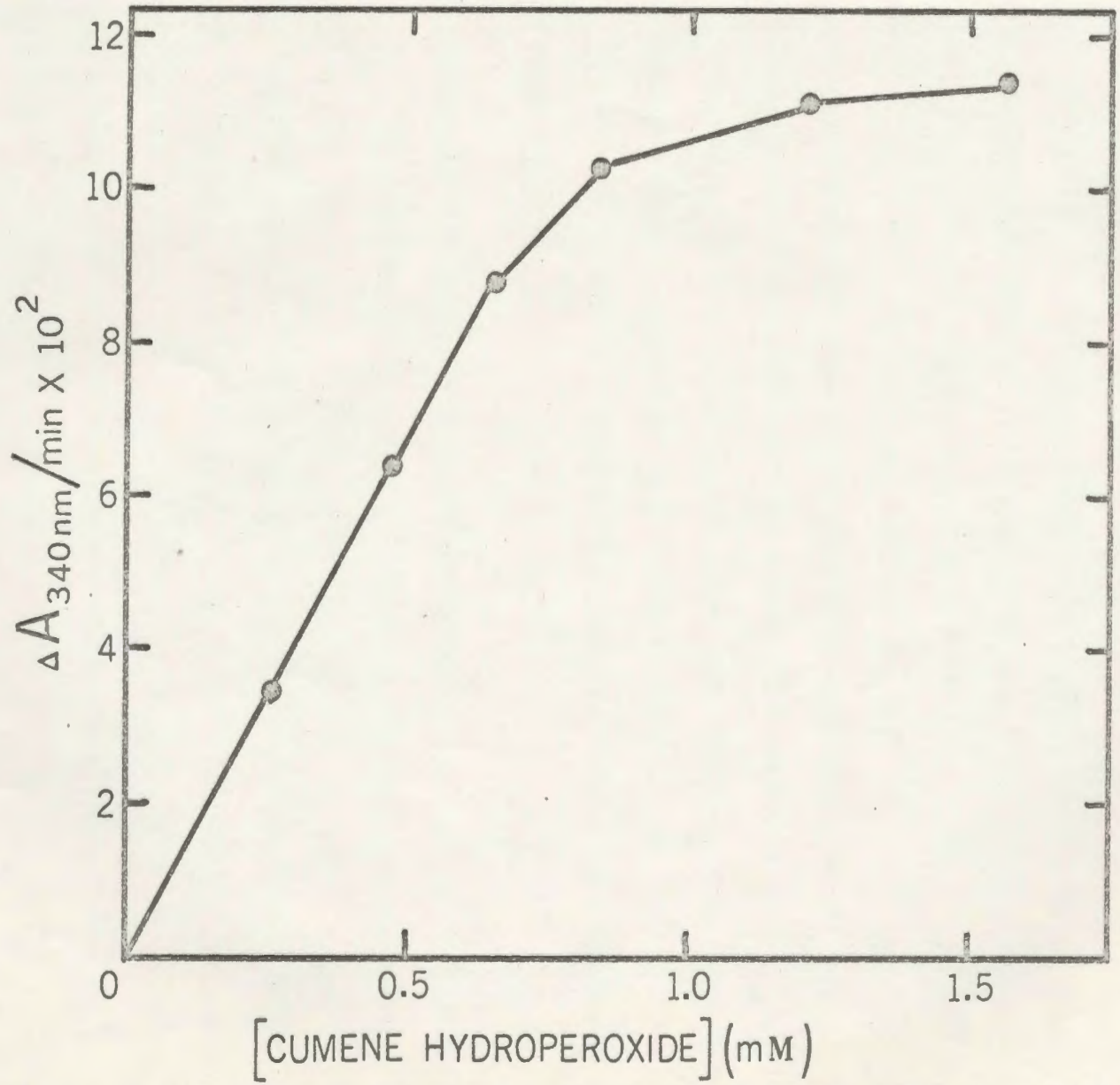
** *Under these assay conditions, the reaction rate was first-order with respect to microsomal protein concentration and first-order with respect to cumene hydroperoxide concentration.*

Figure 9. Effect of increasing microsomal protein concentration on NADPH-peroxidase activity.



The reaction was measured at 340 nm in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, liver microsomal protein, and 0.4 mM cumene hydroperoxide.

Figure 10. Effect of increasing cumene hydroperoxide concentration on microsomal NADPH-peroxidase activity.



The assay medium contained 0.067 M sodium phosphate buffer (pH 7.5), 0.15mM NADPH, 0.9 mg liver microsomal protein, and varying amounts of hydroperoxide in a final volume of 3 ml. Reaction rates were corrected for NADPH oxidation in the absence of hydroperoxide.

presented in Table 13. It is noticed that the 17α-hydroperoxide derivatives of progesterone and pregnenolone were effective substrates for the microsomal enzyme system of both liver and adrenal cortex.

Tissue distribution of microsomal NADPH-peroxidase activity

In Table 14 is shown the tissue distribution of the microsomal NADPH-peroxidase activity. It is seen that the microsomal fractions from tissues which contained high amounts of cytochrome P-450 per milligram protein (e.g. liver, adrenal cortex) were the most active in catalyzing the NADPH-peroxidase reaction whereas microsomes from tissues that contained little or no cytochrome P-450 showed either weak activity or no activity at all. However, a perusal of the data shows that the reaction rates were not strictly proportional to cytochrome P-450 content.

The properties of the NADPH-peroxidase enzyme system of rat liver microsomes were next investigated in detail.

Efficiency of NADPH oxidation by hydroperoxides

Examination of the stoichiometry of the NADPH-peroxidase reaction revealed the oxidation of approximately 0.35 moles of NADPH per mole of cumene hydroperoxide in the presence of liver microsomes under the normal assay conditions. Since NADPH has two reducing equivalents and cumene hydroperoxide has two oxidizing equivalents, this represents a 35% efficiency and indicates a competition between NADPH and certain microsomal constituents for the oxidizing equivalents of the hydroperoxide. Hydrogen donors other than NADPH have been found to be oxidized by hydroperoxides at a similar efficiency in the presence of various heme catalysts (100-102).

TABLE 13

EFFECTIVENESS OF VARIOUS HYDROPEROXIDES IN OXIDIZING NADPH IN THE PRESENCE OF MICROSOMAL FRACTIONS [a]

Hydroperoxide	NADPH-Peroxidase activity [a]	
	Liver microsomes	Adrenocortical microsomes
Progesterone 17α-hydroperoxide	6.7	1.0
Pregnenolone 17α-hydroperoxide	5.8	0.7
Cumene hydroperoxide	4.8	0.7
Cholesterol 7β-hydroperoxide	4.3	-
Allopregnanolone 17α-hydroperoxide	2.9	-

[a] The oxidation of NADPH by hydroperoxides in the presence of microsomal fractions was measured at 340 nm as described in Methods. Reaction rates are expressed as nmoles NADPH oxidized/min/mg protein. The conc. of hydroperoxide used in the assay was 0.05 mM.

TABLE 14

TISSUE DISTRIBUTION OF MICROSOMAL NADPH-PEROXIDASE ACTIVITY [a]

Source of Microsomes	P-450 Content	NADPH-Peroxidase Activity
liver	0.70	48.4
adrenal cortex [b]	0.60	9.7
lung	0.05	3.9
kidney	0.25	2.0
testis	0.02	0.3
small intestine	0.02	0
brain	0	0
heart	0	0
skeletal muscle [c]	0	0

[a] Microsomes from various tissues of the rat were prepared and the P-450 content measured as described in Methods. NADPH-peroxidase activity was determined at 340 nm in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, 1 mg microsomal protein, and 0.8 mM cumene hydroperoxide. Reaction rates were corrected for NADPH oxidation in the absence of hydroperoxide and are expressed as nmoles NADPH oxidized/min/mg protein. P-450 content is expressed as nmoles/mg protein.

[b] Adrenal cortex microsomes were obtained from bovine adrenals.

[c] The hind legs of the rat were used for the source of skeletal muscle.

Inhibition of NADPH-peroxidase activity by various reagents

The effect of various inhibitors on the microsomal oxidation of NADPH by cumene hydroperoxide was next investigated. Cumene hydroperoxide was chosen as the model substrate because its solubility in aqueous medium is sufficient (84) to permit inhibitor studies of the reaction. It was found that the enzyme system was heat labile, being totally inactivated by preincubation of liver microsomes at 80° for 5 minutes (Table 15). Measurement of the activity under a nitrogen or a carbon monoxide atmosphere produced no appreciable inhibition of the reaction rate. NAD^+ produced a weak inhibition whereas 2-phenyl-2-propanol, the hydroxy derivative and main decomposition product of cumene hydroperoxide, exhibited no appreciable effect. Acetone and tert. amyl alcohol, reagents that are known to convert cytochrome P-450 to its inactive P-420 form (103), were potent inhibitors of the reaction.

Evidence for the involvement of NADPH-cytochrome c reductase in NADPH-peroxidase activity

Fig. 11 illustrates the pH dependence of the NADPH-peroxidase activity in 0.1 M sodium phosphate buffer. A pH optimum for the activity of about 8.2 was obtained which was similar to the pH optimum reported by Williams and Kamin (29) for microsomal NADPH-cytochrome c reductase activity.

In Fig. 12 is shown the effect of ionic strength on the microsomal NADPH-peroxidase reaction. It is observed that the activity was stimulated by increasing concentrations of sodium phosphate buffer.

TABLE 15

EFFECT OF VARIOUS INHIBITORS ON NADPH-PEROXIDASE ACTIVITY [a]

Inhibitor	% Inhibition
Heat (80° for 5 min)	100
Nitrogen [b]	9
Carbon Monoxide [c]	10
NAD^+ (0.45 mM)	20
2-Phenyl-2-propanol (0.4 mM)	6
Acetone (5%)	73
tert. Amyl alcohol (7%)	82

[a] NADPH-peroxidase activity was measured at 23° by following the oxidation of NADPH at 340 nm in a 3 ml solution containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, 1 mg liver microsomal protein, specified amounts of various inhibitors, and 0.4 mM cumene hydroperoxide. Reaction rates were corrected for NADPH oxidation in the absence of hydroperoxide. The specific activity of the NADPH system in the absence of inhibitor, in terms of nmoles NADPH oxidized/min/mg protein, was 34.

[b] The contents of an anaerobic cuvette were gassed with N_2 (99.99% pure) for 5 min prior to mixing with hydroperoxide.

[c] The contents of an anaerobic cuvette were gassed with N_2 for 10 min and with CO that had been passed through a deoxygenating medium (83) for 5 min prior to addition of cumene hydroperoxide.

Figure 11. The pH dependence of the microsomal NADPH-peroxidase reaction.

The assay medium contained 0.1 M sodium phosphate buffer of varying pH, 0.15 mM NADPH, 1 mg liver microsomal protein, and 0.4 mM cumene hydroperoxide in a final volume of 3 ml. The pH was routinely checked at the end of the reaction.

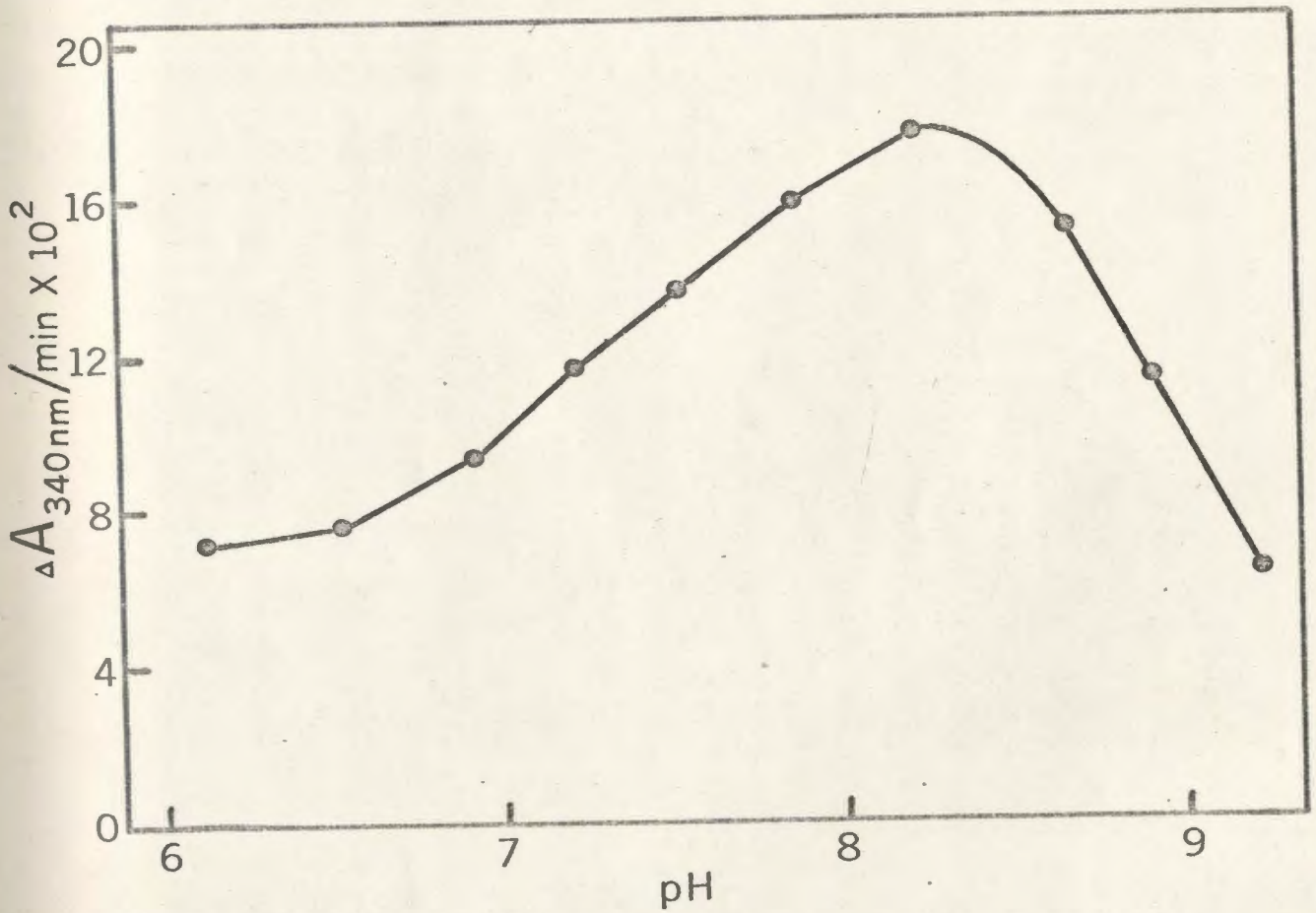
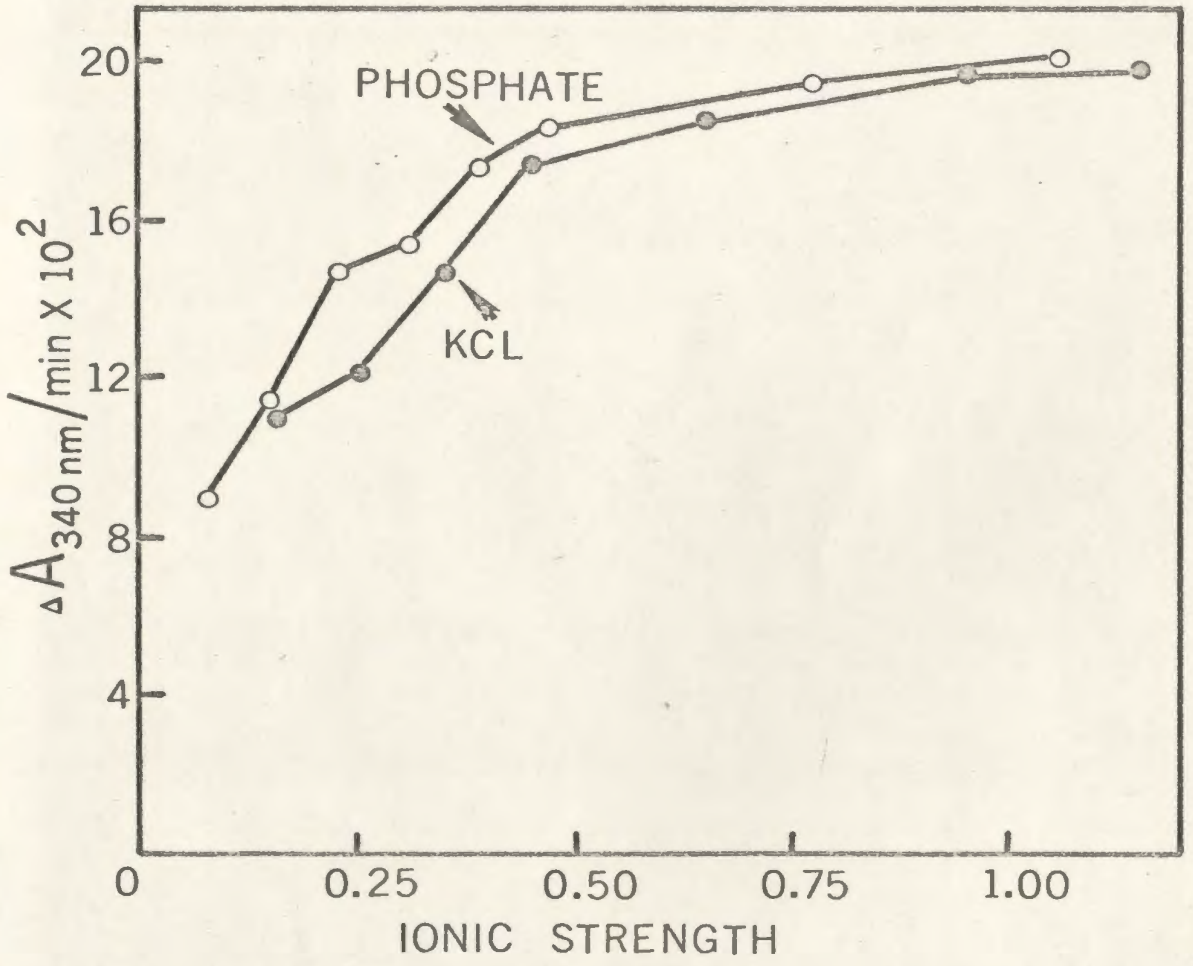


Figure 12. Effect of ionic strength on microsomal NADPH-peroxidase activity.

The reaction medium consisted of 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, 1 mg liver microsomal protein, and 0.4 mM cumene hydroperoxide to which increasing amounts of KCl or additional phosphate was added to obtain desired ionic strengths. Final ionic strength was calculated from the concentrations of phosphate and added KCl.



KCl exhibited an equal stimulatory effect when tested at the same ionic strengths as phosphate. The activity of microsomal NADPH-cytochrome c reductase has also been reported to be stimulated by increasing concentrations of phosphate or KCl (96).

The activity of NADPH-cytochrome c reductase is markedly inhibited by $NADP^+$ and by low concentrations of sulfhydryl reagents such as p-hydroxymercuribenzoate (29, 96). Preincubation of the flavoenzyme with NADPH prior to treatment with p-hydroxymercuribenzoate partially protects the flavoprotein from inactivation. Our results (Table 16) show that microsomal NADPH-peroxidase activity was markedly inhibited by 0.05 mM p-hydroxymercuribenzoate and preincubation of microsomes with NADPH prior to addition of sulfhydryl inhibitor partially protected the enzyme system from inactivation. $NADP^+$ was also found to be an inhibitor of the NADPH-peroxidase reaction.

Antiserum prepared to NADPH-cytochrome c reductase was next used to evaluate further the role of the flavoprotein reductase in the microsomal NADPH-peroxidase reaction. The results (Fig. 13) demonstrate a striking similarity in the pattern of inactivation of NADPH-cytochrome c reductase and NADPH-peroxidase activity by the antiserum. On the other hand, NADH-cytochrome c reductase activity was not affected by the treatment. Furthermore, the antiserum did not inhibit the TMPD-peroxidase reaction, a cytochrome P-450-dependent activity which is assayed by following the P-450-catalyzed rate of oxidation of TMPD by hydroperoxide. It, therefore, appears that the antiserum was a fairly specific inhibitor of NADPH-cytochrome c reductase (31).

TABLE 16

INHIBITION OF NADPH-PEROXIDASE ACTIVITY BY INHIBITORS OF NADPH-CYTOCHROME c REDUCTASE [a]

Inhibitor	% Inhibition
p-Hydroxymercuribenzoate [b] (0.05 mM)	80
p-Hydroxymercuribenzoate after NADPH [c]	35
$NADP^+$ (0.60 mM)	35

[a] NADPH-peroxidase activity was measured in the presence of specified amounts of inhibitors as described in Table 15.

[b] p-Hydroxymercuribenzoate (0.05 mM) was incubated with 1 mg liver microsomal protein in 0.067 M sodium phosphate buffer (pH 7.5) for 2 min directly in the assay cuvette. After incubation, NADPH (0.15 mM) and cumene hydroperoxide (0.4 mM) were added and activity determined in a final vol of 3 ml.

[c] NADPH was incubated with liver microsomes for 1 min prior to addition of p-hydroxymercuribenzoate and hydroperoxide.

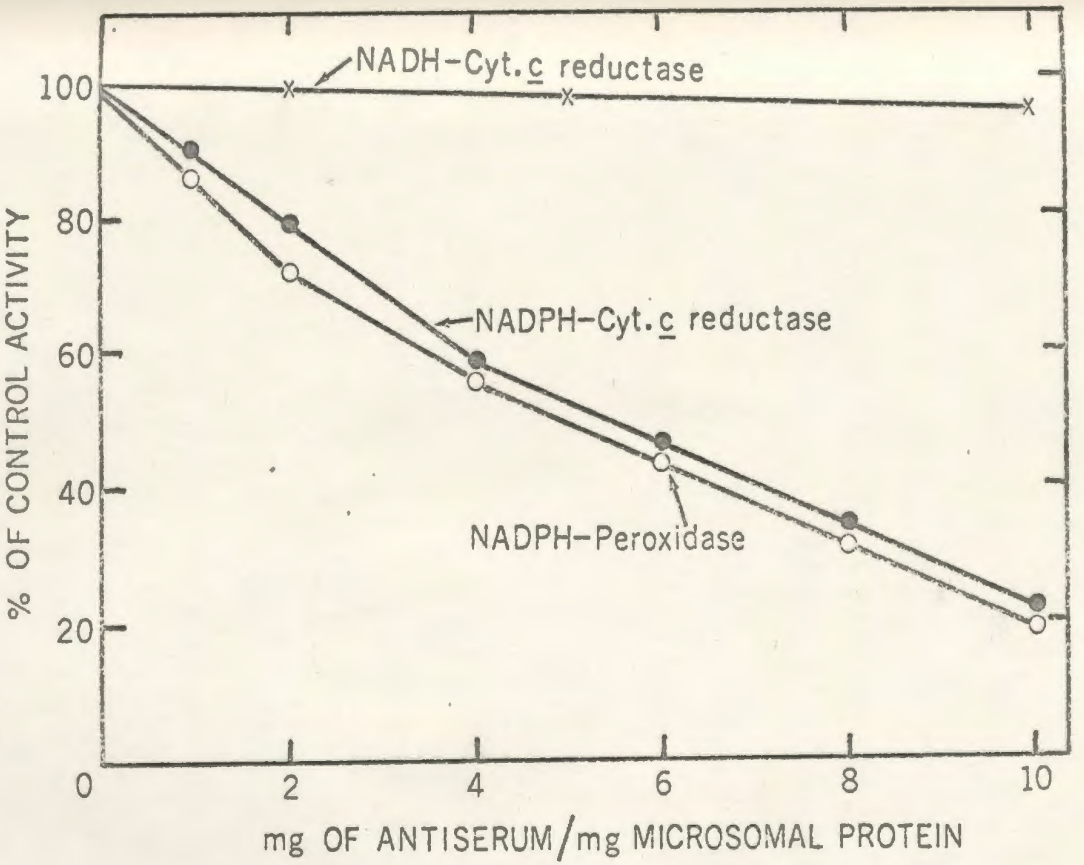


Figure 13. Concomitant inhibition of microsomal NADPH-cytochrome c reductase and NADPH-peroxidase activity by antiserum to NADPH-cytochrome c reductase.

Liver microsomes were incubated with indicated amounts of antiserum at 23° for 5 minutes directly in the assay cuvette in a 1 ml solution containing 0.1 M sodium phosphate buffer (pH 7.5). After incubation the rest of the assay components were added to make up a total volume of 3 ml. For the measurement of NADPH-peroxidase activity, 1 mg microsomal protein and 0.5 mM cumene hydroperoxide was used and for the NADH- and NADPH-cytochrome c reductase activities, 0.05 mg and 0.30 mg microsomal protein, respectively, was employed. The specific activity in the absence of antiserum of the NADPH-peroxidase system expressed as nmoles NADPH oxidized/min/mg protein was 58 and of the NADH- and NADPH-cytochrome c reductase systems, in terms of nmoles cytochrome c reduced/min/mg protein, was 1570 and 89, respectively.

These studies strongly suggest the involvement of NADPH-cytochrome c reductase as one of the electron carriers in the microsomal NADPH-peroxidase enzyme system.

Inhibition of NADPH-peroxidase activity by modifiers of cytochrome P-450

Compounds termed type I, type II, and modified type II substrates interact with microsomal cytochrome P-450 to produce characteristic difference spectra (34, 35). The NADPH-peroxidase enzyme system was found to be sensitive to inhibition by these compounds (Table 17). Steroids that form type I spectra with cytochrome P-450 were found to be effective inhibitors. The type II ligands such as n-octylamine, imidazole, aniline and the modified type II ligands such as corticosterone, phenacetin, and cyanide also produced a strong inhibition.

In Table 18 is shown the effect of various reagents that convert cytochrome P-450 to cytochrome P-420 on microsomal NADPH-peroxidase activity. It is seen that reagents which effected the conversion of cytochrome P-450 to cytochrome P-420 (e.g., detergents, protein denaturants, alcohols) also produced an inactivation of NADPH-peroxidase activity. Detergents such as sodium dodecyl sulfate, sodium deoxycholate, and sodium cholate did not inhibit the activity of the flavoenzyme NADPH-cytochrome c reductase which suggests that the inactivation of NADPH-peroxidase activity by these reagents occurs as a result of the conversion of cytochrome P-450 to its inactive P-420 form and not as a result of the denaturation of the flavoprotein reductase. On the other hand, lubrol WX, urea, and n-propanol in addition to causing the conversion of cytochrome P-450 to cytochrome P-420 also inhibited the activity of NADPH-cytochrome c reductase which suggests

TABLE 17

INHIBITION OF NADPH-PEROXIDASE ACTIVITY BY TYPE I AND TYPE II SUBSTRATES [a]

Type I Substrate	% Inhibition	Type II Substrate	% Inhibition	Modified Type II Substrate	% Inhibition
Androstenedione (0.1 mM)	70	n-Octylamine (0.6 mM)	73	Corticosterone (0.1 mM)	56
Testosterone (0.1 mM)	68	Aniline (5 mM)	66	Phenacetin (1 mM)	50
17β-Estradiol (0.1 mM)	55	Imidazole (2 mM)	66	Potassium cyanide (1 mM)	40
Aminopyrine (5 mM)	55	Pyridine (5 mM)	49		

[a] NADPH-peroxidase activity was measured in the presence of specified conc of type I and type II substrates as described in Table 15.

TABLE 18

INHIBITION OF NADPH-PEROXIDASE ACTIVITY BY REAGENTS THAT CONVERT CYTOCHROME P-450 TO P-420 [a]

P-450 Modifier	% Inhibition		
	NADPH-peroxidase	NADPH-cytochrome c reductase	% P-420 formed
None	0	0	0
Sodium dodecyl sulfate (0.1%)	94	9	98
Sodium deoxycholate (0.5%)	80	9	90
Sodium cholate (1%)	64	9	50
Lubrol WX (1%)	73	29	40
Urea (4 M)	40	48	55
n-Propanol (15%)	97	90	100

[a] Liver microsomes (5 mg protein/ml) were incubated at 23° for 20 min with specified amounts of P-450 modifier in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.4 mM EDTA and 0.05 M sucrose in a final vol of 3 ml. After incubation NADPH-peroxidase and NADPH-cytochrome c reductase activities were measured and the extent of conversion of P-450 to P-420 determined.

that part of the inhibition of the NADPH-peroxidase reaction by these reagents may be due to the inactivation of the flavoprotein reductase.

The inhibition of NADPH-peroxidase activity by various P-450 modifiers suggests the participation of cytochrome P-450 in the reaction.

Inhibition of NADPH-peroxidase activity by cyanide

Cyanide is a modified type II ligand which interacts with cytochrome P-450 to produce a characteristic difference spectrum (34, 35, 57). In addition, cyanide has been reported to inhibit the P-450-dependent hydroxylation of drugs (11) and the demethylation of sterols (57-59) in liver microsomes, the C_{21} hydroxylation of steroids in adrenocortical microsomes (94) and the 11β-hydroxylation of deoxycorticosterone in adrenocortical mitochondria (12). On the other hand, Orrenius (104) found no inhibition by cyanide of aminopyrine demethylation in liver microsomes.

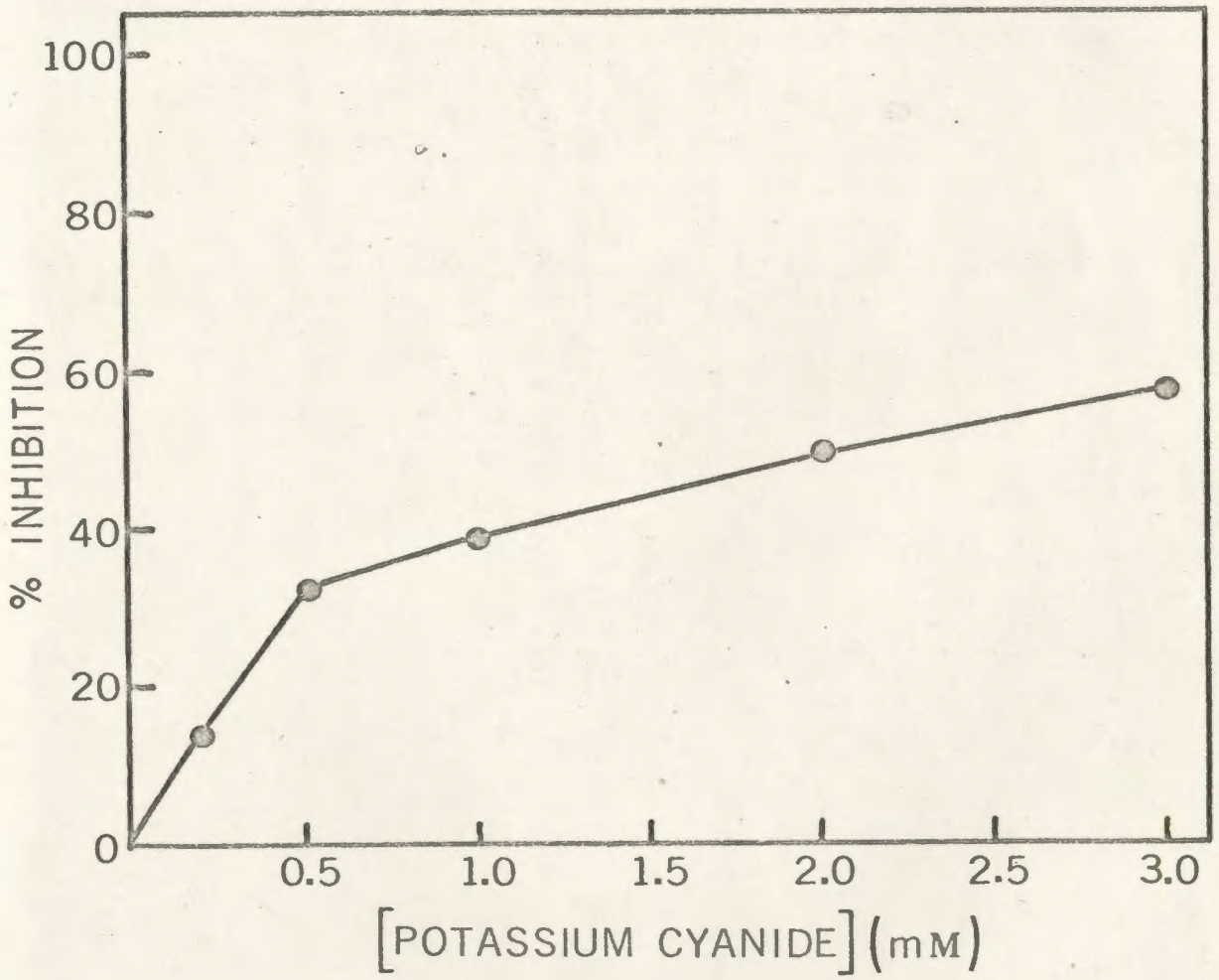
Our results (Fig. 14) demonstrate a 50 percent inhibition of the NADPH-peroxidase reaction at a cyanide concentration of about 2.2 mM. This value correlates very well with the spectral dissociation constant reported for cyanide (K_s = 2.1 mM) in liver microsomes by Schenkman *et al* (34).

Inhibition of microsomal N-demethylase and NADPH-peroxidase activity by steroids

A microsomal electron transport system composed of NADPH-cytochrome *c* reductase and cytochrome P-450 functions in the hydroxylation and demethylation of a wide variety of lipid-soluble compounds. Our results suggest that a similar enzyme system is involved in microsomal NADPH-peroxidase

Figure 14. Cyanide inhibition curve for microsomal NADPH-peroxidase activity.

The NADPH-peroxidase reaction was measured in a 3 ml reaction volume containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, 1 mg liver microsomal protein, varying concentrations of potassium cyanide, and 0.4 mM cumene hydroperoxide.



activity. Steroids are among the physiologically active compounds that inhibit microsomal hydroxylation and demethylation reactions and it was therefore of interest to determine the effects of several steroids on the microsomal N-demethylation of p-chloro-N-methylaniline and to see if similar effects are produced when these steroids interact with the microsomal NADPH-peroxidase enzyme system. The results (Table 19) show that the same concentrations of steroids used to inactivate drug demethylation activity produced similar inhibition of NADPH-peroxidase activity.

Cytochrome b_5 and microsomal NADPH-peroxidase activity

Because of the recent suggestion (32) that cytochrome b_5 may play a role in NADPH-dependent hydroxylation reactions, the role of cytochrome b_5 in the NADPH-peroxidase reaction was investigated with the aid of antibodies prepared to the hemoprotein. Preincubation of liver microsomes with 8 mg antibody per mg protein for 5 minutes and subsequent measurement of NADPH-peroxidase activity revealed no inhibition of the reaction rate. On the other hand, NADH-cytochrome c reductase activity, a cytochrome b_5 -dependent function, was inhibited 60% at the same antibody: protein ratio. These results argue against the participation of cytochrome b_5 in the microsomal NADPH-peroxidase reaction. Omura* (41) recently reported no inhibition by antibody to cytochrome b_5 of aniline or aminopyrine hydroxylation in liver microsomes.

Synergistic effect of NADH on the NADPH-peroxidase reaction rate

Hildebrandt and Estabrook (32) reported the enhancement of NADPH-dependent microsomal hydroxylation reactions by NADH. Therefore, the effect of NADH on the NADPH-dependent peroxidase reaction was investigated. The

* *Work cited in reference 41.*

TABLE 19

INHIBITION OF MICROSOMAL N-DEMETHYLASE AND NADPH-PEROXIDASE ACTIVITY BY STEROIDS [a]

Steroid Inhibitor	% Inhibition	
	N-demethylase	NADPH-peroxidase
Androstenedione	60	67
Progesterone	59	69
17α-hydroxyprogesterone	57	57
Deoxycorticosterone	55	61
Testosterone	50	68
Corticosterone	40	56

[a] The microsomal N-demethylation of p-chloro-N-methylaniline was measured in the presence of 0.1 mM steroid inhibitor by the method of Kupfer and Bruggeman (95). NADPH-peroxidase activity was assayed in the presence of 0.1 mM steroid inhibitor as described in Table 15.

results (Table 20) demonstrate that NADH can support NADPH-dependent peroxidase activity synergistically. In addition, NADH itself can act as an efficient electron donor for the microsomal peroxidase reaction. The properties of the NADH-peroxidase electron transport system of liver microsomes will be described in detail in the next chapter.

Inhibition of NADPH-peroxidase activity by trypsin

Previous studies (38) have shown that treatment of liver microsomes with trypsin solubilizes NADPH-cytochrome c reductase from the microsomal membrane resulting in an inactivation of drug hydroxylation activity. In Table 21 is illustrated the effect of trypsin digestion of liver microsomes on NADPH-peroxidase activity and other microsomal constituents. It is noticed that incubation of liver microsomes with 25 μg trypsin/mg protein at 10° for 15 hr enhanced NADPH-cytochrome c reductase activity by 25% but inhibited the NADPH-peroxidase reaction by 70%. Since trypsin digestion of microsomes converted only 38% of microsomal hemoprotein P-450 into the inactive P-420 form, the conversion to P-420 alone cannot account for the marked inhibition of the activity. The trypsin treatment solubilized 95% of the NADPH-cytochrome c reductase activity and it seems quite probable that part of the inhibition of NADPH-peroxidase activity may be due to the release of the flavoprotein reductase from the microsomal membrane. Approximately 50% of the total microsomal protein was solubilized by the trypsin treatment.

The residual NADPH-peroxidase activity was recovered mainly in the pellet fraction (73%) after centrifugation of the trypsin digest and cytochrome P-450 showed a similar pattern of distribution. In contrast, cytochrome b_5 was recovered almost exclusively in the supernatant fraction. The

TABLE 20

SYNERGISTIC EFFECT OF NADH ON THE NADPH-PEROXIDASE REACTION RATE [a]

Addition	Activity
Microsomes + NADPH + cumene hydroperoxide	34
Microsomes + NADH + cumene hydroperoxide	43
Microsomes + NADPH + NADH + cumene hydroperoxide [b]	52

[a] NAD(P)H-peroxidase activity was measured in a 3 ml reaction vol containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH or NADH, 1 mg liver microsomal protein, and 0.4 mM cumene hydroperoxide. Reaction rates are expressed as nmoles NAD(P)H oxidized/min/mg protein.

[b] In the assay medium, 0.075 mM of each nucleotide was used to make up a total nucleotide conc of 0.15 mM.

TABLE 21

EFFECT OF TRYPSIN DIGESTION OF LIVER MICROSOMES ON NADPH-PEROXIDASE ACTIVITY AND OTHER MICROSOMAL CONSTITUENTS [a]

Microsomal Preparation	NADPH-Peroxidase		NADPH-Cyt. c Reductase		Cyt. P-450		Cyt. P-420	Cyt. b_5	
	Specific activity	Percent recovery	Specific activity	Percent recovery	Specific activity	Percent recovery	Specific activity	Specific activity	Percent recovery
Liver microsomes	34		58		0.79		0.03	0.57	
Trypsin-treated [b] microsomes	11		71		0.45		0.28	0.50	
Supernatant	4	27	119	95	0.15	18	0.29	0.73	95
Pellet	11	73	6	5	0.68	82	0.19	0.04	5

[a] Liver microsomes (10 mg protein/ml) were incubated at 10° with 25 µg trypsin/mg protein for 15 hr in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.3 M sucrose and 2 mM EDTA. Reaction was stopped by addition of trypsin inhibitor (1 mg/mg trypsin). The digest was spun at 105,000 g for 2 hr, pellet was suspended in 0.3 M sucrose-2mM EDTA (pH 7.4) and enzymes of various fractions were assayed. NADPH-peroxidase activity was measured using 0.4 mM cumene hydroperoxide. NADPH-peroxidase and NADPH-cyt. c reductase activities are expressed in terms of nmoles NADPH oxidized/min/mg protein, and nmoles cyt. c reduced/min/mg protein, respectively, whereas cytochrome contents are given in terms of nmoles/mg protein. Percent recoveries in the supernatant and pellet fractions were calculated by taking the activities in the trypsin digest as 100%. Values are expressed as percent of residual activity recoverable. Actual total recoveries were greater than 90%.

[b] Trypsin-treated microsomes refers to a mixture of soluble and particulate materials obtained after treatment of liver microsomes with trypsin as described above.

presence of a weak NADPH-cytochrome c reductase activity in the pellet fraction was sufficient for the catalysis of a slow NADPH-peroxidase activity in the presence of cytochrome P-450 (Table 21). Addition to the pellet fraction of purified NADPH-cytochrome c reductase, prepared by trypsin digestion of liver microsomes (98), did not increase the reaction rate.

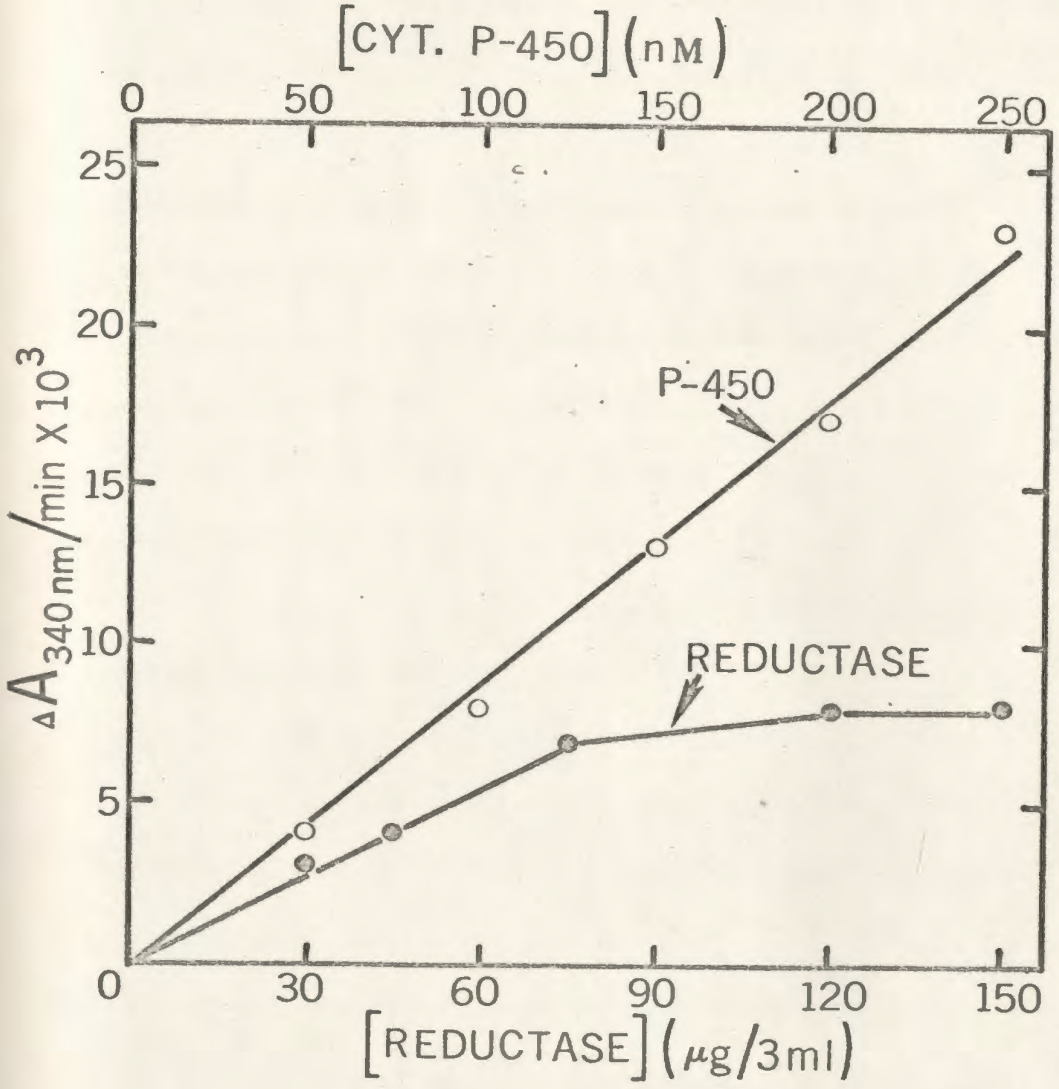
Reconstitution of the microsomal NADPH-peroxidase activity

Since the results in this chapter have implicated NADPH-cytochrome c reductase and cytochrome P-450 as electron carriers in the microsomal NADPH-peroxidase reaction, a study was undertaken to determine whether the membrane-bound enzyme system could be solubilized, resolved into its component parts, and reconstituted. Purified NADPH-cytochrome c reductase was prepared by trypsin digestion of liver microsomes following the procedure of Omura and Takesue (98). The purified preparation catalyzed the reduction of 3.7 μmoles cytochrome c/min/mg protein and was free from contamination by other microsomal electron transfer components. Partially purified cytochrome P-450 was prepared from livers of normal rats by cholate solubilization according to the method of Lu et al (23). The final preparation contained 0.56 nmoles P-450 per mg protein.

The effect on NADPH-peroxidase activity of varying the concentration of purified NADPH-cytochrome c reductase and cytochrome P-450 is shown in Fig. 15. The results indicate that the reaction rate was proportional to the concentration of cytochrome P-450 under conditions where NADPH-cytochrome c reductase was present in excess. On the other hand,

Figure 15. Effect of increasing concentrations of purified NADPH-cytochrome c reductase and cytochrome P-450 on NADPH-peroxidase activity.

The reaction medium consisted of 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, varying concentrations of purified NADPH-cytochrome c reductase or cytochrome P-450, and 0.8 mM cumene hydroperoxide in a final volume of 3 ml. When the concentration of reductase was varied, 0.2 µM P-450 was used and when the concentration of P-450 was varied, 75 µg reductase (specific activity, 3.7 µmoles cytochrome c reduced/min/mg protein) was employed. Reaction rates were corrected for NADPH oxidation in the absence of reductase or P-450, respectively.



when the concentration of purified NADPH-cytochrome c reductase was varied in the presence of 0.2 μM cytochrome P-450, the reaction rate although small showed saturation kinetics with increasing amounts of flavoenzyme. Similar kinetics were obtained when detergent-solubilized NADPH-cytochrome c reductase, prepared by the method of Lu et al (23) was substituted for trypsin-solubilized reductase. However, the cytochrome P-450 preparation was impure and contained a significant amount of NADPH-cytochrome c reductase activity (10 nmoles cytochrome c reduced/min/mg protein). Therefore, more efficient reconstitution of NADPH-peroxidase activity must await further purification of hemoprotein P-450.

A comparison of the effectiveness of liver microsomes and the reconstituted enzyme system in catalyzing the NADPH-peroxidase reaction is shown in Table 22. The specific activity of the reconstituted enzyme system using either trypsin-solubilized or detergent-solubilized reductase was about 25% of that originally present in liver microsomes. Addition to the reconstituted system of either trypsin-solubilized or detergent-solubilized cytochrome b_5 (0.2 μM) did not increase the reaction rate.

Lu and coworkers (23, 52) have resolved the liver microsomal hydroxylating enzyme system into fractions containing cytochrome P-450, NADPH-cytochrome c reductase, and lipid. The active lipid component has been identified as phosphatidylcholine (56). The effect of microsomal lipid or phosphatidylcholine on the NADPH-peroxidase activity of the reconstituted enzyme system is presented in Table 22. The results indicate a slight increase in the reaction rate after addition of 0.05 mg of microsomal lipid or phosphatidylcholine. Addition of larger amounts of lipid did not increase the activity any further.

TABLE 22

A COMPARISON OF NADPH-PEROXIDASE ACTIVITY IN LIVER MICROSOMES AND THE RECONSTITUTED ENZYME SYSTEM [a]

Addition	NADPH-Peroxidase Activity
Liver microsomes	79
Detergent-solubilized NADPH-cyt. c reductase + P-450	18
Detergent-solubilized NADPH-cyt. c reductase + P-450 + 0.05 mg microsomal lipid[b]	20
Detergent-solubilized NADPH-cyt. c reductase + P-450 + 0.05 mg phosphatidylcholine[b]	20
Trypsin-solubilized NADPH-cyt. c reductase + P-450	18

[a] NADPH-peroxidase activity in liver microsomes was measured at 340 nm in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH, 1 mg microsomal protein, and 0.8 mM cumene hydroperoxide. The reaction medium for the reconstituted system was identical except that 0.5 μM purified P-450 plus an excess of either detergent-solubilized or trypsin-solubilized NADPH-cyt. c reductase were substituted for liver microsomes. The effect of lipid on the reaction rate was tested by pre-incubation of lipid with the reductase and P-450 for 5 min prior to assay. Reaction rates are expressed in terms of nmoles NADPH oxidized/min/nmole P-450.

[b] Microsomal lipid prepared by the method of Lu et al (23) and phosphatidylcholine (Sigma) were solubilized by sonication (see Methods) and added as an aqueous solution.

Stimulation of NADPH-peroxidase activity by phenobarbital treatment

Phenobarbital treatment of rats has been shown to markedly increase drug hydroxylation activity and the specific content of cytochrome P-450 in liver microsomes without appreciably affecting the concentration of cytochrome $\underline{b}_5$. The effect of phenobarbital pretreatment of rats on microsomal NADPH-peroxidase activity and other components of liver microsomes is illustrated in Table 23. It is noticed that the specific activity of the NADPH-peroxidase enzyme system was enhanced 7.5-fold when cumene hydroperoxide was employed as substrate and 2.7-fold when progesterone 17α-hydroperoxide was used as substrate. This activation was accompanied by a 2.3-fold enhancement of NADPH-cytochrome $\underline{c}$ reductase activity and a 3-fold increase in cytochrome P-450 concentration. The specific content of cytochrome $\underline{b}_5$ showed only a 30% increase.

Oxidation of NADPH-reduced cytochrome P-450 by hydroperoxides

Since our results have implicated cytochrome P-450 as an electron transfer component in the microsomal NADPH-peroxidase reaction, it was of considerable interest to determine whether the reduced form of the hemoprotein could be oxidized by hydroperoxides. It was necessary to carry out reduction experiments in the absence of oxygen since the reduced form of cytochrome P-450 in the microsomal membrane is highly autoxidizable. Accordingly, microsomal cytochrome P-450 was reduced with NADPH in the presence of carbon monoxide which binds only the ferrous form of P-450, and the reduced hemoprotein was then allowed to react with known quantities of hydroperoxide under strict anaerobic conditions. The results (Table 24) show that after treatment with 0.16 mM cumene

TABLE 23

STIMULATION OF MICROSOMAL NADPH-PEROXIDASE ACTIVITY BY PHENOBARBITAL TREATMENT [a]

Activity	Control	Phenobarbital-induced
NADPH-peroxidase		
(1) cumene hydroperoxide	4.8	36.0
(2) progesterone 17α-hydroperoxide	5.3	14.5
NADPH-cytochrome *c* reductase	57.0	131.0
Cytochrome P-450	0.6	1.8
Cytochrome b_5	0.40	0.51

[a] Male Sprague-Dawley rats (250-300 g) were injected intraperitoneally with sodium phenobarbital (50 mg/kg) twice daily for 5 days. Control rats received an equivalent vol of 0.9% NaCl. Animals were sacrificed 24 hr after the last injection, microsomes were prepared from perfused livers and enzyme activities determined on the pooled livers of 3 rats as described in Methods. NADPH-peroxidase activity is expressed as nmoles NADPH oxidized/min/mg protein, NADPH-cyt. *c* reductase as nmoles cyt. *c* reduced/min/mg protein, and cytochrome P-450 and b_5 contents as nmoles/mg protein.

TABLE 24

OXIDATION OF THE NADPH-REDUCED CYTOCHROME P-450-CO COMPLEX BY CUMENE HYDROPEROXIDE [a]

Addition	ΔA(450-490 nm)
Microsomes + NADPH + CO	0.097
Microsomes + NADPH + CO + 0.16 mM cumene hydroperoxide	0.057
Microsomes + NADPH + CO + 0.16 mM cumene hydroperoxide + sodium dithionite	0.096
Microsomes + NADPH + CO + 0.40 mM cumene hydroperoxide	0.003

[a] To a reference cuvette and an anaerobic sample cuvette was added 3 ml of a mixture of liver microsomes (2 mg protein/ml) containing 0.1 M sodium phosphate buffer (pH 7.5) and 0.45 mM NADPH. Into the sidearm of the sample cuvette was placed a solution of cumene hydroperoxide. The sample cuvette was flushed with nitrogen (99.99% pure) for 20 min and then with CO that had been passed through a deoxygenating medium (83) for 5 min and the CO-difference spectrum was recorded using a Perkin-Elmer 356 dual wavelength spectrophotometer. The contents of the sample cuvette were then mixed with hydroperoxide (final conc. 0.16 mM and 0.40 mM, respectively) and the difference spectrum recorded. A few grains of sodium dithionite were added to the sample cuvette containing 0.16 mM cumene hydroperoxide and the spectrum again measured. The reduction of cytochrome P-450 was determined at 450 nm relative to 490 nm.

hydroperoxide, the reduced P-450-CO complex was decreased to 58% of the control value and after reaction with 0.4 mM cumene hydroperoxide, the hemoprotein-CO complex disappeared completely. That the disappearance of the P-450-CO complex upon treatment with cumene hydroperoxide was not due to the destruction of the heme moiety of the hemoprotein was evidenced by the reappearance of a fully-developed P-450-CO complex upon reduction by sodium dithionite. The initial rate of oxidation of the reduced P-450-CO complex at 450 nm could not be measured accurately since the reaction took place instantaneously even when low concentrations of hydroperoxide were employed.

These results suggest that the ferrous form of cytochrome P-450 can be oxidized very rapidly by cumene hydroperoxide.

CHAPTER IV

THE MICROSOMAL NADH-PEROXIDASE ELECTRON TRANSPORT SYSTEM

CHAPTER IV: THE MICROSOMAL NADH-PEROXIDASE ELECTRON TRANSPORT SYSTEM

This chapter will examine the role of various electron transport components in the microsomal NADH-peroxidase enzyme system.

MATERIALS AND METHODS

Materials

Trypsin, soybean trypsin inhibitor, nucleotides, and p-hydroxymercuribenzoate were obtained from Sigma. Phenacetin was supplied by British Drug Houses, Toronto. All other reagents were of the highest grade commercially available.

Assay of NADH-peroxidase activity

Microsomal NADH-peroxidase activity was measured at 23° by following the rate of oxidation of NADH by hydroperoxides in the first min of reaction at 340 nm in the presence of microsomal fractions. The assay medium contained, unless otherwise stated, 0.067 M sodium phosphate buffer (pH 7.5), 0.27 M sucrose, 2 mg microsomal protein, 0.15 mM NADH, and 0.05 mM hydroperoxide in a final vol of 3 ml. Reaction rates were routinely corrected for NADH oxidation in the absence of hydroperoxide and were calculated using an extinction coefficient for NADH of 6.22 $cm^{-1}mM^{-1}$. Measurements were normally carried out with a Beckman DB-G double beam spectrophotometer. Turbidity in the sample solutions was balanced by using a reference cuvette containing 0.067 M sodium phosphate buffer (pH 7.5) and the same amount of microsomal protein as employed in the sample cuvette. Reactions which produced low velocities or those which

utilized large quantities of protein were measured with a Perkin-Elmer 356 double beam spectrophotometer in the split beam mode.

Determination of the K_m for NADH in the NADH-peroxidase reaction

The K_m for NADH in the microsomal NADH-peroxidase reaction was estimated by following the rate of oxidation of NADH at 340 nm on a Perkin-Elmer 356 recording spectrophotometer with a full-scale deflection of 0.1 absorbance units. The reaction medium consisted of 0.1 M sodium phosphate buffer (pH 7.5), 0.17 mg liver microsomal protein, varying amounts of NADH, and 0.8 mM cumene hydroperoxide in a final vol of 3 ml. Reaction rates were corrected for NADH oxidation in the absence of cumene hydroperoxide.

Enzyme assays

NADPH- or NADH-cytochrome c reductase activity was measured at 23° by following the rate of reduction of cytochrome c (50 μM) at 550 nm in a 3 ml solution containing 0.033 M sodium phosphate buffer (pH 7.5), 0.15 mM NADPH or NADH, and a suitable amount of enzyme. NADH-cytochrome b_5 reductase activity was measured by following the rate of reduction of purified cytochrome b_5 (3.7 μM) at 424 nm in a 3 ml solution containing 0.033 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, and enzyme. NADH-ferricyanide reductase activity was determined at 420 nm in a 3 ml reaction vol containing 0.033 M sodium phosphate buffer (pH 7.5), 1 mM potassium ferricyanide, 0.15 mM NADH, and enzyme. The millimolar extinction differences (cm^{-1} mM^{-1}) between reduced and oxidized electron acceptors used in calculating reaction rates were 21 at 550 nm for

cytochrome c, 1.02 at 420 nm for potassium ferricyanide, and 100 at 424 nm for cytochrome b_5 (98).

Purification of microsomal components

Cytochrome b_5 was prepared from liver microsomes by either trypsin digestion (98) or detergent solubilization (99). NADH-cytochrome b_5 reductase was prepared by either lysosomal digestion of liver microsomes according to Takesue and Omura (105, 106) or by detergent solubilization as described by Spatz and Strittmatter (107). Partially purified cytochrome P-450 was prepared by cholate solubilization of liver microsomes according to Lu et al (23). Microsomal lipid was prepared by the method of Lu and coworkers (52).

Preparation of lipid micelles

Phosphatidylcholine, lysophosphatidylcholine, and phosphatidylcholine-lysophosphatidylcholine micelles were prepared by sonic oscillation in aqueous medium under nitrogen as described in Methods section in Chapter III.

Preparation of antibodies

Antibody to NADH-cytochrome b_5 reductase prepared by the method of Takesue and Omura (106) and antibody to cytochrome b_5 prepared according to Fukushima et al (97) was kindly supplied by Professor T. Omura, Kyushu University. Antiserum to NADPH-cytochrome c reductase prepared by the method of Glazer et al (31) was kindly donated by Professor J.B. Schenkman, Yale University.

RESULTS

The NADH-peroxidase assay

NADH-peroxidase activity was followed by monitoring the rate of oxidation of NADH by hydroperoxides at 340 nm in the presence of microsomal fractions. Using cumene hydroperoxide as substrate and rat liver microsomes as the enzyme source, the reaction rate was found to be first-order with respect to microsomal protein concentration (Fig. 16). Using cumene hydroperoxide as substrate, an apparent K_m value of about 0.6 mM was obtained * (Fig. 17). Under these assay conditions, the rate of NADH oxidation was calculated to be approximately 37 nmoles/min/mg protein/μmole cumene hydroperoxide**.

Measurement of the K_m for NADH in the microsomal NADH-peroxidase reaction showed that an NADH concentration of 6 μM gave an identical initial reaction rate (47 nmoles NADH oxidized/min/mg protein/μmole cumene hydroperoxide) to that obtained when higher concentrations of NADH were employed. It appears, therefore, that the K_m for NADH is smaller than 3 μM. Strittmatter and Velick (40) reported a K_m for NADH of 2.7 μM for purified NADH-cytochrome b_5 reductase.

Hydroperoxide specificity of microsomal NADH-peroxidase

The hydroperoxide specificity of microsomal NADH-peroxidase is

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** A similar K_m value was obtained using a Lineweaver-Burke plot.*

*** Under these assay conditions, the reaction rate was first-order with respect to microsomal protein concentration and first-order with respect to cumene hydroperoxide concentration.*

Figure 16. Effect of increasing microsomal protein concentration on NADH-peroxidase activity.

The reaction was measured at 340 nm in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, specified concentrations of liver microsomal protein, and 0.8 mM cumene hydroperoxide.

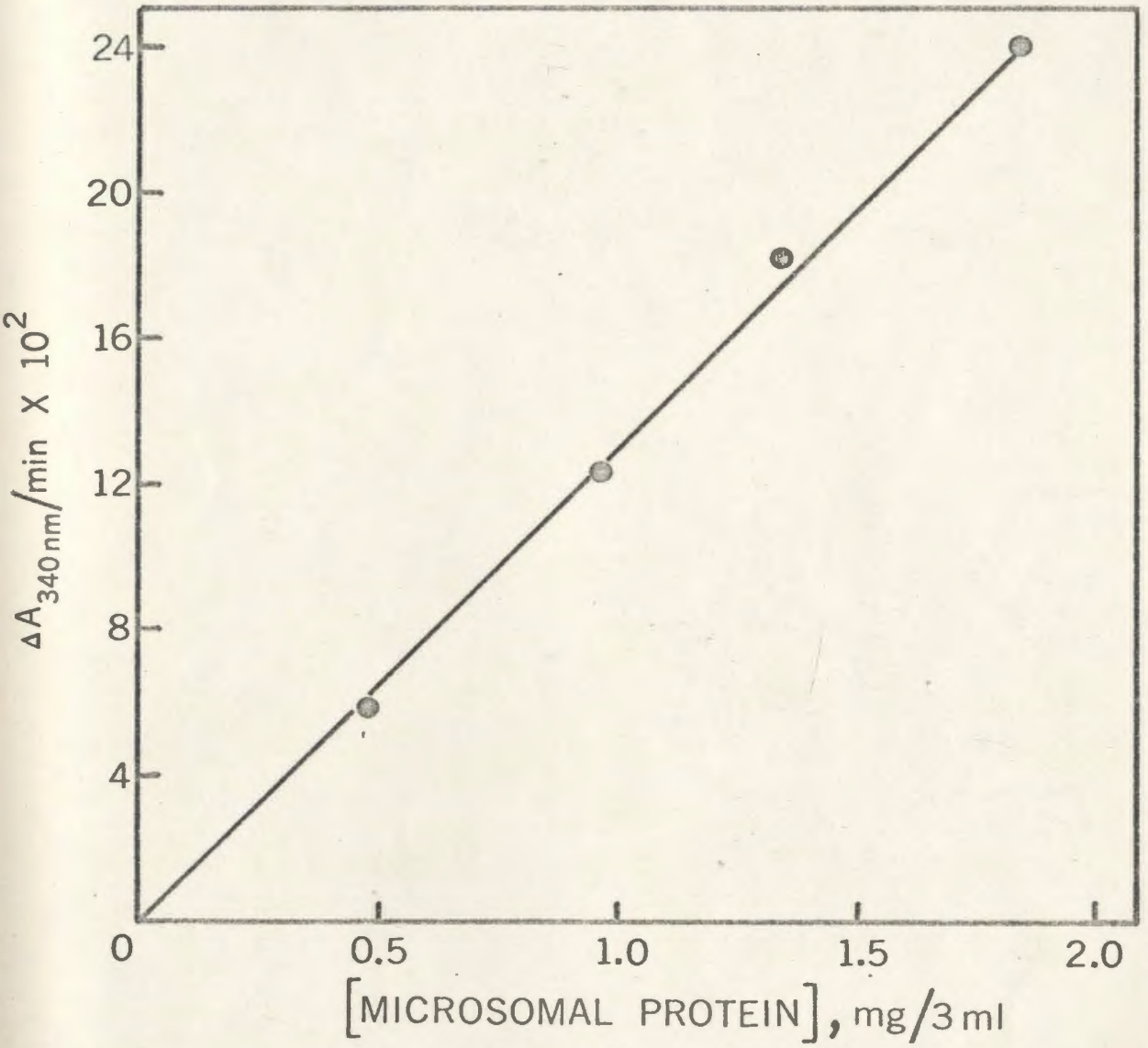
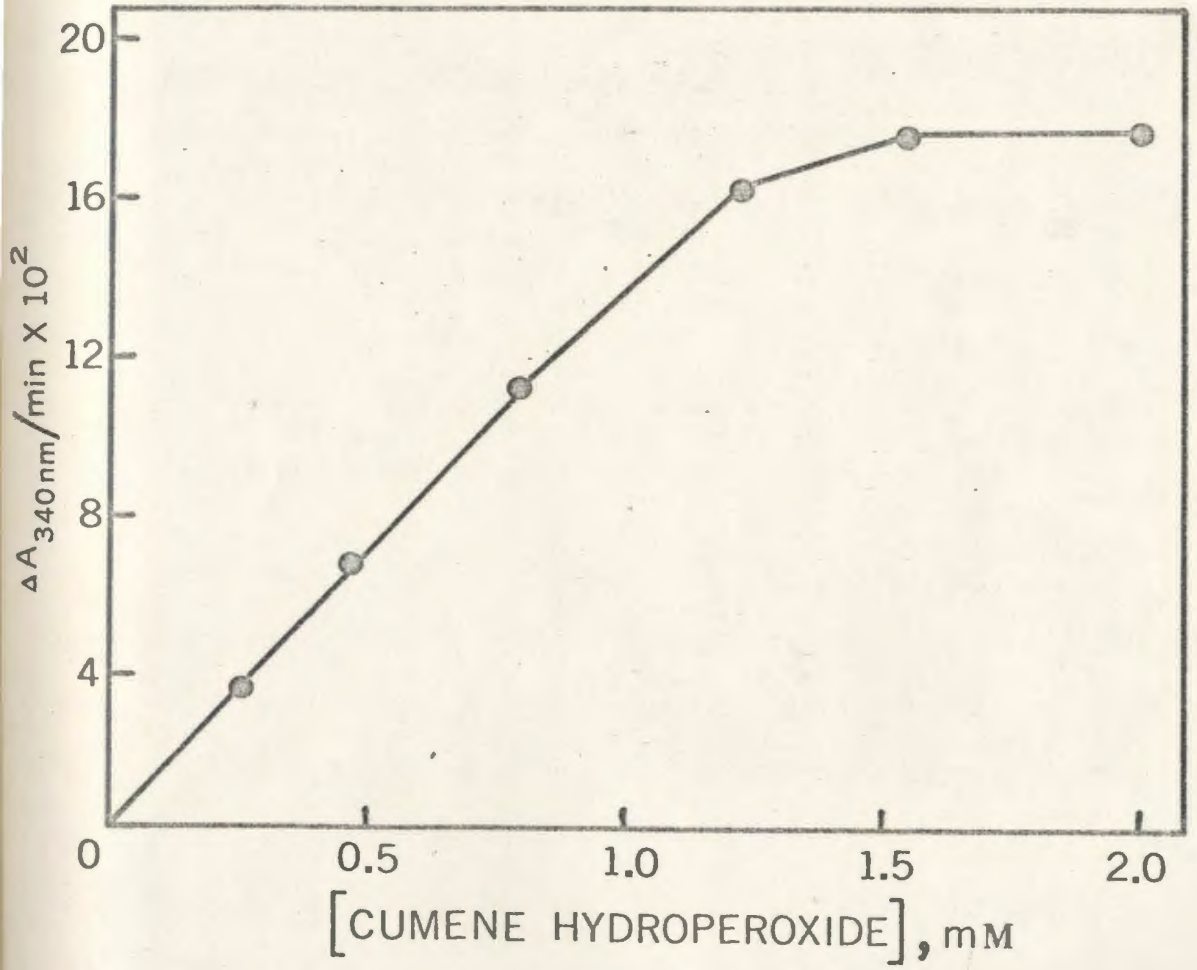


Figure 17. Effect of increasing cumene hydroperoxide concentration on microsomal NADH-peroxidase activity.

The assay medium contained 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 0.9 mg liver microsomal protein, and varying amounts of hydroperoxide in a final volume of 3 ml. Reaction rates were corrected for NADH oxidation in the absence of hydroperoxide.



presented in Table 25. The 17α-hydroperoxide derivatives of progesterone and pregnenolone were found to be effective substrates for the microsomal enzyme system of both liver and adrenal cortex.

Tissue distribution of microsomal NADH-peroxidase activity

In Table 26 is shown the tissue distribution of the microsomal NADH-peroxidase activity. It is observed that the microsomal fractions of tissues which contained high amounts of cytochrome P-450 per mg protein (e.g. liver, adrenal cortex) were the most active in catalyzing the NADH-peroxidase reaction whereas microsomes from tissues that contained little or no cytochrome P-450 were either weakly active or completely inactive. However, the reaction rates were not strictly proportional to cytochrome P-450 content.

The properties of the NADH-peroxidase enzyme system of rat liver microsomes were next examined in detail.

Efficiency of NADH oxidation by hydroperoxides

Examination of the stoichiometry of the NADH-peroxidase reaction revealed the oxidation of approximately 0.5 moles of NADH per mole of cumene hydroperoxide in the presence of liver microsomes under the normal assay conditions. This represents a 50% efficiency and indicates a competition between NADH and certain microsomal constituents for the oxidizing equivalents of the hydroperoxide. Hydrogen donors other than NADH have been found to be oxidized by hydroperoxides at a similar efficiency in the presence of various heme catalysts (100-102).

TABLE 25

HYDROPEROXIDE SPECIFICITY OF MICROSOMAL NADH-PEROXIDASE[a]

Hydroperoxide	NADH-Peroxidase Activity	
	liver microsomes	adrenocortical microsomes
Progesterone 17α-hydroperoxide	6.8	1.2
Pregnenolone 17α-hydroperoxide	6.3	0.8
Cumene hydroperoxide	5.8	0.8
Cholesterol 7β-hydroperoxide	3.9	-
Allopregnanolone 17α-hydroperoxide	2.9	-

[a] The oxidation of NADH by hydroperoxides in the presence of microsomal fractions was measured at 340 nm as described in Methods. Reaction rates are expressed as nmoles NADH oxidized/min/mg protein. A hydroperoxide conc. of 0.05 mM was employed.

TABLE 26

TISSUE DISTRIBUTION OF MICROSOMAL NADH-PEROXIDASE ACTIVITY [a]

Source of Microsomes	P-450 Content	NADH-Peroxidase Activity
Liver	0.70	80.8
Adrenal cortex [b]	0.60	4.8
Kidney	0.25	3.4
Lung	0.05	2.9
Testis	0.02	0
Small Intestine	0.02	0
Brain	0	0
Heart	0	0
Skeletal muscle [c]	0	0

[a] Microsomes from various tissues of the rat were prepared and the P-450 content (nmoles/mg protein) determined. NADH-peroxidase activity was measured at 340 nm in a 3 ml solution containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 1 mg microsomal protein, and 0.8 mM cumene hydroperoxide. Reaction rates were corrected for NADH oxidation in the absence of hydroperoxide and are expressed as nmoles NADH oxidized/min/mg protein.

[b] Adrenal cortex microsomes were obtained from bovine adrenals.

[c] The hind legs of the rat were used for the source of skeletal muscle.

Effect of pH and ionic strength on NADH-peroxidase activity

The pH dependence of the microsomal NADH-peroxidase activity is illustrated in Fig. 18. A maximal reaction rate in 0.1 M sodium phosphate buffer was obtained between pH 7.7 and 7.8.

In Fig. 19 is shown the effect of ionic strength on the microsomal NADH-peroxidase reaction. It is seen that the activity was stimsulated by increasing concentrations of sodium phosphate buffer. KCl exhibited a similar stimulatory effect when tested at the same ionic strengths as phosphate.

Inhibition of NADH-peroxidase activity by various reagents

The effect of various inhibitors on the microsomal oxidation of NADH by cumene hydroperoxide was next examined. It was found that the enzyme system was heat labile, being totally inactivated by preincubation of liver microsomes at 80° for 5 min (Table 27). Measurement of the activity under a nitrogen or a carbon monoxide atmosphere produced no appreciable inhibition. NAD^+ and 2-phenyl-2-propanol did not affect the reaction rate. Acetone and tert. amyl alcohol, reagents that are known to convert cytochrome P-450 to its inactive P-420 form (103), were potent inhibitors of the reaction.

Evidence for the involvement of NADH-cytochrome b_5 reductase in NADH-peroxidase activity

The activity of microsomal NADH-cytochrome b_5 reductase is markedly inhibited by low concentrations of sulfhydryl reagents such as p-mercuribenzoate (108, 109). Preincubation of the flavoenzyme with NADH prior to treatment with p-mercuribenzoate partially protects the flavoprotein

Figure 18. The pH dependence of the microsomal NADH-peroxidase reaction.

The assay medium consisted of 0.1 M sodium phosphate buffer of varying pH, 1 mg liver microsomal protein, 0.15 mM NADH, and 0.4 mM cumene hydroperoxide in a final volume of 3 ml. The pH was routinely checked at the end of the reaction.

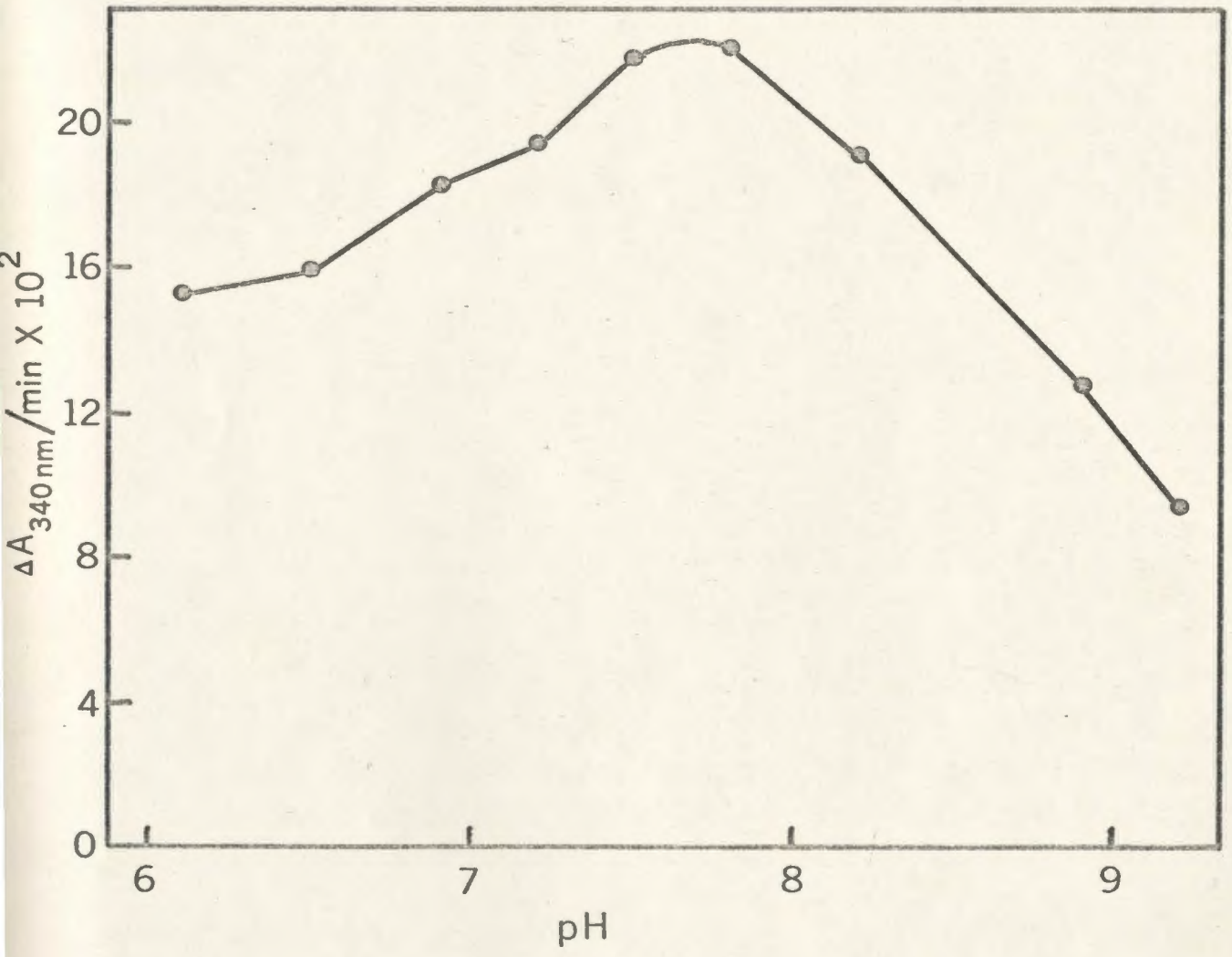


Figure 19. Effect of ionic strength on NADH-peroxidase activity.

The reaction medium contained 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 1 mg liver microsomal protein, and 0.4 mM cumene hydroperoxide to which increasing amounts of KCl or additional phosphate was added to obtain desired ionic strengths. Final ionic strength was calculated from the concentrations of phosphate and added KCl.

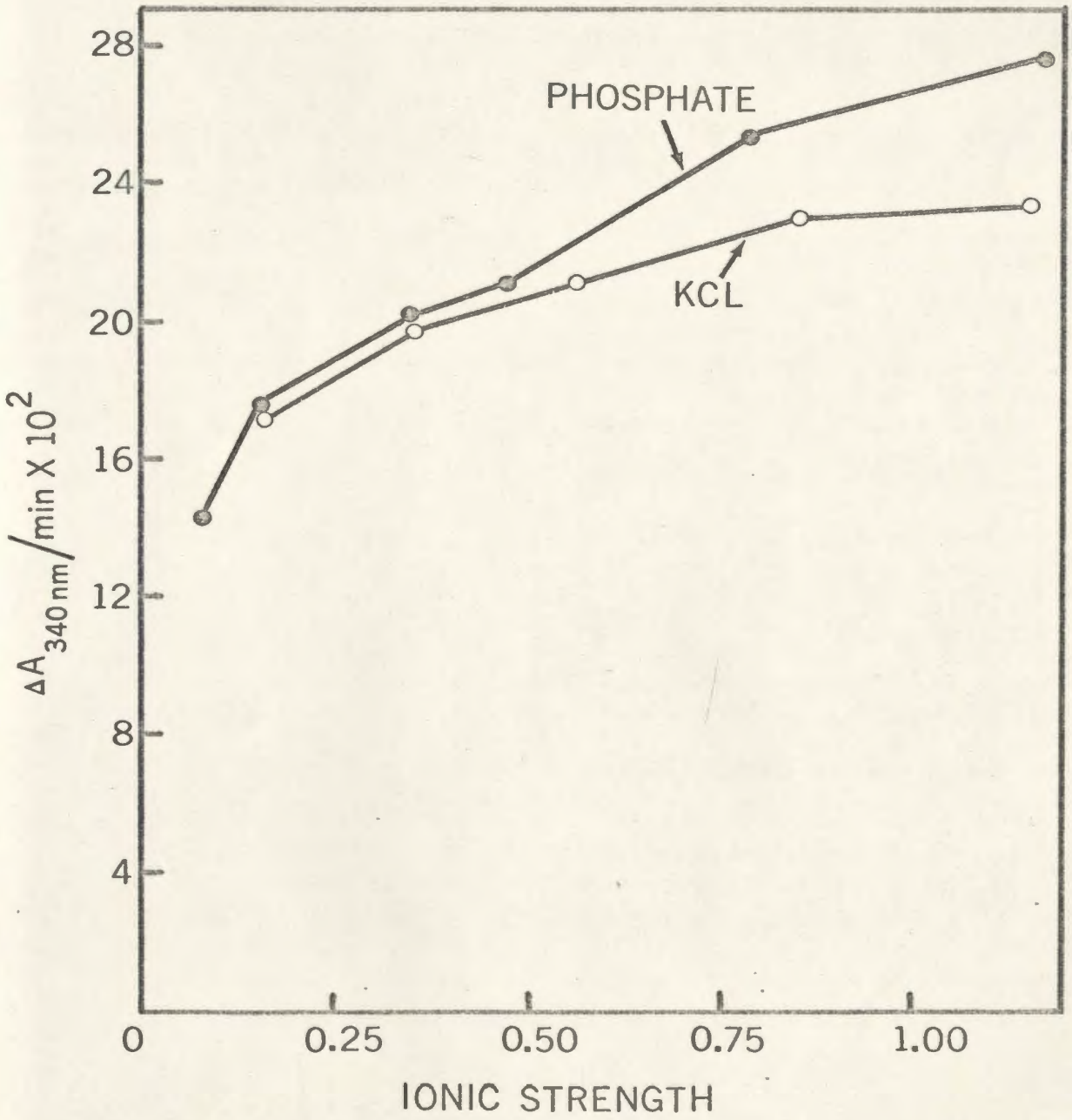


TABLE 27

EFFECT OF VARIOUS INHIBITORS ON NADH-PEROXIDASE ACTIVITY [a]

Inhibitor	% Inhibition
Heat (80° for 5 min)	100
Nitrogen [b]	8
Carbon monoxide [c]	7
NAD^+ (0.45 mM)	8
2-Phenyl-2-propanol (0.4 mM)	8
tert. Amyl alcohol (7%)	98
Acetone (5%)	73

[a] The assay medium contained 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 1 mg liver microsomal protein, specified amounts of inhibitor, and 0.4 mM cumene hydroperoxide in a final vol of 3 ml. The specific activity of the NADH-peroxidase system in the absence of inhibitor, in terms of nmoles NADH oxidized/min/mg protein, was 42.

[b] The contents of an anaerobic cuvette were gassed with nitrogen (99.99% pure) for 10 min prior to addition of cumene hydroperoxide.

[c] The contents of an anaerobic cuvette were gassed with nitrogen for 10 min and CO that had been passed through a deoxygenation medium (83) for 5 min prior to addition of cumene hydroperoxide.

from inactivation. The results (Table 28) show that microsomal NADH-peroxidase activity was markedly inhibited by 0.05 mM p-hydroxymercuribenzoate and preincubation of microsomes with NADH prior to addition of sulfhydryl inhibitor partially protected the enzyme system from inactivation.

Antibody prepared to NADH-cytochrome b_5 reductase was next used to evaluate further the role of the flavoprotein reductase in the microsomal NADH-peroxidase reaction. The results (Table 28) indicate a 30% inhibition of the reaction rate by 20 mg antibody per mg microsomal protein. NADH-ferricyanide reductase activity, a function associated with the enzyme NADH-cytochrome b_5 reductase (106), was inhibited 50% at the same antibody: protein ratio. On the other hand, NADPH-cytochrome c reductase was not affected by the antibody. Unfortunately, the limited supply of antibody precluded an extensive analysis of the inhibitory effect.

Although not conclusive, these results suggest the involvement of NADH-cytochrome b_5 reductase as one of the electron carriers in the microsomal NADH-peroxidase enzyme system.

Inhibition of NADH-peroxidase activity by modifiers of cytochrome P-450

It is well established that type I, type II, and modified type II substrates interact with microsomal cytochrome P-450 to produce characteristic spectral changes (34, 35). The NADH-peroxidase enzyme system was found to be sensitive to inhibition by these substances (Table 29). Steroids forming type I spectra with microsomal P-450 were effective inhibitors as were the type II and modified type II compounds. Other steroid substrates

TABLE 28

INHIBITION OF NADH-PEROXIDASE ACTIVITY BY INHIBITORS OF NADH-CYTOCHROME b_5 REDUCTASE [a]

Inhibitor	% Inhibition
p-Hydroxymercuribenzoate (0.05 mM)	81
p-Hydroxymercuribenzoate after NADH [b]	55
Antibody to NADH-cyt. b_5 reductase [c] (20 mg/mg microsomal protein)	30

[a] NADH-peroxidase activity was measured in the presence of specified amounts of inhibitor as described in Table 27. p-Hydroxymercuribenzoate (0.05 mM) was incubated with 1 mg liver microsomal protein in 0.067 M sodium phosphate buffer (pH 7.5) for 2 min directly in the assay cuvette. After incubation, NADH (0.15 mM) and cumene hydroperoxide (0.4 mM, final conc.) were added and activity determined in a final vol of 3 ml.

[b] Liver microsomes were incubated with NADH for 1 min prior to addition of p-hydroxymercuribenzoate and hydroperoxide.

[c] Microsomes were incubated with indicated amount of antibody at 23° for 5 min directly in the assay cuvette in a 1 ml solution containing 0.1 M sodium phosphate buffer (pH 7.5). After incubation the rest of the assay components were added to make up a total vol of 3 ml. The cumene hydroperoxide conc. used was 0.8 mM.

TABLE 29

INHIBITION OF NADH-PEROXIDASE ACTIVITY BY P-450 MODIFIERS[a]

Type I Compound	% Inhibition
Androstenedione (0.1 mM)	70
Testosterone (0.1 mM)	49
17β-Estradiol (0.1 mM)	40
Aminopyrine (5 mM)	25
Type II Ligand	
Imidazole (2 mM)	81
n-Octylamine (0.5 mM)	73
Pyridine (5 mM)	58
Aniline (5 mM)	62
Modified Type II Compound	
Potassium Cyanide (1 mM)	58
Phenacetin (1 mM)	57
Corticosterone (0.1 mM)	45
Steroid Inhibitor	
Progesterone (0.1 mM)	55
Deoxycorticosterone (0.1 mM)	49
17α-Hydroxyprogesterone (0.1 mM)	40

[a] NADH-peroxidase activity was measured in the presence of specified amounts of various inhibitors as described in Table 27.

such as progesterone, 17α-hydroxyprogesterone, and deoxycorticosterone were also found to act as efficient inhibiting agents.

The effect of various reagents that convert cytochrome P-450 to P-420 on microsomal NADH-peroxidase activity is shown in Table 30. It is observed that reagents which effected the conversion of cytochrome P-450 to P-420 (e.g. detergents, alcohols, protein denaturants) produced a parallel inhibition of NADH-peroxidase activity. These P-450 modifiers did not inhibit the activity of the flavoenzyme NADH-cytochrome $\underline{b}_5$ reductase to any significant extent, indicating that the diminution in NADH-peroxidase activity occurred as a consequence of the conversion of P-450 to P-420 rather than as a result of an inactivation of the flavoprotein reductase.

The inhibition of NADH-peroxidase activity by various P-450 modifiers suggests the participation of cytochrome P-450 in the microsomal NADH-peroxidase system.

Inhibition of NADH-peroxidase activity by cyanide

Gaylor and associates (57) examined the binding of the modified type II ligand, cyanide, to the oxidized forms of cytochrome P-450 in liver microsomes. Two binding constants for cyanide of 0.5 mM and 2.5 mM were obtained suggesting the presence in liver microsomes of two different species of cytochrome P-450. The species of P-450 that exhibited the higher affinity for cyanide was postulated to be involved in the NADH-dependent sterol demethylation activity, a reaction that is inhibited 50% by 0.5 mM cyanide (57-59). Cyanide was found to be an effective inhibitor of the NADH-peroxidase reaction (Fig. 20), a 50% inhibition occurring at a cyanide

TABLE 30

INHIBITION OF NADH-PEROXIDASE ACTIVITY BY REAGENTS THAT CONVERT CYTOCHROME P-450 TO P-420 [a]

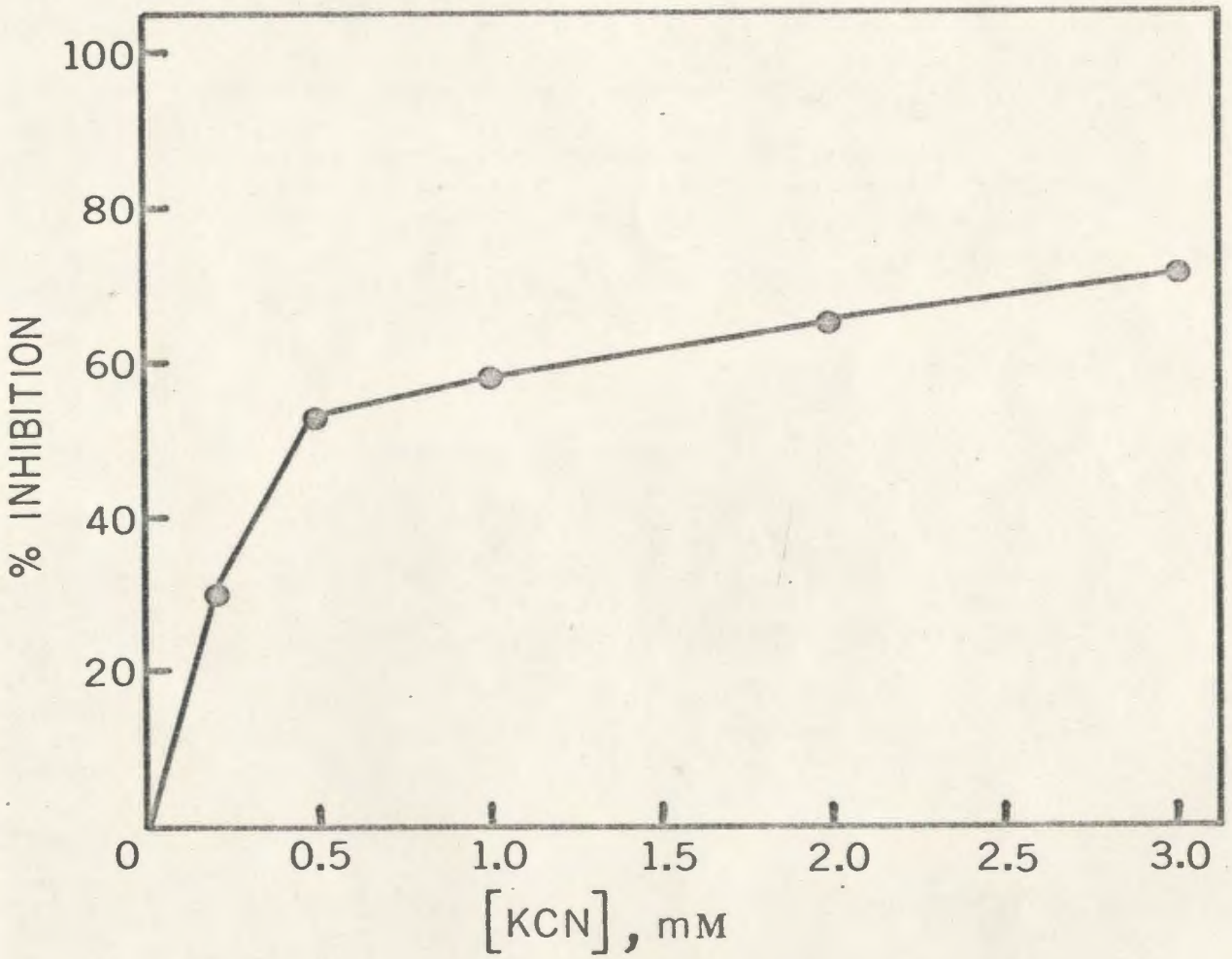
P-450 Modifier	% Inhibition	% P-420 Formed
None	0	0
Sodium dodecyl sulfate (0.1 %)	95	98
Sodium deoxycholate (0.5%)	86	90
Sodium cholate (1%)	71	50
Lubrol WX (1%)	75	40
Urea (4 M)	62	55
n-Propanol (15%)	98	100
Trypsin [b]	45	43

[a] Liver microsomes (5 mg protein/ml) were incubated at 23° for 20 min with specified amounts of P-450 modifier in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.4 mM EDTA and 0.05 M sucrose in a final vol of 3 ml. NADH-peroxidase and NADH-cyt. b_5 reductase activities and the extent of conversion of P-450 to P-420 were determined.

[b] Liver microsomes at a protein conc. of 10 mg/ml were incubated at 15° for 15 hr with trypsin (25 µg/mg protein) in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.3 M sucrose and 2 mM EDTA. NADH-peroxidase activity and the extent of conversion of P-450 to P-420 were determined.

Figure 20. Cyanide inhibition curve for microsomal NADH-peroxidase activity.

The reaction medium consisted of 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 1 mg liver microsomal protein, varying concentrations of potassium cyanide, and 0.4 mM cumene hydroperoxide.



concentration of about 0.45 mM. These results are suggestive of an involvement in the NADH-peroxidase reaction of the P-450 species that exhibits the higher affinity for cyanide.

Stimulation of NADH-peroxidase activity by phenobarbital treatment

The effect of phenobarbital pretreatment of rats on microsomal NADH-peroxidase activity and other components of liver microsomes is illustrated in Table 31. The specific activity of the NADH-peroxidase enzyme system was enhanced 10-fold when cumene hydroperoxide was employed as substrate and 2.7-fold when progesterone 17α-hydroperoxide was used as substrate. Cytochrome P-450 showed a 3-fold increase in specific content whereas the cytochrome $\underline{b}_5$ level was only slightly elevated. On the other hand, NADH-cytochrome $\underline{c}$ reductase activity, a cytochrome $\underline{b}_5$-dependent function, was diminished 1.7-fold by the phenobarbital treatment.

Cytochrome $\underline{b}_5$ and microsomal NADH-peroxidase activity

Since cytochrome $\underline{b}_5$ is one of the electron carriers that can accept reducing equivalents from NADH via NADH-cytochrome $\underline{b}_5$ reductase, it was of considerable interest to determine whether the hemoprotein plays a role in the microsomal NADH-peroxidase enzyme system. When purified cytochrome $\underline{b}_5$ (0.4 μM final conc.) prepared by either trypsin solubilization (98) or detergent solubilization (99) was added to the NADH-peroxidase assay medium and the microsomal activity measured, no change in the reaction rate was observed.

Antibody prepared against cytochrome $\underline{b}_5$ was next used to evaluate further the role of the hemoprotein in the reaction. Preincubation of liver microsomes with 6 mg antibody per mg protein for 5 min and subsequent

TABLE 31

STIMULATION OF MICROSOMAL NADH-PEROXIDASE ACTIVITY BY PHENOBARBITAL TREATMENT [a]

Activity	Control	Phenobarbital-Induced
NADH-peroxidase		
1) cumene hydroperoxide	4.8	48.0
2) progesterone 17α-hydroperoxide	5.3	14.5
NADH-cytochrome *c* reductase	449.0	268.0
Cytochrome P-450	0.6	1.8
Cytochrome b_5	0.4	0.51

[a] Male Sprague-Dawley rats (250-300 g) were injected intraperitoneally with sodium phenobarbital (50 mg/kg) twice daily for 5 days. Control rats received an equivalent vol of 0.9% NaCl. Animals were sacrificed 24 hr after the last injection, microsomes were prepared from perfused livers and enzyme activities determined on the pooled livers of 3 rats as described in Methods. NADH-peroxidase and NADH-cyt. *c* reductase activities are expressed as nmoles NADH oxidized/min/mg protein and nmoles cyt. *c* reduced/min/mg protein, respectively, whereas cyt. b_5 and P-450 contents are given as nmoles/mg protein.

measurement of NADH-peroxidase activity by the normal procedure produced no inhibition of the reaction rate. On the other hand, this same antibody: protein ratio inhibited NADH-cytochrome c reductase activity, a cytochrome b_5-dependent function, by 45%.

These results argue against the participation of cytochrome b_5 in the NADH-peroxidase reaction.

NADPH-cytochrome c reductase and NADH-peroxidase activity

The activity of NADPH-cytochrome c reductase is markedly inhibited by $NADP^+$ and by antibody prepared against the flavoenzyme. These modifiers were previously found to be potent inhibitors of the NADPH-dependent peroxidase activity (see Chapter III). When tested in their ability to inhibit the NADH-dependent peroxidase activity, $NADP^+$ (0.6 mM) and antibody to the flavoprotein (8 mg/mg microsomal protein) were found to be completely ineffective in inhibiting the reaction rate. These results indicate that the flavoenzyme does not participate in the NADH-dependent peroxidase reaction.

Effect of trypsin on microsomal NADH-peroxidase activity

Previous investigators (38) have demonstrated that treatment of liver microsomes with trypsin completely solubilizes NADPH-cytochrome c reductase and cytochrome b_5 from the microsomal membrane but leaves the major portion of NADH-cytochrome b_5 reductase and hemoprotein P-450 still intact. Accompanying the solubilization of cytochrome b_5 is a drastic reduction in NADH-cytochrome c reductase activity.

The present study has utilized proteolytic digestion as a means

of further identifying the microsomal components involved in the NADH-peroxidase enzyme system. The results (Table 32) show that incubation of microsomal fraction at 10° with 25 μg trypsin/mg protein for 15 hr resulted in a 48% inhibition of NADH-peroxidase activity. This inactivation was probably due to the conversion of cytochrome P-450 to P-420 since the trypsin treatment converted 38% of the hemoprotein into the inactive form. NADH-cytochrome c reductase activity was inhibited 96% by the trypsin digestion presumably as a result of the solubilization of cytochrome b_5. On the other hand, NADH-ferricyanide reductase activity, a function which is associated with the enzyme NADH-cytochrome b_5 reductase, was only slightly diminished.

The residual NADH-peroxidase activity was recovered mainly in the pellet fraction (70%) after centrifugation of the tryptic digest. NADH-ferricyanide reductase activity and cytochrome P-450 showed a similar pattern of distribution whereas cytochrome b_5 was recovered exclusively in the supernatant fraction. The treatment solubilized over 95% of the NADPH-cytochrome c reductase activity and about 50% of the total microsomal protein. Addition to the pellet fraction of an excess of either purified NADPH-cytochrome c reductase or cytochrome b_5, prepared by trypsin digestion of liver microsomes (98), did not increase the NADH-peroxidase activity.

Reconstitution of the microsomal NADH-peroxidase activity

Since our results have implicated NADH-cytochrome b_5 reductase and cytochrome P-450 as electron carriers in the microsomal NADH-peroxidase enzyme system, an attempt was made to solubilize and resolve the enzyme system into its component parts and to reconstitute the activity. Purified

TABLE 32

EFFECT OF TRYPSIN DIGESTION OF LIVER MICROSOMES ON NADH-PEROXIDASE ACTIVITY AND OTHER MICROSOMAL CONSTITUENTS [a]

Microsomal Preparation	NADH-Peroxidase		NADH-Cyt. c Reductase	NADH-Ferricyanide Reductase		Cyt. P-450		Cyt. P-420	Cyt. b_5	
	Specific Activity	Percent Recovery	Specific Activity	Specific Activity	Percent Recovery	Specific Activity	Percent Recovery	Specific Activity	Specific Activity	Percent Recovery
Liver microsomes	44		985	4.2		0.79		0.03	0.57	
Trypsin-treated[b] microsomes	23		31	3.8		0.45		0.28	0.50	
Supernatant	13	30	17	1.3	27	0.15	18	0.29	0.73	95
Pellet	24	70	43	3.7	73	0.68	82	0.19	0.04	5

[a] Liver microsomes (10 mg protein/ml) were incubated at 10° with 25 µg trypsin/mg protein for 15 hr in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.3 M sucrose and 2 mM EDTA. Reaction was stopped by addition of trypsin inhibitor (1 mg/mg trypsin). The digest was spun at 105,000 g for 2 hr, pellet was suspended in 0.3 M sucrose-2 mM EDTA (pH 7.4) and enzymes of various fractions assayed. NADH-peroxidase activity was measured using 0.4 mM cumene hydroperoxide. NADH-peroxidase, NADH-cyt. c reductase, and NADH-ferricyanide reductase activities are expressed as nmoles NADH oxidized/min/mg protein, nmoles cyt. c reduced/min/mg protein, and µmoles ferricyanide reduced/min/mg protein, respectively, and cytochrome contents are given as nmoles/mg protein. % recoveries in the supernatant and pellet fractions were calculated by taking the activities in the trypsin digest as 100%. Values are expressed as % of residual activity recoverable. Actual total recoveries were greater than 90%.

[b] Trypsin-treated microsomes refers to a mixture of soluble and particulate materials obtained after treatment of liver microsomes with trypsin as described above.

NADH-cytochrome b_5 reductase was prepared by lysosomal digestion of liver microsomes according to Takesue and Omura (105, 106). The purified preparation catalyzed the reduction of 100 nmoles cytochrome b_5/min/mg protein when 3.7 μM purified cytochrome b_5 was employed in the assay medium. Partially purified cytochrome P-450 was prepared by cholate solubilization of liver microsomes following the method of Lu *et al* (23). The final preparation contained 0.56 nmoles P-450 per mg protein.

The effect of varying the concentration of purified NADH-cytochrome b_5 reductase and cytochrome P-450 on NADH-peroxidase activity is shown in Fig. 21. The reaction rate was found to be proportional to the concentration of the reductase and of cytochrome P-450. Similar kinetics were obtained when detergent-solubilized NADH-cytochrome b_5 reductase was substituted for lysosomal-solubilized enzyme. However, the cytochrome P-450 preparation was not pure and contained a significant amount of NADH-cytochrome b_5 reductase activity (15 nmoles cytochrome b_5 reduced/min/mg protein). The presence of this enzyme in the P-450 preparation may account for the low stimulation of NADH-peroxidase activity upon addition of increasing amounts of purified flavoenzyme (Fig. 21). It is, therefore, apparent that more efficient reconstitution of the NADH-peroxidase activity must await further purification of cytochrome P-450.

A comparison of the effectiveness of liver microsomes and the reconstituted enzyme system in catalyzing the NADH-peroxidase reaction is shown in Table 33. The specific activity of the reconstituted system using either detergent-solubilized or lysosomal-solubilized reductase was about 25% of that originally present in liver microsomes. Addition to the reconstituted system of either trypsin-solubilized or detergent-solubilized

Figure 21. Effect of increasing concentrations of purified NADH-cytochrome b_5 reductase and cytochrome P-450 on NADH-peroxidase activity.

The assay medium contained 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, varying concentrations of purified NADH-cytochrome b_5 reductase or cytochrome P-450, and 0.8 mM cumene hydroperoxide in a final volume of 3 ml. When the concentration of reductase was varied, 0.2 µM P-450 was used and when the P-450 concentration was varied, 130 µg reductase (specific activity, 100 nmoles cytochrome b_5 reduced/min/mg protein) was employed. Reaction rates were corrected for NADH oxidation in the absence of reductase or P-450, respectively.

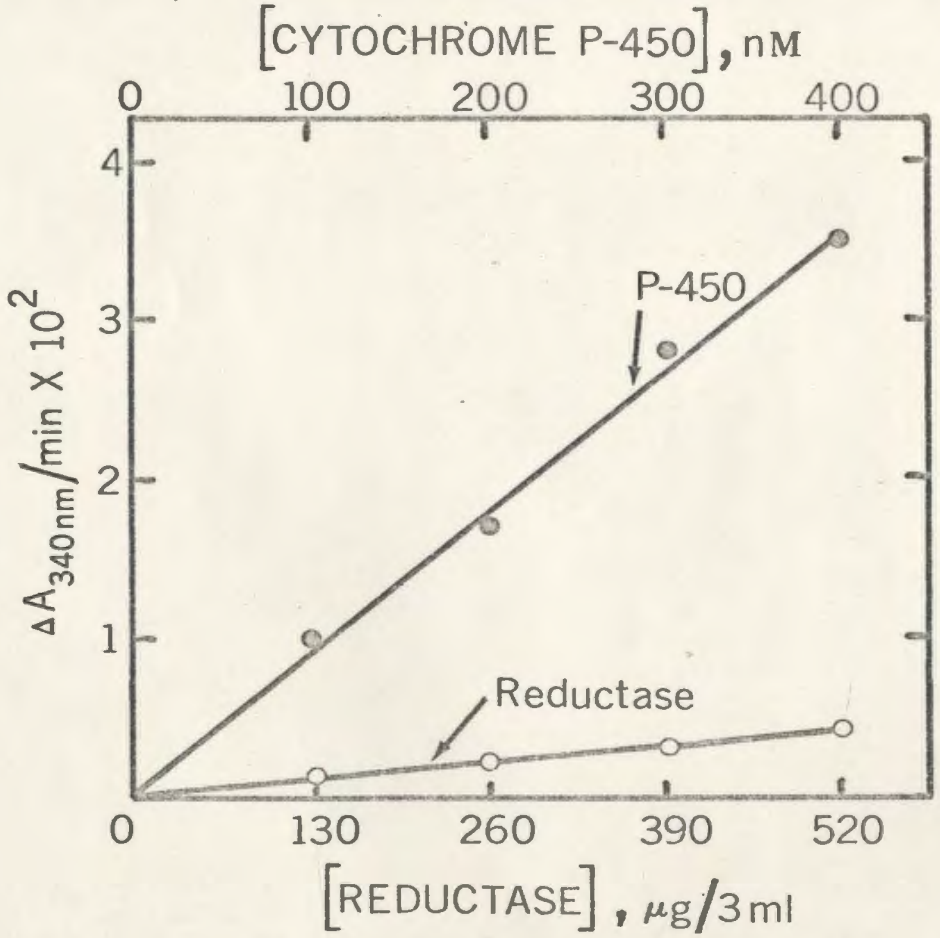


TABLE 33

A COMPARISON OF NADH-PEROXIDASE ACTIVITY IN LIVER MICROSOMES AND THE RECONSTITUTED ENZYME SYSTEM [a]

Addition	NADH-Peroxidase Activity
Liver microsomes	87
Detergent-solubilized NADH-cyt. b_5 reductase + P-450	20
Detergent-solubilized NADH-cyt. b_5 reductase + P-450 + 1.0 mg phosphatidylcholine-lysophosphatidylcholine micelles [b]	23
Lysosomal-solubilized NADH-cyt. b_5 reductase + P-450	15

[a] NADH-peroxidase activity in liver microsomes was measured at 340 nm in a 3 ml system containing 0.067 M sodium phosphate buffer (pH 7.5), 0.15 mM NADH, 1 mg microsomal protein, and 0.8 mM cumene hydroperoxide. In the reconstituted system, 0.5 μM purified P-450 plus an excess of either detergent-solubilized or lysosomal-solubilized NADH-cyt. b_5 reductase were substituted for liver microsomes. Reaction rates are expressed as nmoles NADH oxidized/min/nmole P-450.

[b] Phosphatidylcholine-lysophosphatidylcholine micelles containing an equal amount of each component were prepared in aqueous medium by sonication as described in Methods. The micelles were preincubated with the reductase and P-450 for 5 min prior to addition of NADH and hydroperoxide.

cytochrome b_5 (0.4 μM) did not enhance the reaction rate.

Jones and Wakil (110) have demonstrated a requirement for phosphatidylcholine-lysophosphatidylcholine lipid by microsomal NADH-cytochrome c reductase. The effect of phosphatidylcholine-lysophosphatidylcholine micelles on the NADH-peroxidase activity of the reconstituted system is presented in Table 33. The reaction rate was slightly enhanced by the addition of these lipid micelles. Microsomal lipid (0.2 mg) prepared by the method of Lu et al (52) had no stimulatory effect.

Oxidation of NADH-reduced cytochrome P-450 by hydroperoxides

Our results have implicated cytochrome P-450 as an electron carrier in the microsomal NADH-peroxidase reaction and it was therefore necessary to determine whether the reduced form of the hemoprotein could be oxidized by hydroperoxides. Accordingly, microsomal cytochrome P-450 was reduced with NADH in the presence of carbon monoxide which binds only the ferrous form of P-450 and the reduced hemoprotein was allowed to react with known quantities of hydroperoxide under strict anaerobic conditions. The results (Table 34) indicate that after treatment with 0.16 mM cumene hydroperoxide, the reduced P-450-CO complex was decreased to 38% of the control value and after reaction with 0.4 mM cumene hydroperoxide, the hemoprotein-CO complex was oxidized completely. The disappearance of the P-450-CO complex upon treatment with cumene hydroperoxide was not due to the destruction of the hemoprotein since a fully developed P-450-CO complex reappeared upon reduction by sodium dithionite. The initial rate of oxidation of the complex at 450 nm was too rapid to be measured by conventional methods even when low concentrations of hydroperoxide were employed.

TABLE 34

OXIDATION OF THE NADH-REDUCED CYTOCHROME P-450-CO COMPLEX BY CUMENE HYDROPEROXIDE [a]

Addition	ΔA (450-490 nm)
Microsomes + NADH + CO	0.075
Microsomes + NADH + CO + 0.16 mM cumene hydroperoxide	0.028
Microsomes + NADH + CO + 0.16 mM cumene hydroperoxide + sodium dithionite	0.073
Microsomes + NADH + CO + 0.4 mM cumene hydroperoxide	0

[a] To a reference cuvette and an anaerobic sample cuvette was added 3 ml of a mixture of liver microsomes (2 mg protein/ml) containing 0.1 M sodium phosphate buffer (pH 7.5) and 0.45 mM NADH. Into the sidearm of the sample cuvette was placed a solution of cumene hydroperoxide. The sample cuvette was flushed with nitrogen for 20 min and with CO that had been passed through a deoxygenating medium (83) for 5 min and the CO-difference spectrum was recorded. The contents of the sample cuvette were then mixed with hydroperoxide (final conc. 0.16 mM and 0.40 mM, respectively) and the difference spectrum recorded. A few grains of sodium dithionite were added to the sample cuvette containing 0.16 mM cumene hydroperoxide and the spectrum again was measured. The reduction of cytochrome P-450 was determined at 450 nm relative to 490 nm.

These results indicate that the NADH-reduced form of cytochrome P-450 can be oxidized very rapidly by cumene hydroperoxide.

DISCUSSION

Cytochrome P-450 as a microsomal peroxidase in steroid hydroperoxide decomposition

In an effort to determine the mechanism by which various hydroperoxides are decomposed within the cell and in seeking to establish the possible role of organic hydroperoxides as transient species in biological hydroxylation reactions, the decomposition of steroid and other organic hydroperoxides by hepatic and adrenocortical microsomes using TMPD as an electron donor has been examined. Strong evidence has been presented that cytochrome P-450 is the microsomal peroxidase responsible for the decomposition of these hydroperoxides.

Cytochrome P-450 is the oxygen-activating pigment involved in many hydroxylation and demethylation reactions. A prerequisite for enzymic hydroxylation is that the substrate be bound to the hemoprotein catalyst during the course of the hydroxylation reaction. The reactive area of cytochrome P-450, i.e., the vicinity of the heme, appears to be buried in a highly hydrophobic portion of P-450 protein or phospholipid of the microsomal membrane and this hydrophobic environment seems to be essential for substrate hydroxylation (37). Reagents such as proteases, certain detergents, acid or base, phospholipases, alcohols, sulfhydryl reagents can alter the hydrophobic environment of the heme or the conformation of cytochrome P-450 and convert the hemoprotein into its inactive P-420 form. This modification is usually* accompanied by an inactivation of hydroxylating activity (11, 42). In this study the peroxidase activity of microsomal fractions was inhibited

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* *An enzyme system from Pseudomonas aminovorans contains an enzymically active cytochrome P-420-type hemoprotein which catalyzes the NAD(P)H-dependent oxidation of amines in the presence of oxygen (124).*

by reagents that convert cytochrome P-450 to cytochrome P-420. Since cytochrome P-420 does not exhibit substrate binding (45, 85), this could explain its ineffectiveness in catalyzing the peroxidase reaction with cumene hydroperoxide and steroid hydroperoxides as substrates.

In contrast, cytochrome P-420 was a very active peroxidase towards linoleic acid hydroperoxide (89). Since fatty acids such as linoleic acid have a strong binding affinity for cytochrome P-450 as well as other proteins in general (111), the effectiveness of cytochrome P-420 in acting as a peroxidase for linoleic acid hydroperoxide may be due to the avid binding of the hydroperoxide to the hemoprotein catalyst which could then result in effective peroxidase action. Furthermore, fatty acid hydroperoxides are unique among the hydroperoxides in that they can act as effective substrates for a wide variety of heme compounds (e.g. cytochrome P-450, cytochrome P-420, hematin, methemoglobin, cytochrome c) whereas most steroid hydroperoxides and cumene hydroperoxide seem to be effective only with hemoprotein P-450, with the exception of progesterone 17α-hydroperoxide which is also a very good substrate for hematin.

The peroxidase activity of microsomal fractions was inhibited by compounds that form type I, type II,and modified type II difference spectra with cytochrome P-450. The type II ligands were generally more inhibitory than the type I and modified type II compounds. A possible explanation for this result lies in the different binding sites for type I, type II and modified type II compounds on the P-450 molecule. It is generally recognized that the heme iron is the active site which is responsible for the peroxidase activity of hemoproteins. Therefore, type II ligands which bind directly to

the heme iron of P-450 to form a hemichrome (35) would be expected to exert a more pronounced inhibition than type I or modified type II compounds which interact with some other portion of the P-450 molecule (35).

Cytochrome P-450 in microsomes exists both in a free state and in a form bound to endogenous substrate (i.e. steroids). A prerequisite for peroxidase activity is that the hydroperoxide be bound to the hemoprotein peroxidase at some stage of catalysis. Accordingly, the ease with which hydroperoxide can bind to a particular form of P-450 should determine the effectiveness of that form in catalyzing the peroxidase reaction. To what extent the substrate-bound form of P-450 is active in catalyzing hydroperoxide decomposition will most likely depend on the precise site on the P-450 molecule at which the substrate is attached. For example, if the substrate is bound directly to the heme iron of P-450 as are type II ligands, then it may seriously interfere with the interaction of the hydroperoxide and the hemoprotein and may thereby inhibit catalysis of the peroxidase reaction, unless the hydroperoxide can displace the substrate from its binding site. If the substrate is bound at a site other than the heme iron residue, such as the type I or the modified type II binding site, inhibition of peroxidase activity will occur only when the substrate interferes with the binding of the hydroperoxide to the "active center" of P-450.

Cytochrome P-450 has been isolated from hepatic microsomes and from adrenocortical mitochondria and microsomes as a mixture of two forms: high spin P-450 in which the Fe^{3+} heme is detectable in a high spin state and low spin P-450 in which the heme iron is in a low spin

state (16, 85, 86). Interaction of cytochrome P-450 with various steroid or drug substrates appears to cause a shift in the proportion of one spin state to the other, leading to the characteristic induced type I (high spin) or type II (low spin) difference spectra. The two forms of P-450 in adrenocortical mitochondria are in fact able to catalyze completely separate enzymic reactions, the low spin form functioning in 11β-hydroxylation and the high spin form being responsible for side chain cleavage activity (11). Low spin cytochrome P-450 appears to be responsible for the peroxidase activity of liver and adrenocortical microsomes since both of these fractions contain the predominantly low spin form of the cytochrome (45, 85). Inhibitor studies with n-octylamine reported in this study further strengthen this conclusion.

The level of cytochrome P-450 in liver microsomes is markedly increased by treatment of animals with various drugs such as phenobarbital or with polycyclic carcinogens such as 3-methylcholanthrene (41). In this study the peroxidase activity in liver microsomes of phenobarbital-induced rats showed a similar increase to the rise in P-450 content when progesterone 17α-hydroperoxide was used as substrate. In contrast, the specific activity of microsomal peroxidase with cumene hydroperoxide as substrate was enhanced markedly by phenobarbital treatment, an increase which was several times more than could be accounted for by the rise in P-450 content. Several possible explanations could account for the difference in the rates of decomposition of the two hydroperoxides by microsomes from phenobarbital-treated rats: 1) the hydroperoxides bind at a different catalytic site on the P-450 molecule; 2) phenobarbital treatment changes the hydrophobic

environment of the P-450 heme (46) or/and the conformation of cytochrome P-450 and modifies the hydroperoxide-binding site in such a manner as to make the hemoprotein selectively more active as a peroxidase towards cumene hydroperoxide. After 3-methylcholanthrene treatment, the specific activity of microsomal peroxidase using progesterone 17α-hydroperoxide as substrate increased proportionally to P-450 content whereas with cumene hydroperoxide as substrate, the peroxidase activity showed a substantial decrease. Therefore, it appears that 3-methylcholanthrene-induced P-450 is just as effective a peroxidase for progesterone 17α-hydroperoxide, but is a less effective peroxidase for cumene hydroperoxide, than is normal P-450.

This induction study indicates that differences exist in the selectivity and rates of metabolism of various hydroperoxides by the different forms of P-450 in liver microsomes from induced animals. Differences in the rates of hydroxylation of various drugs and steroids by induced forms of liver microsomal P-450 have been reported (7, 45, 104). Environmentally-induced changes in drug metabolism (e.g. changes in nutritional or hormonal status, stress, alteration of atmospheric pressure, fasting, administration of various drugs and carcinogens) have been associated with changes in hepatic microsomal P-450 content (45) and what effect these environmental factors will have on microsomal TMPD-peroxidase activity remains to be determined.

Mitton et al (112) found that cholesterol 5α- and 7α-hydroperoxides* were converted in significant yield to the corresponding hydroxy derivatives by an aqueous liver extract. Incubation of cumene hydroperoxide and the 17α-hydroperoxides of progesterone and pregnenolone with

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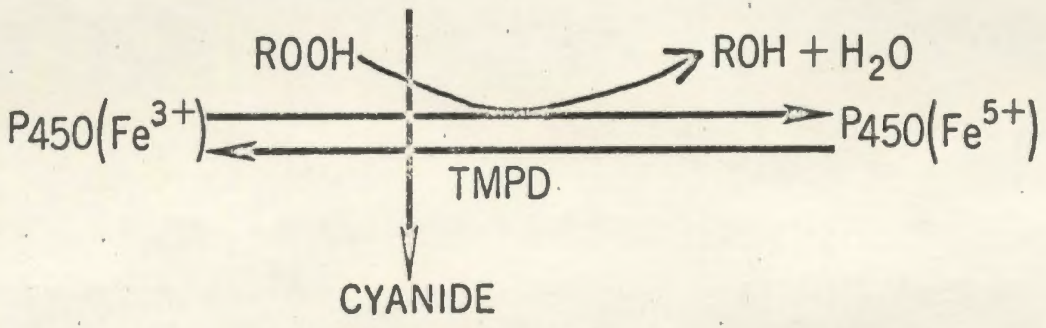
* *cholesterol 5α-hydroperoxide = cholest-6-en-3B-ol 5α-hydroperoxide*
cholesterol 7α-hydroperoxide = cholest-5-en-3B-ol 7α-hydroperoxide

hepatic or adrenocortical microsomes also gives rise to the corresponding hydroxy derivatives as the major products. Thus, a general mechanism for microsomal TMPD-peroxidase activity is proposed in scheme 2. It is suggested that ferric hemoprotein P-450 initially reduces hydroperoxide (ROOH) to a hydroxy derivative (ROH) and in the process the hemoprotein is converted to a higher oxidation state, the perferryl (Fe^{5+}) form, which is responsible for the oxidation of TMPD to Wurster's blue. Cyanide complexes with the ferric form of P-450 and inhibits formation of the perferryl form thus inhibiting peroxidase activity. Perferryl forms of hemoproteins have been implicated in the peroxidase activities of various hemoproteins (74, 113).

Investigation of the metabolites formed when 17α-hydroperoxide derivatives of progesterone and pregnenolone interact with microsomal cytochrome P-450 preparations revealed two pathways of decomposition: 1) a decomposition of the hydroperoxides by hepatic microsomal P-450 resulting in a complex range of degradation products formed presumably by the interaction of free radical alkoxy or peroxy intermediates; formation of such reactive intermediates may account for the destruction of the hemoprotein during the course of the reaction; 2) a catalytically-controlled reduction of the hydroperoxides by adrenocortical microsomal P-450 resulting in the formation of the corresponding 17α-hydroxy analogs together with a small amount of degraded C_{19}-17-ketosteroids; this pathway of decomposition did not result in the destruction of the hemoprotein.

Destruction of intracellular cytochrome P-450 by endogenous hydroperoxide may have undesired physiological consequences, However, in the



Scheme 2. Mechanism for microsomal TMPD-peroxidase activity

cell cytochrome P-450 may be protected by a variety of hydrogen donors such as NADPH, NADH, GSH, ascorbate, and vitamin E as well as by natural substrates (i.e. steroids, fatty acids) which compete with hydroperoxide for the "active site" on the hemoprotein.

Several "activated oxygen" species such as hydroxyl radicals, superoxide ions, "oxenoid" species, steroidal alkoxy radicals, and epoxide species have been proposed as initial intermediates in the cytochrome P-450-catalyzed hydroxylation mechanism (44, 60-62, 114, 115). Hydroperoxide intermediates have been proposed in cholesterol side chain cleavage (67-70), steroid 11β-hydroxylation (63), and in the hydroxylation of fluorene, tetralin, and other organic compounds (64-66) and these reactions apparently involve the participation of cytochrome P-450. Therefore, our finding that pregnene 17α-hydroperoxides are converted to their respective 17α-hydroxy analogs by adrenocortical microsomal P-450 preparations at comparable rates as found for certain hydroxylation reactions (C-21 hydroxylation of 17α-hydroxy progesterone) could be of particular physiological significance.

The formation of degraded C_{19}-17-ketosteroids from the 17α-hydroperoxides could also be of importance since this reaction could offer possibly a new biosynthetic route for the production of the androgen hormones in steroidogenic tissue (116). In addition, our discovery that progesterone 17α-hydroperoxide can be converted to 17α, 21-dihydroxyprogesterone by adrenocortical microsomes in the presence of NADPH or NADH may open up a new pathway via which the corticosteroid hormones may be biosynthesized.

It is evident that further studies on the role of steroid hydroperoxides as possible precursors of the steroid hormones in endocrine tissue are required. In particular, answers to the question of their

formation in active hydroxylase systems are called for and this particular area of research remains a challenge for the future.

The microsomal NADPH-peroxidase electron transport system

The role of various electron transport components in the microsomal NADPH-peroxidase enzyme system has been investigated. Several lines of evidence for the involvement of a similar electron transport system in NADPH-peroxidase activity and in substrate hydroxylation reactions in liver microsomes have been found: 1) both activities require the participation of NADPH-cytochrome c reductase and cytochrome P-450; 2) both systems are inhibited by antibody to NADPH-cytochrome c reductase (31); 3) both reactions are stimulated by increasing ionic strength (32); 4) the activities are sensitive to inhibition by low concentrations of sulfhydryl reagents (33); 5) $NADP^+$ causes an inhibition of both reactions; 6) the activities are inhibited to a similar extent by steroids; 7) type I and type II substrates cause a marked inhibition; 8) reagents that convert cytochrome P-450 to cytochrome P-420 produce inactivation; 9) both reactions are sensitive to inhibition by trypsin; 10) *in vivo* phenobarbital treatment causes a marked stimulation of both activities (117); 11) the K_m for NADPH in both systems is smaller than 3 μM (28).

Gaylor and collaborators (57) investigated the binding of the modified type II ligand, cyanide, to the oxidized forms of cytochrome P-450 in liver microsomes. Two binding constants for cyanide of 0.5 mM and 2.5 mM were obtained suggesting the presence in liver microsomes of two

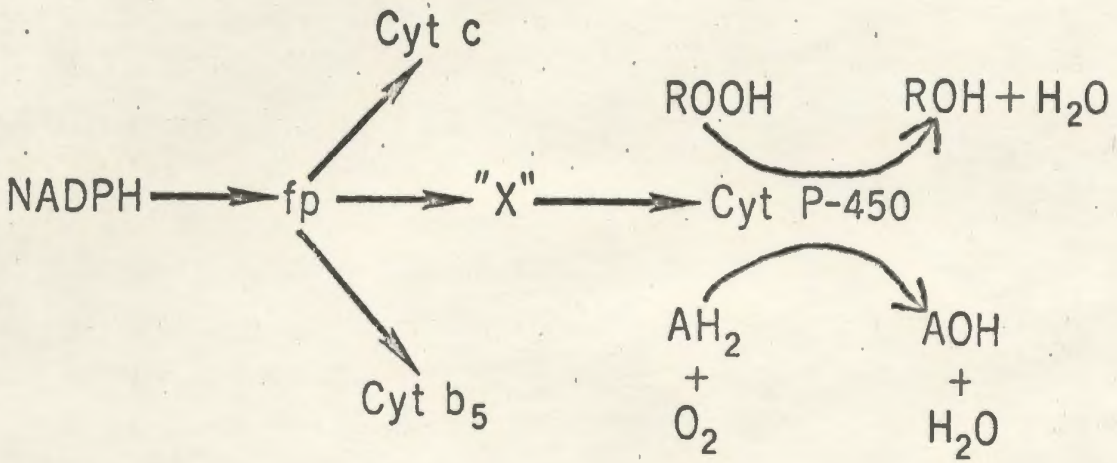
different species of oxidized P-450 (48, 57-59). Our results have demonstrated a 50% inhibition of NADPH-peroxidase activity by 2.2 mM cyanide suggesting the involvement in the reaction of the P-450 species that exhibits the lower affinity for cyanide. Gaylor and his group proposed that the P-450 species exhibiting the higher affinity for cyanide is involved in the NADH-dependent sterol demethylation activity in liver microsomes, a reaction that is inhibited 50% by 0.5 mM cyanide. NADPH can also be used as an electron donor for sterol demethylation activity (58,59) and it will be interesting to see which of the two cyanide-sensitive forms of P-450 is involved in the NADPH-dependent demethylation reaction.

Inhibitor studies with antibody to NADPH-cytochrome c reductase revealed that changes in the activity of the flavoenzyme were paralleled by identical changes in NADPH-peroxidase activity. This finding coupled to the similarities in the response to various treatments of the flavoprotein reductase activity and NADPH-peroxidase activity suggests that the reduction of cytochrome P-450 by NADPH-cytochrome c reductase may be the rate-determining step in the overall NADPH-peroxidase reaction (see scheme 4).

Attempted reconstitution of the microsomal NADPH-peroxidase activity revealed that the enzyme system was labile to various solubilization treatments. The cytochrome P-450 preparation when mixed with trypsin-solubilized NADPH-cytochrome c reductase gave essentially the same reaction rate as the P-450 preparation mixed with detergent-solubilized flavoenzyme. Orrenius and collaborators (38) have suggested that solubilization of NADPH-cytochrome c reductase from microsomes by trypsin is

responsible for the decreased rate of reduction of cytochrome P-450 and the subsequent inhibition of drug hydroxylation activity in trypsin-treated microsomes. A lipase-solubilized flavoenzyme preparation of Williams and Kamin (29) was also shown to be ineffective in reducing partially purified cytochrome P-450 and this reconstituted system lacked the ability to catalyze the ω-hydroxylation of fatty acids (52). It, therefore, appears that the low NADPH-peroxidase activity in our reconstituted system may be the result of the diminution in ability of solubilized NADPH-cytochrome c reductase to reduce cytochrome P-450. Microsomal lipid or phosphatidylcholine was found to enhance slightly the NADPH-peroxidase activity of the reconstituted system and it will be interesting to see what sort of phospholipid requirement exists for the enzyme system in intact liver microsomes.

Incubation of cumene hydroperoxide with hepatic microsomal fractions fortified with NADPH results in the formation of the corresponding hydroxy derivative as the major product. The conversion of hydroperoxides to alcohols by microsomal cytochrome P-450 appears to be an inherent characteristic of the hemoprotein. A scheme of NADPH-dependent microsomal electron transport accommodating the reduction of hydroperoxides to hydroxy derivatives is proposed in scheme 3. It is generally assumed that NADPH supplies reducing equivalents to cytochrome P-450 via the flavoprotein NADPH-cytochrome c reductase (f_p) either directly or through some unknown electron carrier "X" (32). The flavoprotein is also presumed to mediate the transfer of electrons from NADPH to cytochrome b_5. In the presence of oxygen, reduced cytochrome P-450 catalyses the hydroxylation of various steroid and drug substrates (AH_2) to yield hydroxylated product (AOH) and water.



Scheme 3. Scheme of NADPH-dependent microsomal electron transport

Cytochrome P-450 can also act as a microsomal peroxidase (scheme 3) converting hydroperoxide (ROOH) to the corresponding alcohol (ROH), a reaction that does not require the presence of oxygen and is not sensitive to carbon monoxide. The insensitivity of NADPH-peroxidase activity to carbon monoxide is consistent with the observations of Chen and Lin (64) who found no apparent inhibition by CO of the NADPH-dependent conversion of tetralin hydroperoxide to tetralol. However, carbon monoxide was found to inhibit the hydroxylation of tetralin to tetralol, a reaction involving tetralin hydroperoxide as an intermediate (64). Therefore, it appears that in hydroxylation reactions involving hydroperoxides as intermediates, the conversion of substrate to the transient hydroperoxide is sensitive to inhibition by carbon monoxide whereas the reduction of the hydroperoxide to the corresponding alcohol is insensitive to carbon monoxide. Several other P-450-dependent reactions have been found to be unaffected by carbon monoxide (27, 58, 59) and hence it appears that CO inhibition by itself is not always a reliable criterion for P-450 participation.

The pathway presented in scheme 3 implies that hydroperoxides are capable of oxidizing reduced cytochrome P-450, a conclusion that has been verified by experiments carried out in this study. In this respect, hydroperoxides act in a similar manner to oxygen. The reduction of hydroperoxides to the corresponding hydroxy derivatives requires two reducing equivalents as does the process of substrate hydroxylation whereas the reduction of ferric cytochrome P-450 by NADPH is a one-electron process (32). Hildebrandt and Estabrook (32) suggested that the second electron

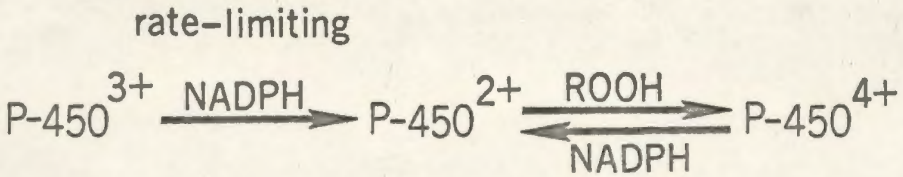
in microsomal hydroxylation reactions is donated by reduced cytochrome $\underline{b}_5$ whereas Holtzmann and Carr (118) postulated a second reduced cytochrome P-450 molecule as being the electron donor.

A tentative mechanism for microsomal NADPH-peroxidase activity is proposed in scheme 4. It is suggested that the first step in the reaction sequence is the reduction of ferric P-450 to ferrous P-450 by NADPH via NADPH-cytochrome $\underline{c}$ reductase. The second stage involves the interaction of hydroperoxide (ROOH) with ferrous P-450 to form ferryl P-450. Ferryl P-450 can accept two electrons from NADPH to yield regenerated ferrous P-450. The conversion of ferrous P-450 to ferryl P-450 may proceed by one of two possible routes: 1) via an intermediate ferric P-450 state; or 2) as a single step two-electron oxidation analogous to the reaction of ferrous horseradish peroxidase with hydrogen peroxide (119).

The microsomal NADH-peroxidase electron transport system

The role of various electron transport components in the microsomal NADH-peroxidase enzyme system has been investigated. Evidence has been presented for the participation of NADH-cytochrome $\underline{b}_5$ reductase and cytochrome P-450 as electron carriers in the reaction.

A comparison of NADH-peroxidase activity and cytochrome P-450 content in microsomal fractions from various tissues revealed that hepatic microsomes contained the highest amount of P-450 per mg protein and were the most active in catalyzing the NADH-peroxidase reaction. Microsomes from tissues which contained little or no cytochrome P-450 were either weakly active or completely inactive. However, the activity of bovine adrenocortical microsomes was very low as compared to liver microsomes, both



Scheme 4. Mechanism for microsomal NADPH-peroxidase activity

fractions containing similar P-450 levels. These results are consistent with previous observations which showed a much lower TMPD-peroxidase and NADPH-peroxidase activity in adrenocortical microsomes as compared to liver microsomes, indicating that the two hemoproteins possess different peroxidase properties.

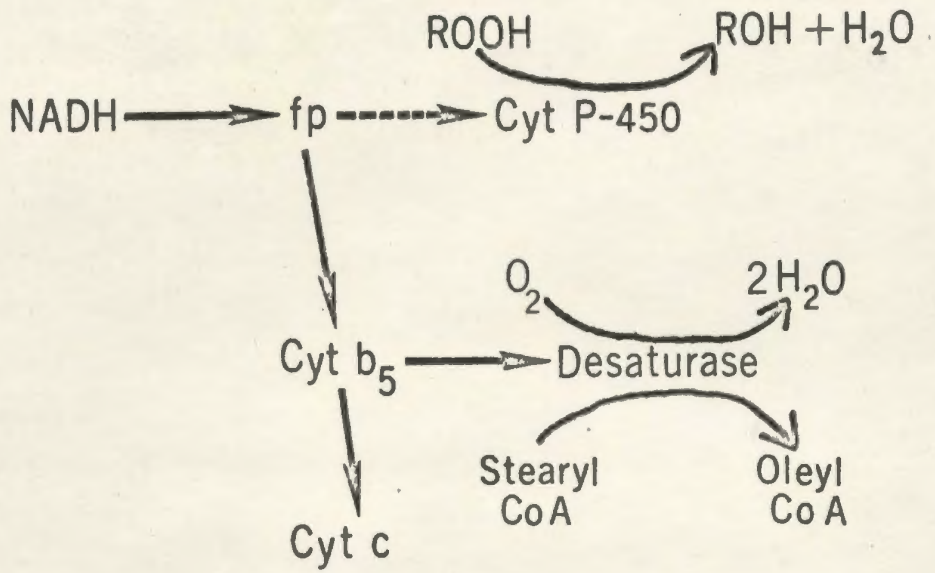
The NADH-peroxidase activity in liver microsomes of phenobarbital-induced rats showed a parallel increase to the rise in P-450 content when progesterone 17α-hydroperoxide was employed as substrate. In contrast, the specific activity of the enzyme system with cumene hydroperoxide as substrate was enhanced several times more than could be accounted for by the rise in P-450 level. These results are consistent with previous observations which showed a drastic increase in TMPD-peroxidase activity and NADPH-peroxidase activity using cumene hydroperoxide as substrate but only a moderate increase with progesterone 17α-hydroperoxide following phenobarbital treatment.

Attempted reconstitution of the microsomal NADH-peroxidase activity revealed that the enzyme system was labile to various solubilization treatments. The use in the reconstituted system of a cytochrome P-450 preparation plus detergent-solubilized NADH-cytochrome b_5 reductase gave essentially the same reaction rate as cytochrome P-450 plus lysosomal-solubilized flavoenzyme. The rate-limiting step in the overall reaction could not be determined with certainty but one would expect the reduction of ferric cytochrome P-450 to be the control point of the reaction, analogous to the situation occurring in the NADPH-peroxidase enzyme system. Therefore, the low NADH-peroxidase activity observed in the reconstituted system may reflect the diminution in ability of solubilized

NADH-cytochrome b_5 reductase to reduce cytochrome P-450. Addition to the reconstituted system of phosphatidylcholine-lysophosphatidylcholine micelles produced a slight increase in the reaction rate. Phospholipid requirements have been shown for several microsomal enzymes (52, 110) and it seems quite probable that the NADH-peroxidase enzyme system of intact liver microsomes also requires phospholipid for maximum catalytic activity.

Incubation of cumene hydroperoxide with microsomal fractions fortified with NADH resulted in the formation of the corresponding hydroxy derivative as the major product. A scheme of NADH-dependent microsomal electron transport is proposed in scheme 5. It is suggested that NADH supplies reducing equivalents to cytochrome P-450 via the flavoenzyme NADH-cytochrome b_5 reductase (fp) either directly or through some unknown electron carrier. Reduced cytochrome P-450 then acts as a microsomal peroxidase converting hydroperoxide (ROOH) to the corresponding alcohol (ROH), a reaction that does not require oxygen and is insensitive to inhibition by carbon monoxide. This scheme implies that hydroperoxides are capable of oxidizing NADH-reduced cytochrome P-450, a conclusion supported by the results in this study.

NADH-cytochrome b_5 reductase can also mediate the channelling of electrons from NADH to cytochrome b_5. Reduced cytochrome b_5 then transfers reducing equivalents to a desaturase enzyme which converts stearyl coenzyme A to oleyl coenzyme A in the presence of oxygen (120-123). Reduced cytochrome b_5 can also channel electrons to externally added cytochrome c. Both the fatty acid desaturase enzyme system and NADH-cytochrome c reductase require the presence of phospholipid (110, 120). The terminal desaturase appears to be a different enzyme from cytochrome P-450 (120-123).



Scheme 5. Scheme of NADH-dependent microsomal electron transport

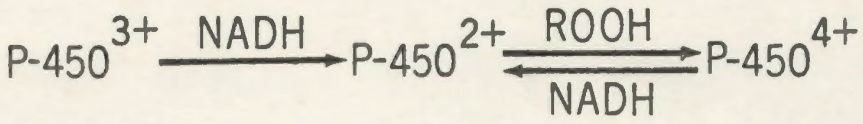
A tentative mechanism for microsomal NADH-peroxidase activity is proposed in scheme 6. It is suggested that the first step in the reaction sequence is the reduction of ferric P-450 to ferrous P-450 by NADH via NADH-cytochrome b_5 reductase. The second stage involves the interaction of hydroperoxide (ROOH) with ferrous P-450 to form ferryl P-450. Ferryl P-450 can accept two electrons from NADH to yield regenerated ferrous P-450. The conversion of ferrous P-450 to ferryl P-450 may proceed either via an intermediate ferric P-450 state or as a single step two-electron oxidation analogous to the reaction of ferrous horseradish peroxidase with hydrogen peroxide (119).

Evidence for a similar electron transport system operating in NADH-peroxidase activity and in NADH-dependent sterol demethylation includes: 1) utilization in both systems of NADH as the preferred electron donor; 2) involvement of a cytochrome P-450 species that exhibits a high affinity for cyanide; 3) insensitivity of both reactions to inhibition by carbon monoxide; 4) no decrease in both reaction rates after removal of cytochrome b_5 by trypsin digestion (58, 59). *In vivo* phenobarbital treatment markedly enhanced the NADH-peroxidase reaction rate and it will be interesting to see what effect this treatment has on the sterol demethylation reaction.

Conclusion

A summary of the properties of the TMPD-peroxidase, NADPH-peroxidase, and NADH-peroxidase enzyme systems is presented in Table 35. Evidence suggesting the involvement of two distinct forms of cytochrome P-450 in the NADPH-peroxidase and NADH-peroxidase enzyme systems will now be discussed.

First of all, the TMPD-peroxidase reaction is a measure of the



Scheme 6. Mechanism for microsomal NADH-peroxidase activity

TABLE 35

SUMMARY OF THE PROPERTIES OF THE TMPD-PEROXIDASE, NADPH-PEROXIDASE, AND NADH-PEROXIDASE ENZYME SYSTEMS[a]

Property	TMPD-Peroxidase	NADPH-Peroxidase	NADH-Peroxidase
Stoichiometry [b]	0.25	0.35	0.50
Reaction rate [c]	73	28	37
K_m for electron donor	40 μM	< 3 μM	< 3 μM
K_m for cumene hydroperoxide	-	0.4 mM	0.6 mM
pH optimum	6.5-7.7	8.2	7.7-7.8
Induction by phenobarbital [d]	19-fold	7.5-fold	10-fold
Concentration of cyanide required for 50% inhibition	0.8 mM	2.2 mM	0.45 mM
Concentration of n-octylamine required for 50% inhibition	0.09 mM	0.24 mM	0.07 mM
Inhibition by N_2	-	-	-
Inhibition by CO	-	-	-
Involvement of NADPH-cytochrome c reductase	-	+	-
Involvement of NADH-cytochrome b_5 reductase	-	-	+
Involvement of cytochrome P-450	+	+	+
Involvement of cytochrome b_5	-	-	-

[a] To keep results consistent, the same batch of liver microsomes was used in obtaining the data for each of the properties of the three enzyme systems.

[b] Stoichiometry expressed as moles electron donor oxidized/mole cumene hydroperoxide in presence of liver microsomes.

[c] Reaction rates expressed as nmoles electron donor oxidized/min/mg protein/μmole cumene hydroperoxide. One reducing equivalent is utilized when TMPD is oxidized to Wurster's blue whereas two reducing equivalents are used up when NAD(P)H is oxidized to $NAD(P)^+$.

[d] Rats were injected intraperitoneally with sodium phenobarbital (50 mg/kg) twice daily for 5 days.

peroxidase activity of all the various forms of cytochrome P-450 in liver microsomes. On the other hand, the NADPH-peroxidase and NADH-peroxidase reaction measures only the peroxidase activity of NADPH-reducible and NADH-reducible cytochrome P-450, respectively. The TMPD-peroxidase, NADPH-peroxidase, and NADH-peroxidase reactions were enhanced 19-fold, 7.5-fold, and 10-fold, respectively, following phenobarbital treatment. Combining the increases in the latter two activities gives a total increase of 17.5-fold, a value which is similar to the 19-fold stimulation observed for the TMPD-peroxidase reaction.

Secondly, the P-450 species involved in the NADH-peroxidase enzyme system has a high affinity for cyanide and n-octylamine, the P-450 species which functions in the NADPH-peroxidase reaction exhibits a low affinity for cyanide and n-octylamine, and the cytochrome P-450 species which participate in the TMPD-peroxidase reaction have an intermediate affinity for the inhibitory agents. These results are suggestive of the presence of two different forms of cytochrome P-450 in liver microsomes, one form functioning in NADPH-dependent activities and the other playing a role in NADH-supported reactions.

Challenges for the future include isolation and purification of the various forms of cytochrome P-450, determination of their electron donor and substrate specificities, and reconstitution of functional NADPH- and NADH-supported cytochrome P-450-dependent enzyme activities.

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PUBLICATIONS ARISING FROM THIS WORK -

Abstracts -

1. Hrycay, E.G. and O'Brien, P.J. (1972). Cytochrome P-450 as a microsomal peroxidase in steroid hormone biosynthesis. Abstracts of 8th FEBS Meeting, no. 844, Amsterdam.

2. Hrycay, E.G., and O'Brien, P.J. (1973). Cytochrome P-450 as an electron carrier in microsomal NAD(P)H-peroxidase activity. Abstracts of 9th International Congress of Biochemistry, Stockholm, in press.

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