Drug Metabolism and Drug Toxicity

Editors

Jerry R. Mitchell, M. D., Ph. D.
Marjorie G. Horning, Ph. D,

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Jerry R. Mitchell, M.D., Ph.D.
Professor of Chemical Toxicology and Internal Medicine
Baylor College of Medicine
Houston, Texas

Marjorie G. Horning, Ph.D.
Professor of Biochemistry
Baylor College of Medicine
Houston, Texas

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Preface

For many years the metabolism of drugs was equated with detoxification and inactivation. Indeed the first major monograph in the field by R. Tecwyn Williams in 1947 was titled *Detoxication Mechanisms—The Metabolism of Drugs and Allied Organic Chemicals.* Since the early 1960s, however, it has been increasingly recognized that drugs and environmental chemicals can be metabolized to toxic and/or pharmacologically active as well as inactive metabolites. This volume was initiated by the Drug Metabolism Division of the American Society for Pharmacology and Experimental Therapeutics in response to a perceived need for a textbook in drug metabolism and disposition that incorporated current principles and methods for studying metabolic transformations in relation to toxicology.

The book has been organized into six sections. The first section discusses metabolic pathways; that is, specific enzymes or enzyme complexes that are responsible for the metabolism of drugs and environmental chemicals. The first chapter presents a status report on the cytochrome-P_{450} complex. The oxygenation of organic nitrogen and sulfur compounds and halogenated hydrocarbons as well as the role of activated oxygen in chemical toxicity are discussed in the following chapters.

The second section is concerned with metabolism and toxicity at the cellular and organ level. Studies on the metabolic pathways involved in toxicity in isolated mammalian cells are presented in the chapter by Smith and Orrenius. In the following chapter by Bend, the role of tissues and more specifically the role of certain types of cells in these extrahepatic tissues in chemically mediated toxicity is discussed.

Although drug metabolism and drug toxicity can be directed or moderated by enzymic reaction and cellular responses, environmental and genetic factors also are important. The influence of genetic factors and the use of recombinant DNA technology to define genetic differences in drug metabolizing enzymes is discussed by Nebert.

The relationship of metabolism to toxicity and carcinogenicity is discussed in section three. The importance of DNA repair in preventing toxicity in human cells is reviewed by McCormick. Thorgeirsson discusses the importance of several metabolic pathways in converting the aromatic amides into carcinogenic or inactive metabolites.

The fourth section consists of three chapters (Benet, Wilkinson, and Gillette) that discuss quantitative approaches which help to define the relationship between drug metabolism and drug toxicity. The basic principles of pharmacokinetics are reviewed by Benet; the problems involved in applying these principles in studies of toxicity in whole animals and man are discussed by Wilkinson and Gillette.

The pathophysiological mechanisms by which reactive metabolites interact with cells and cell constituents are discussed in section five. Trump and colleagues review current concepts about the role of sodium and calcium regulation in the pathogenesis of lethal injury in cell culture studies. They categorize the major mechanisms resulting in lethal cell injury and discuss their possible extrapolation to lethal injury from chemically reactive metabolites. Mitchell and colleagues focus on the chemical nature of individual reactive metabolites; they critique the relative importance of alkylation and peroxidation reactions with tissue macromolecules, the validity of the current data base and conclusions drawn, and known pathophysiological interactions with glutathione and calcium. Lauterburg and colleagues conclude the section with a review of the dynamic regulation of glutathione homeostasis *in vivo.* They

examine current opinions about glutathione compartments in the liver, about excretion of glutathione disulfide into blood, about the role of the liver in exporting glutathione, and about the extrapolation of data obtained *in vitro* to the dynamic state that exists *in vivo*.

The last section is concerned with methods for the isolation, separation, quantitation, and identification of metabolites. Perfused organs provide a simple model for studying the relationship of pharmacokinetic parameters of metabolism to drug toxicity. The methodology and applications of perfusion techniques in toxicity studies are described by Pang. Procedures for profiling drug metabolites by high pressure liquid chromatography and gas chromatography, as well as the information that can be obtained from these profiles are discussed by M. G. Horning and colleagues. The final chapter by E. C. Horning and colleagues discusses the identification of metabolites by mass spectrometric analysis. The many recent developments that have made the identification of polar, nonvolatile, and high molecular weight compounds possible are reviewed.

The intent of the editors in compiling this book was to document the role of drug metabolism in various toxic responses (mutagenicity, carcinogenicity, cytotoxicity, cell death) that may follow drug administration or chemical exposure. Considerable progress has been made in understanding the mechanisms by which metabolites can elicit a toxic response. However, information in this interdisciplinary field can only be described as fragmentary. It is hoped that the experimental approaches for investigating toxicity that are described in this book will be helpful both to graduate students and established investigators in pulling together important information and concepts from the different disciplines. For this reason the book is selective in its approach rather than comprehensive, with emphasis placed particularly on several topics either not reviewed adequately elsewhere or reviewed in the literature of disciplines not commonly followed by most pharmacologists, toxicologists, and biochemists. We also hope that the book will stimulate readers to develop new ideas and experimental approaches for solving many of the fascinating and important problems that drug toxicity presents.

Contributors

M. W. Anders, *Department of Pharmacology, University of Rochester, 601 Elmwood Avenue, Rochester, New York 14642*

John R. Bend, *Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, North Carolina 27709*

Leslie Z. Benet, *Department of Pharmacy, University of California, San Francisco, California 94143*

Irene K. Berezesky, *Department of Pathology, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201*

James S. Bus, *Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, North Carolina 27709*

D. I. Carroll, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

G. B. Corcoran, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Ronald W. Estabrook, *Department of Biochemistry, University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235*

James E. Gibson, *Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, North Carolina 27709*

James R. Gillette, *Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205*

Irene B. Glowinski, *Laboratory of Carcinogen Metabolism, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205*

Evan C. Horning, *Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Marjorie G. Horning, *Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

H. Hughes, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Khingkan Lertratanangkoon, *Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Bernhard M. Lauterburg, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Veronica M. Maher, *Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824*

J. Justin McCormick, *Carcinogenesis Laboratory, Michigan State University, Fee Hall, East Lansing, Michigan 48824*

Michael E. McManus, *Laboratory of Carcinogen Metabolism, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205*

Jerry R. Mitchell, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Daniel W. Nebert, *Laboratory of Developmental Pharmacology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205*

Masahiko Negishi, *Laboratory of Developmental Pharmacology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205*

J. G. Nowlin, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Sten Orrenius, *Department of Forensic Medicine, Karolinska Institutet, S-104 01 Stockholm, Sweden*

K. Sandy Pang, *Faculty of Pharmacy, University of Toronto, 19 Russel Street, Toronto, Ontario M5S 1A1, Canada*

Long-Sheng Sheng, *Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Cosette J. Serabjit-Singh, *Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, North Carolina 27709*

Charles V. Smith, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Martyn T. Smith, *Department of Forensic Medicine, Karolinska Institutet, S-104 01 Stockholm, Sweden*

R. N. Stillwell, *Department of Medicine and Institute for Lipid Research, Baylor College of Medicine, Houston, Texas 77030*

Snorri S. Thorgeirsson, *Laboratory of Carcinogen Metabolism, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205*

Benjamin F. Trump, *Department of Pathology, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201*

Grant R. Wilkinson, *Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232*

D. M. Ziegler, *Clayton Foundation Biochemical Institute and Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712*

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Cytochrome P-450 and Oxygenation Reactions: A Status Report

Ronald W. Estabrook

Department of Biochemistry, University of Texas Health Science Center, Dallas, Texas 75235

Oxygen is the key to the survival of life as we know it. The suitability of oxygen to play this unique role in biological and chemical reactions has been eloquently discussed by P. George (25). Molecular oxygen, a gas, is a diradical of relatively low reactivity. However, it can serve as an electron acceptor, wherein it progressively undergoes reduction to the protonated four-electron reduced product, water. During the course of this stepwise reduction (Fig. 1), there is sequential formation of the free radical, the superoxide anion (or its protonated form, the perhydroxyl radical), hydrogen peroxide, and the hydroxyl radical. In addition, oxygen at its two-electron reduced state, in the presence of a metal ion, can give rise to an electrophilic atom of oxygen containing six electrons—the so-called oxenoid species of oxygen (33).

Considerable interest has focused on the reactivity of these intermediary states of oxygen reduction, in particular, the oxenoid species and the hydroxyl radical, in regard to possible mechanisms for oxygenase reactions. Indeed, the shared characteristic of monooxygenase reactions is the "activation of oxygen"; therefore, a better understanding of the reactions of oxygen will be a necessary framework on which to construct the complex chemistry associated with these biologically important reactions.

REACTIONS OF OXYGEN IN BIOLOGICAL SYSTEMS

The diversity of reactions in which oxygen participates is extensive. Central to higher organisms is the need for the transport of oxygen from the lungs to the tissues where oxygen is utilized. An elegant series of reactions involving the reduced hemeprotein, hemoglobin, have been a topic for investigation since the beginnings of biochemistry. Today we have detailed knowledge of the chemistry of this "oxygen transferase" and its companion hemeprotein, myoglobin. Much of what we know about the chemistry of oxygen reactions with other cellular hemeproteins is based on these fundamental studies with hemoglobin.

When oxygen is presented to a cell that is active in metabolism, it can undergo a number of fates. Most prominent is the reduction of oxygen to water by the mitochondrial enzyme, cytochrome *c* oxidase. The critical role that the mitochondrial electron transport system plays in the generation of cellular energy (ATP) dictates the avidity of this enzyme system for the limited amount of oxygen available to the cell. Indeed, it has been estimated that over 90% of the oxygen utilization by life on this planet occurs as a result of the action of cytochrome oxidase (66). Despite

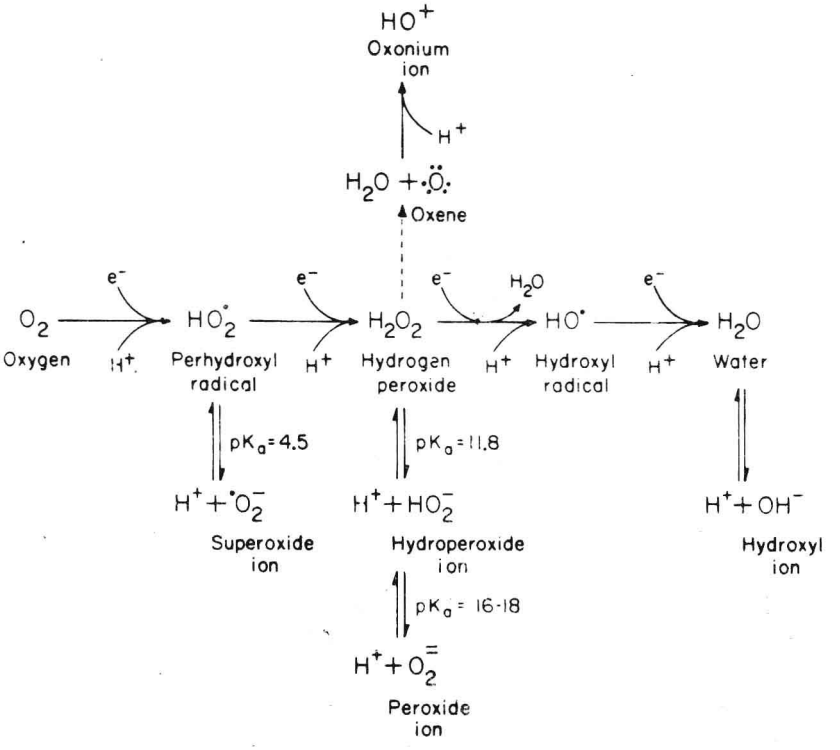

FIG. 1. Steps in the sequential reduction of oxygen.

major efforts to better understand the reactions of molecular oxygen with this hemeprotein–copper complex, our knowledge is still rudimentary, and only recently have some of the intermediates of oxygen reduction been observed during electron transport associated with the reaction of cytochrome oxidase and oxygen (8).

Other reactions of oxygen with enzymes in mammalian cells can involve special flavoproteins or metal-containing proteins. Generally, these catalyze the oxidation of specific substrates concomitant with the formation of hydrogen peroxide (many times with the superoxide anion as an intermediate), e.g., xanthine oxidase or *d*-gluconolactone oxidase. In recent years, however, considerable interest has turned to understanding the reaction of molecular oxygen with a class of enzymes that catalyze "oxygen fixation," i.e., oxygenases. In this case, molecular oxygen can be demonstrated to be incorporated into the substrate molecule that is oxidized (35,36). The requisite enzymes may contain a metal ion, such as iron or copper, or they may be hemeproteins. The reaction may be of the "dioxygenase" type, whereby both atoms of molecular oxygen are incorporated into the oxidized substrate, or they may be "monooxygenases" or mixed-function oxidases (49), where only one atom of molecular oxygen is found in the product, and the other atom is presumed to be simultaneously reduced to water.

A monooxygenase that has gained increasing attention during the last 20 years is the hemeprotein, cytochrome P-450. Although a relative newcomer to the inventory of cellular hemeproteins, cytochrome P-450 is now recognized to play a key role in the oxidative biotransformation of a diversity of "endogenous substrates," such as steroids and fatty acids, as well as "exogenous chemicals," including many drugs and other xe-

nobiotics. For this reason, the reactions catalyzed by cytochrome P-450 may regulate the duration of effectiveness of many pharmacologically active agents or the rate of biotransformation of steroid hormones essential for cellular homeostasis. However, substrate oxidations catalyzed by cytochrome P-450 frequently have the undesired consequence of leading to highly reactive metabolites responsible for cellular toxicity, many times leading to the modification of the genomic information essential for maintenance of the characteristics of a cell. In this case, cell death can occur or there can be initiation of uncontrolled cell growth, as seen with many neoplasias. Thus, an understanding of those factors that regulate and control the action of cytochrome P-450 is of importance for determining the consequences when new agents are introduced for therapeutic purposes or when new chemicals are produced for use in the expansion of our standard of living.

The main theme of this chapter is a status report on our present knowledge of mammalian cytochrome P-450 with particular emphasis on the enzymes responsible for the metabolism of xenobiotics.

CYTOCHROME P-450

Properties of Cytochrome P-450

Cytochrome P-450 is a hemeprotein containing one molecule of iron–protoporphyrin IX as its prosthetic group (53,54). It is readily identified by a pronounced absorbance band at 450 nm in the Soret region of the visible spectrum when the carbon monoxide adduct of the reduced hemeprotein is formed—hence the name P-450. This property of cytochrome P-450 is attributed to the presence of a thiolate group as a ligand of the hemeprotein (39,59). The ability to observe a spectral absorbance band at 450 nm, displaced from the absorbance bands of comparable carbon monoxide complexes of other hemeproteins (55), has provided a simple and convenient method for readily determining the concentration of this hemeprotein in various tissue fractions (19).

The hemeprotein, P-450, as isolated from mammalian tissues, has a molecular weight ranging from about 48,000 to 56,000. Since cytochrome P-450 of mammalian tissues is associated with membrane structures, it has hydrophobic characteristics that require the use of detergents and stabilizing agents for its solubilization. This property of P-450 had rendered it refractory to isolation and purification for many years, since it is readily denatured to a protein hemochromogen called P-420 (54). In the last 10 years, great advances have been made in establishing procedures for the purification of a number of different cytochromes P-450 from mammalian tissues (10).

One can classify the cytochromes P-450 into two general categories, as shown in Fig. 2. Class A represents the types of P-450 that require an FAD-containing flavoprotein and an iron–sulfur protein for electron transport from reduced pyridine nucleotide (NADPH) to the hemeprotein (20). This class of P-450 is most frequently found associated with the mitochondria of steroid-metabolizing tissues and also is typical of the P-450s associated with bacteria (30,61). The P-450s of class A are usually involved in the metabolism of endogenous compounds such as cholesterol, deoxycorticosterone, or cholecalciferol and generally possess a high degree of substrate specificity. The class B types of cytochromes P-450 require an unusual type of flavopro-

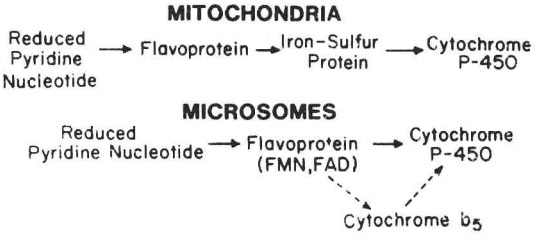

FIG. 2. Two general categories of cytochrome P-450. The specific reduced pyridine nucleotide interacting flavoproteins required for the transfer of electrons are designated, and the role of the iron–sulfur protein is indicated.

tein, which contains both FAD and FMN as prosthetic groups (43,75,77), for the transfer of reducing equivalents from reduced pyridine nucleotide to the hemeprotein. In general, this class of P-450s is found associated with the microsomal fraction of tissue homogenates in close association with another hemeprotein, termed cytochrome b_5. Recent studies have also demonstrated the presence of a similar type of P-450 associated with the nucleus of some tissues (6). Although cytochrome P-450 is widely distributed in nature, most studies for the isolation and purification of the pigment have been carried out using tissues such as the liver or adrenal, principally because of the high concentration of the hemeprotein in these tissues.

Concentration of Cytochrome P-450

In the liver of a rat or rabbit, the concentration of cytochrome P-450 is about 30 to 40 nmole per gram wet weight of tissue, i.e., the equivalent of 30 to 40 μM (17). Following treatment of animals with chemicals known to induce P-450 (see below), the concentration of cellular P-450 can increase three- to fourfold, attaining levels in excess of 0.1 mM. Thus, cytochrome P-450 is the major intracellular pigment of organs such as the liver, frequently approaching the concentration of myoglobin seen in tissues such as heart and muscle. In a tissue such as liver, the vast majority of the P-450 is associated with the endoplasmic reticulum, and it can be readily separated from other cellular organelles by differential centrifugation of the "microsomal fraction." This is in contrast to the intracellular distribution of P-450 in a tissue such as the adrenal cortex. In this case, approximately 75% of the P-450s are localized in the mitochondrion.

In the microsomal fraction of the livers of untreated rodents, the concentration of cytochrome P-450 is about 0.6 to 1.0 nmole per milligram of microsomal protein. Following treatment of the animal with a drug such as phenobarbital, this concentration can increase to as much as 5 nmole of P-450 per milligram of microsomal protein. In the latter case, it can be calculated that the hemeprotein, P-450, represents as much as 25% of the protein of the endoplasmic reticulum. Indeed, it is fair to say that P-450 is a major contributor to the structure of these membranes in liver tissue.

Induction of Cytochrome P-450

Shortly after the discovery of the role of cytochrome P-450 in the oxidative transformation of many drugs and steroids (11,16), Remmer and Merker (64) recognized that this hemeprotein may be responsible for the development of the drug tolerance seen following repeated treatment of animals with various barbiturates. Today we know that the intracellular concentration of cytochrome P-450 can easily be perturbed by a wide variety of agents, including diet, drug, or other xenobiotic treatment of the animal and events that modify the pattern of heme biosynthesis or degradation.

An example of the response of P-450 of liver to treatment of rats with phenobarbital is shown in Fig. 3. In this case, the concentration of cytochrome P-450 was determined spectrophotometrically in both a homogenate of liver and the microsomal fraction following daily treatment of animals by the intraperitoneal injection of the drug. It should be noted that the concentration of P-450 starts increasing within a short time following initiation of treatment and that repeated injections of phenobarbital continue the onset of increased synthesis of P-450 until intracellular levels four- to fivefold higher than that seen in the untreated animal are obtained (also see above). This property of enhanced enzyme synthesis has served as a convenient method for the enrichment of the tissue concentration of P-450 for subsequent studies on the isolation and purification of the various types of the hemeprotein (63).

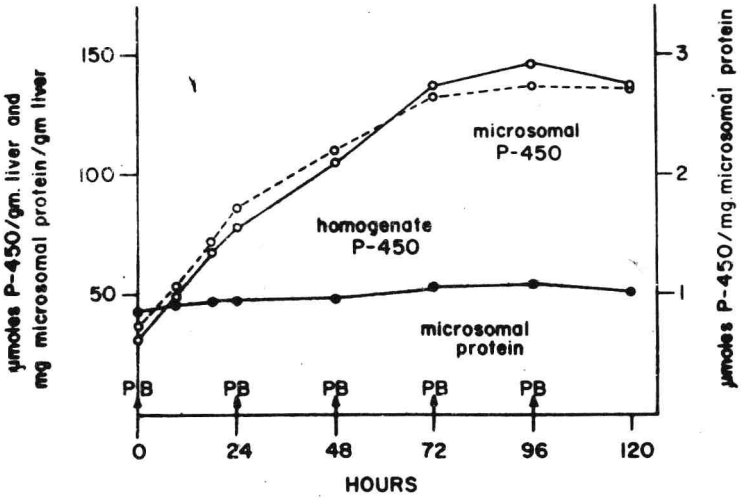

FIG. 3. Changes in the concentration of cytochrome P-450 of liver following repeated treatment of rats by the daily intraperitoneal injection of phenobarbital.

Multiplicity of P-450s

The term P-450 is the generic name for a family of hemeproteins that can be identified by differences in their physical and immunoreactive properties. The number of known isozymes of P-450 continues to grow as more refined means of recognition of unique proteins are developed.

Early studies to examine the response of animals to different "inducing agents" suggested the presence of more than one enzyme responsible for the metabolism of the many different types of compounds oxidatively transformed by cytochrome P-450 (9). That this implied different P-450s was initially confirmed by spectrophotometric studies in which differences in the reaction of carbon monoxide or ethyl isocyanide with the hemeprotein were noted (1,37,48). As shown in Fig. 4, difference spectra of the CO-reactive hemeproteins present in liver microsomes from animals pretreated with either phenobarbital or 3-methylcholanthrene show the presence of a small but measurable difference in the location of the absorbance maxima; i.e., the former is located at 450 nm, and the latter at 448 nm. For comparison,

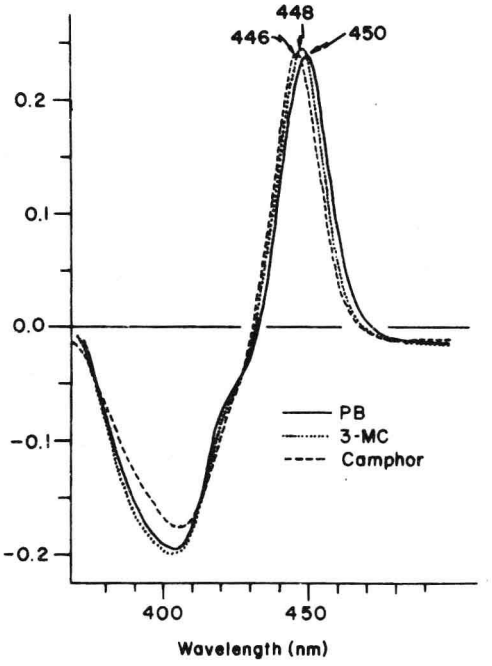

FIG. 4. Differences in the absorbance band maxima of the carbon monoxide complexes of different cytochromes P-450 as measured by difference spectrophotometry. The concentrations of cytochrome P-450 in liver microsomes from rats treated with phenobarbital or 3-methylcholanthrene have been adjusted to be equal to that of the P-450 isolated from *P. putida*.

the difference spectrum of the P-450 isolated and purified from the bacterium *Pseudomonas putida* is shown, illustrating that this P-450 has its absorbance band maximum at 446 nm.

This difference in the location of the absorbance band maxima of the CO adduct of the reduced hemeprotein, as measured by difference spectrophotometry, has been used as the basis for one type of nomenclature to distinguish different P-450s. Thus, the terminology of P-448, P-450, etc. was introduced. One must recognize, however, the imprecision of this method and the care and special instrumentation required in order to obtain meaningful results.

A second type of nomenclature, based on the inducing agent used to treat the animal, has also been used by some, e.g., $P\text{-}450_{PB}$ or $P\text{-}450_{MC}$. A third type of nomenclature has developed based on the presence of P-450s with various organs, such as $P\text{-}450_{K}$ for the pigment of kidney cortex microsomes. Further, some prefer to name P-450s based on their specific functions, such as the $P\text{-}450_{SCC}$ or $P\text{-}450_{11\beta}$, representing those hemeproteins of the adrenal cortex functional for the side chain cleavage of cholesterol or the 11-β hydroxylation of deoxycorticosterone, respectively, or $P\text{-}450_{cam}$ for the hemeprotein isolated from *P. putida* responsible for the initial step in the oxidative metabolism of camphor. At this time, no systematic method of nomenclature has yet been accepted by the workers in this area of research.

A major development in the characterization of multiple forms of P-450 occurred when the methodologies were established for the isolation and purification of the hemeproteins from their membrane environment (47,73). Application of the technique of polyacrylamide gel electrophoresis of detergent-solubilized proteins to monitor the purity of the isolated hemeproteins showed readily discernible differences in the physical properties of different P-450s (34). Following the nomenclature convention for isozymes, a more discriminating type of nomenclature for the various P-450s has developed (34). Based on their relative electrophoretic mobility on SDS gels, the P-450s are named $P\text{-}450_{LM2}$ or $P\text{-}450_{LM4}$, etc., where LM stands for the source, in this case liver microsomes, and the numeral stands for the relative position on the gel, with the lower number representing the lower-molecular-weight form. This approach has expanded our knowledge of the inventory of various P-450s from the limited few noted spectrophotometrically and permits a comparison of various P-450s isolated and purified in different laboratories.

Seen in recent years is a proliferation of studies directed to the isolation of P-450s from many different sources. This has led to the nonuniform pattern of nomenclature described above. The result has been very confusing to investigators not intimately concerned with the details of P-450 isolation and characterization. As the procedures for the purification of P-450 become more sophisticated, this problem is certain to be exacerbated; e.g., recent reports show that at least 11 distinct types of P-450 can now be isolated from the livers of untreated rabbits (2).

The ability to recognize different P-450s posed the important question of the relative concentration of each type of P-450 in a tissue and the commonality of P-450s isolated from different sources. The availability of pure proteins permitted the development of antibodies that would react preferentially with a specific type of P-450 (71). Although only a limited number of antibodies to different P-450s are now available, the power of this approach has already been demonstrated (70) for evaluating the content and composition of a tissue's inventory of different P-450s (Table 1). The exciting possibility of developing monoclonal antibodies is now being tested (58), and this method will markedly expand the usefulness of this approach for the identity of different P-450s.

The last year has seen a surge of activity applying the techniques of molecular biology to the study of P-450. The ability to prepare unique species of cDNA that will be useful

TABLE 1. *Antibody titer of P-450 content of liver microsomes*

	Total P-450 (nmole/mg protein)	Percent of total P-450[a]			
		$P\text{-}450_a$	$P\text{-}450_b$	$P\text{-}450_c$	Unknown
Adult male					
Control	1.11	3	2	1	94
Phenobarbital	2.51	1	43	1	55
3-Methylcholanthrene	1.93	3	1	68	28
Adult female					
Control	0.89	6	1	1	92
Phenobarbital	1.72	8	43	2	47
3-Methylcholanthrene	1.38	14	1	74	11

[a] Data from the paper of Thomas et al. (72). The nomenclature for designation of specific P-450s and the use of antibodies for the quantitation of the various forms of the hemeprotein is that described by Thomas et al. (72).

as probes to evaluate the genomic content of cellular information for a specific type of P-450, supported by knowledge of the nucleotide sequence coding for any P-450, offers a major step in furthering our knowledge of the extent of and basis for the multiplicity of P-450s (5,14,24,52).

Catalytic Cycle of Cytochrome P-450

Central to our understanding of the role of cytochrome P-450 in the oxidative metabolism of many drugs and other xenobiotics is the need to know the various steps in the catalytic function of this hemeprotein. A schematic representation of our present understanding of the sequence of reactions occurring during catalysis is presented in Fig. 5 together with other electron transfer reactions of liver microsomes that might be related to the function of cytochrome P-450. These reactions may be briefly summarized as follows.

Oxidized cytochrome P-450 (designated Fe^{+3}) can react reversibly with a molecule of substrate to be metabolized (designated as R) to form the equivalent of an enzyme–substrate complex. Many factors can influence the affinity of different P-450s for different substrates and the associated equilibrium constant for this reaction.

The complex of oxidized hemeprotein and substrate can accept an electron transferred from NADPH via the flavoprotein, NADPH–cytochrome P-450 reductase (designated as fp_1).

The resultant complex of the reduced (ferrous) hemeprotein, designated by Fe^{+2}, and substrate can react with oxygen to form a ternary complex called oxycytochrome P-450. A number of isoelectronic structures for this intermediate can be written, indicating the potential for distribution of electrons with any of the three components. In the absence of oxygen and presence of carbon monoxide, the complex of reduced cytochrome P-450 and substrate can react to form the ternary complex of hemeprotein, substrate, and carbon monoxide. It is this product that is characterized spectrophotometrically by the appearance of an absorbance band at about 450 nm.

The one-electron reduced ternary complex of oxygen, substrate, and hemeprotein can then be transformed in at least three ways:

1. The molecule of oxygen associated with this complex can dissociate or be displaced, regenerating the complex of substrate and reduced hemeprotein.
2. The bound molecule of oxygen can dissociate in a form equivalent to its one-electron state of reduction (the superoxide anion), thereby reforming the complex of oxidized cytochrome P-450 with substrate. The superoxide formed in this type

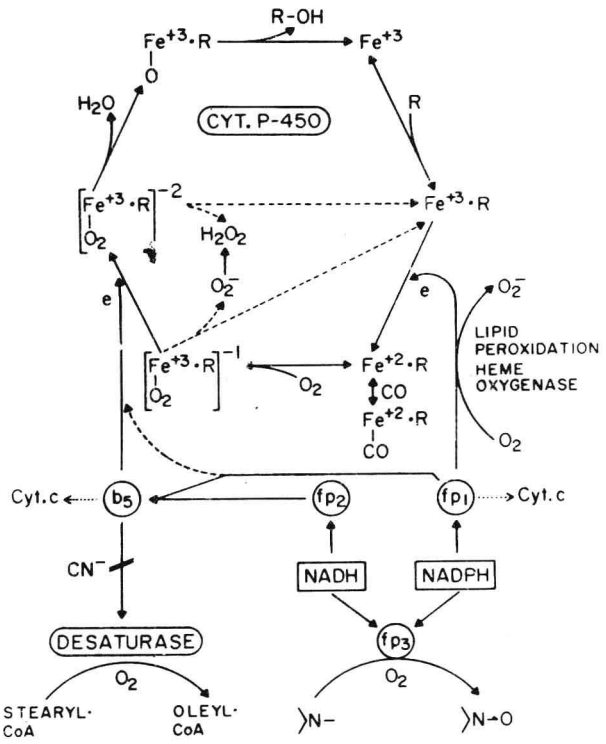

FIG. 5. The catalytic cycle of cytochrome P-450 and associated electron transfer reactions of liver microsomes.

of reaction can then dismutate (after protonation), giving rise to hydrogen peroxide. It has been suggested that this is the dominant pathway operative during the NADPH oxidase activity of liver microsomes, where hydrogen peroxide is formed concomitant with the oxidation of NADPH.

3. Oxycytochrome P-450 may accept an additional electron to form the equivalent of a two-electron reduced complex of oxygen, substrate, and hemeprotein. This intermediate has been termed peroxycytochrome P-450. The source of the electron for this reduction step is a matter of controversy. It has been proposed that reduced cytochrome b_5 can function as a suitable electron donor for this reaction. Alternatively, it has been proposed that the flavoprotein, NADPH–cytochrome P-450 reductase, because of its unique property of possessing two different flavin prosthetic groups, might better serve as the source of electrons for the reduction of oxycytochrome P-450. The possibility must also be considered that peroxycytochrome P-450 can dissociate, after protonation, to form hydrogen peroxide, although no direct experimental evidence has been reported in support of this hypothesis.

The key reactions of "oxygen activation" occur subsequent to the formation of peroxycytochrome P-450. Considerable interest now centers on these reactions. At least two possibilities can be seriously considered on the basis of present evidence:

1. It has been proposed that peroxycytochrome P-450 can be protonated, followed by removal of a molecule of water, to generate a complex of substrate and hemeprotein with an atom of oxygen that is highly electrophilic, the so-called oxenoid species of oxygen (see Fig. 1). Once