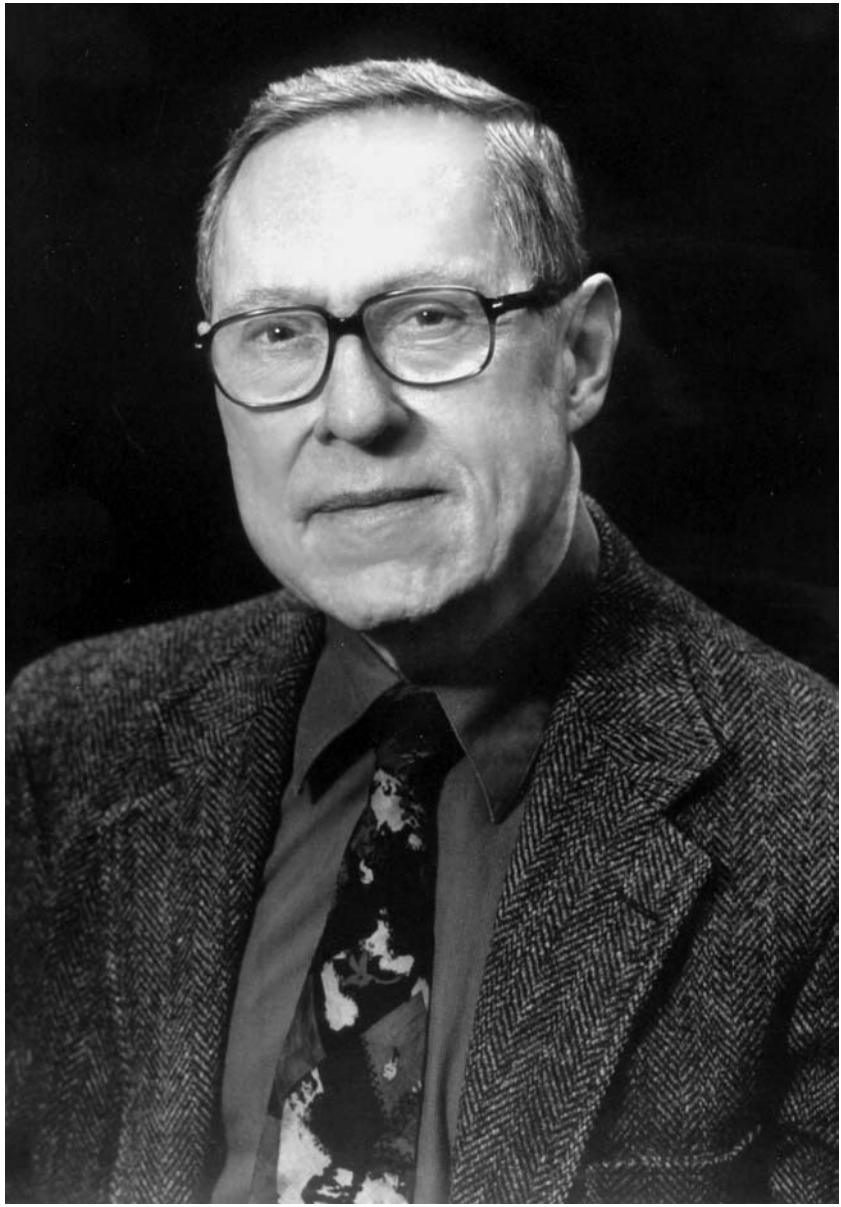

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CYTOCHROME P450: Nature's Most Versatile Biological Catalyst

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Key Words resolution and reconstitution of the microsomal P450 system, purification and characterization of multiple P450s, coupling of reductase and P450 reaction cycles, oxygen activation to give multiple functional oxidants

■ **Abstract** The author describes studies that led to the resolution and reconstitution of the cytochrome P450 enzyme system in microsomal membranes. The review indicates how purification and characterization of the cytochromes led to rigorous evidence for multiple isoforms of the oxygenases with distinct chemical and physical properties and different but somewhat overlapping substrate specificities. Present knowledge of the individual steps in the P450 and reductase reaction cycles is summarized, including evidence for the generation of multiple functional oxidants that may contribute to the exceptional diversity of the reactions catalyzed.

BACKGROUND

To my knowledge, my forebears, who migrated to the state of Colorado in the United States from Germany (via Russia) and from the Netherlands, had no scientific credentials. However, my father and my paternal grandmother were highly interested in reading about scientific advances despite having had no formal training, and it was my good fortune that my parents were fully supportive, even pleasantly surprised, at my own scientific bent. I also had the benefit of exposure to rigorous courses in the Denver public schools. Our teachers frequently told us that the schools in our city were ranked among the ten best in the country. We did not ask for documentation of that fact, but the science courses in my high school were challenging and so interesting that I considered a future career in several such fields. Geology particularly intrigued me, possibly because from our classroom windows we could see in the foothills of the Rocky Mountains the formations we were studying. The chemistry courses, however, opened a new world to me, and I knew I would continue to pursue some branch of this subject. That turned out to be the relatively new field of biochemistry, which I first learned about as an undergraduate at the University of Colorado, Boulder. Professor Reuben Gustavson, whose

training had been in steroid biochemistry at the University of Chicago, taught the freshman chemistry course, in which he included examples of problems in biology waiting to be solved by the application of chemistry. His enthusiasm and engrossing stories about the early history of science and the personalities involved made the subject come alive. I readily accepted his invitation to join his small research group working on estrogen metabolism, and, at his recommendation, spent the summer following my junior year at the University of Chicago in the laboratory of Professor F.C. Koch. From my experience, I am convinced that research in some field of scholarly endeavor is as crucial to undergraduate education as the usual didactic studies.

GRADUATE STUDY

Present-day academic institutions advertise widely to attract the best qualified faculty members, postdoctoral associates, and even medical and graduate students, and they spend much effort in interviewing and impressing applicants. It was much simpler in 1943, when I had a brief discussion with Dr. Gustavson about my desire to enter graduate school. He suggested a few top institutions and we decided on the University of Illinois. As a result of his correspondence with Professor William C. Rose, Head of the Biochemistry Division in the Chemistry Department there, I moved to Urbana in September as a Graduate Research Assistant, and I undertook a laboratory project a few weeks later.

There were no laboratory rotations in the 1940s, but after considering several attractive possibilities, I chose Professor Will Rose as my mentor. I paraphrase here what I have written at greater length elsewhere about his personality and accomplishments (1). Rose's research interests included the intermediary metabolism of amino acids, creatine, uric acid, and related compounds, and he was renowned for the discovery, isolation, and identification of a new amino acid, α-amino-β-hydroxy-*n*-butyric acid, which he named threonine. This was the culmination of experiments in which rats failed to grow on diets containing the 19 previously known amino acids. When I arrived in Urbana, the identity of the 10 amino acids essential for growth in rats and the 8 essential for the maintenance of nitrogen equilibrium in the human (that is, male graduate students) was already known. It fell to my lot to isolate or synthesize, purify, and analyze amino acids and then feed them to my fellow students enlisted as human guinea pigs to establish the quantitative requirements for the essential amino acids and the availability of their D-isomers or *N*-acetyl derivatives. In those days, the recruits were grateful for the free synthetic diets, the dollar a day they were paid, and the prospect of seeing their initials in Rose's widely read publications, but they required my constant encouragement because the rations were unpalatable. I have often remarked that any skills I developed to persuade my recalcitrant fellow students to continue in these difficult experiments (and therefore lead to completion of my Ph.D. thesis) became useful many years later when, as chair of a biochemistry department, I had to deal

with faculty colleagues having contrary views. Rose's students were somewhat in awe of the professor, probably wondering whether they could meet his exacting standards or hope to emulate the seeming ease with which he succeeded in all his professional endeavors. I learned in time that behind his somewhat reserved and formal manner was genuine warmth and an understanding that young scientists develop their full potential only by profiting from their mistakes; we became close friends until his death at age 98.

FACULTY AND SABBATICAL POSITIONS

Following completion of my Ph.D. thesis in 1946, I stayed at the University of Illinois for an additional year as a postdoctoral fellow, in part because I was courting another student, Mary Lou Newburn, whom I later married. A year later, I accepted an attractive faculty position elsewhere. Again, to illustrate the simplicity of the process at that time, Professor Rose heard of a suitable vacancy at the University of Pennsylvania and wrote to Professor D. Wright Wilson, Chairman of the Department of Physiological Chemistry, as it was then called, in the School of Medicine, on my behalf. Dr. Wilson, a man of few words, took me to a brief lunch at a national meeting in Chicago, offered me the position, and I accepted it never having been in Philadelphia or having met other faculty members in his department. He and I had faith in each other, and no time or expense was devoted to a more formal interview at that institution. However, when I arrived in Philadelphia some months later and learned from him, to my dismay, that I had been hired as a nutritionist (whereas my intent was to pursue intermediary metabolism as a biochemist), I realized that we had exchanged too few words previously. Before long, he accepted my career plan, but in my opinion our present system of thoroughly interviewing numerous applicants is much more likely to lead to success. Furthermore, it is fairer to potential candidates not having personal connections.

The Physiological Chemistry faculty at the University of Pennsylvania was among the first in this country to work with radioactive carbon-14 as a tracer, and several of the senior members were widely known for their studies: John Buchanan on purine biosynthesis, Wilson on pyrimidine biosynthesis, and Samuel Gurin on fatty acid β-oxidation. In addition, Otto Meyerhof, the famous biochemist who had come to the department as a refugee from Germany, was continuing his investigations on energy relationships in glycolysis. I undertook studies on amino acid metabolism, beginning with leucine, the oxidation of which is difficult because classical β-oxidation is not possible owing to the branched structure of the intermediate, isovaleryl-CoA. We discovered that a novel ATP-dependent CO_2 fixation was involved that led to intermediates in acetoacetate and cholesterol synthesis (2–6), and I have pursued unusual oxidative reactions ever since.

Another advantage of my position at Pennsylvania was the generosity of Dr. Wilson in allowing me to be absent for a year (although I had not yet earned a

sabbatical leave) to gain knowledge of enzymology in the laboratory of Professor Severo Ochoa, then Chairman of Pharmacology at the School of Medicine of New York University. As mentioned elsewhere (1), Ochoa's facilities were crowded and had limited equipment, but it was an exciting place to pursue research. Ochoa's scientific insight was supplemented by daily discussions with luminaries such as Otto Loewi, Ephraim Racker, Sarah Ratner, and a legion of visiting postdoctoral fellows, present and former students, and sabbatical guests from all corners of the world. The environment was ideal for a visitor to master enzymology as an essential tool to pursue the complexities of intermediary metabolism.

In 1955, I accepted an offer of a full professorship in Biological Chemistry at the University of Michigan, and moved with my wife and children Larry and Susan to Ann Arbor, not without regrets at leaving my friends and colleagues at the University of Pennsylvania. Michigan has now been a permanent and supportive home for my scientific career for almost 50 years, during 20 of which I served as chair of my department. As described below, my research interests gradually turned from amino acid metabolism, biotin function in CO_2 fixation, and pyruvate kinase properties and function to cytochrome P450 and its role in the metabolism of drugs and many other foreign compounds, as well as substrates of physiological importance.

Because of my increasing interest in mechanistic aspects of enzyme catalysis, I spent a sabbatical leave in 1961–62 with Professor Vladimir Prelog, Director of the Organic Chemistry Laboratory of the Eidgenössische Technische Hochschule (Swiss Federal Institute of Technology) in Zürich. Well known for his investigations on natural products and his outstanding contributions to stereochemistry (1), he had started to investigate enzyme stereoselectivity, and I began to work on hydride transfer from decalin ketones by oxidoreductases. The *Curvularia* enzymes that we employed proved to be difficult to purify, and we eventually found that a more suitable enzyme for our studies was the 3-oxoacyl-acyl carrier protein reductase component of a fatty acid synthetase (7). Many of my colleagues hesitate to take sabbatical leaves, believing they can't be spared from the day-to-day operations of their laboratories and institutions. On the contrary, more important insights may often be gained at a distance from our usual overloaded schedules. In my own case, the opportunity to reflect on my future research plans in a different setting undoubtedly contributed to my striking out in new directions.

My mentors differed in their personal characteristics and research interests, but all were completely dedicated to science. Severo Ochoa, for example, stated in a personal essay entitled "The Pursuit of a Hobby" (8) that in his life biochemistry had been his "only and real hobby." In that connection, I recall being at the University of Sheffield when Hans Krebs, who had built an excellent department there before his move to the chair at Oxford, returned to give a seminar. During the question period, a student asked Sir Hans to what he owed the secret of his success. He modestly replied, "Luck." When the applause died down, he became more serious and said, "I had a certain amount of luck in my life, but then I made a correlation—the harder I worked, the luckier I got."

OMEGA OXIDATION: A TRAIL LEADING TO RESOLUTION AND RECONSTITUTION OF THE LIVER MICROSOMAL P450-CONTAINING ENZYME SYSTEM

Although most biological oxidations occur according to the predictions of known chemical principles, those that do not are often found to involve particularly interesting cofactors, such as previously unsuspected metals or organic coenzymes. In other instances, novel functions of amino acid residues in the enzymes are discovered, thus altering our concepts of biological catalysis. I have long been intrigued by difficult oxidations of unfunctionalized alkyl groups, as in the conversion of the side chain of leucine to acetoacetate (as described above); the anabolism and catabolism of poorly soluble lipids; the degradation of natural products such as terpenoids; and the transformations of some chemically unreactive "foreign" substances such as drugs, solvents, and pesticides to products that may be more or less toxic than their precursors. Even the highly inert alkanes in petroleum have been known for many years to undergo microbial oxidation.

In the late 1950s, I picked fatty acid ω-oxidation, in which the attack occurs at the least reactive position, the terminal methyl group, as a model for such difficult oxidations. In 1932, Verkade et al. (9) in the Netherlands had discovered this unexpected conversion when they fed fatty acids of intermediate chain length to dogs and to human volunteers and isolated the resulting urinary dicarboxylic acids. Halina Den, a graduate student in my laboratory, was able to show that a ^{14}C-labeled α,α-dimethyl-substituted fatty acid underwent terminal oxidation in liver tissue (10), but the instability and insolubility of the enzyme system prevented further progress.

We then turned to the microbial oxidation of hydrocarbons as even more inert substrates. A postdoctoral associate from Illinois, James Baptist, isolated from soil samples a strain of *Pseudomonas oleovorans*, called the "gasoline bug" by our colleagues, which grew well on alkanes such as hexane. Cell-free extracts were soon obtained that required NADH for the aerobic conversion of octane to octanol (11, 12) and, of particular interest, the ω-oxidation of fatty acids as demonstrated by Masamichi Kusunose and his wife Emi, visitors from Japan (13). After the successful resolution of the enzyme system into three enzyme fractions by Bill Peterson (14), the components were eventually purified and characterized as rubredoxin, a red nonheme iron protein (15) previously only known to occur in anaerobic bacteria; a flavoprotein containing FAD and functioning as NADH-rubredoxin reductase that was characterized by Tetsufumi (Ted) Ueda (16, 17); and the ω-hydroxylase, an almost colorless protein that aggregated extensively and was activated by the addition of ferrous ions (18, 19). The properties of the bacterial hydroxylase made it a difficult candidate for further mechanistic studies, but it has continued to be investigated by others, who have established that it contains a nonheme diiron cluster (20).

In the hope that our findings with bacteria would be applicable to mammalian metabolism, we returned to the liver system we had abandoned approximately

ten years previously. Fortunately, Anthony Lu joined my research group in 1966 as a postdoctoral associate upon completion of his graduate studies in biochemistry at the University of North Carolina. My immediate impression was that this extremely capable young scientist deserved to be given a suitably challenging problem. I doubt that I made it clear just how challenging the hepatic microsomal system would be, but I advised him to begin with the methods that had succeeded with the pseudomonad. The lack of success with this approach did not discourage either of us, nor did the dearth of knowledge at that time about membrane-bound enzymes. After more than two years, Anthony's dedicated efforts eventually resulted in solubilization of the catalytically active rabbit liver microsomal ω-hydroxylation system by the use of various detergents with glycerol and other agents to prevent enzyme denaturation. As is now well known, ion exchange column chromatography resolved the system into three components, which upon recombination under controlled conditions, catalyzed the ω-hydroxylation of ^{14}C-labeled lauric acid (21, 22). As shown in Figure 1, these included a reddish-brown fraction that we soon identified, to our surprise and considerable delight, as cytochrome P450 by the spectral change upon reaction with carbon monoxide after dithionite reduction, and a yellow fraction containing NADPH-cytochrome P450 reductase that was fully active in electron transfer to P450, unlike preparations isolated by others after solubilization by protease treatment, with loss of the hydrophobic peptide at the NH_2-terminus. The third fraction contained an active component that was colorless, heat-stable, and extractable by organic solvents. This was later found by Henry Strobel, another talented postdoctoral associate from North Carolina, to contain microsomal phospholipids, of which phosphatidylcholine was the most effective (23). Thus, we had in our hands the solubilized, reconstituted enzyme system that would allow us to purify and characterize the enzymes involved. A variety of drugs, including aminopyrine, benzphetamine, hexobarbital, ethylmorphine, norcodeine, and *p*-nitroanisole, were also found to be oxidized by the reconstituted system (24), and, of much interest, Robert Kaschnitz (25) and Wilfried Duppel & Jean-Michel Lebeault (26) found that the same methods used with rabbit liver were successful with human liver and with *Candida tropicalis*, respectively. Our findings were greatly aided by previous knowledge that the microsomal CO-binding pigment of unknown function (27–29) had been characterized as a *b* type cytochrome by Omura & Sato (30). In addition, it was known that this catalyst in hepatic microsomes is involved in the hydroxylation of several steroids and drugs, as established in pioneering photochemical action spectroscopic experiments by Omura et al. in 1965 (31).

In his Bernard Brodie Award Lecture, Anthony Lu (32) has also commented on our limited knowledge of membrane-bound enzymes in the early days and the challenge of working on mammalian cytochrome P450. To indicate the many important questions remaining at that time, a brief summary of the proceedings of the first Symposium on Microsomes and Drug Oxidations held in Bethesda, Maryland, in 1968 (33) is in order. The idea came from the Committee on Drug Safety, Drug Research Board of the National Academy of Sciences. Organized by

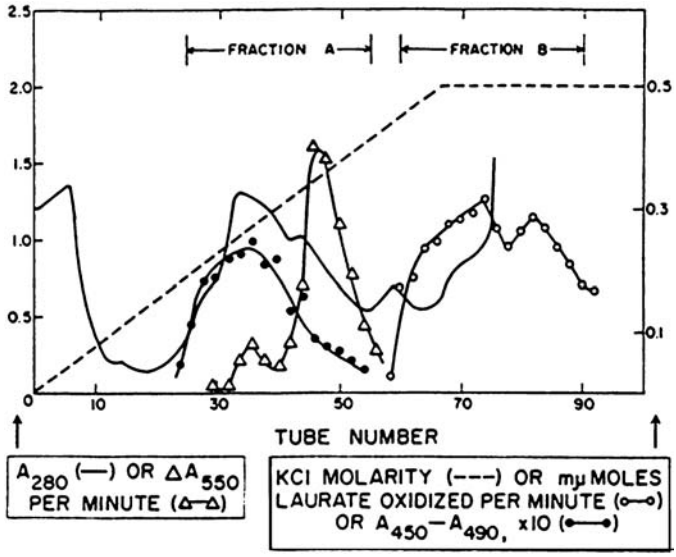

Figure 1 Resolution of ω-hydroxylation enzyme system into three components by DEAE-cellulose column chromatography of rabbit liver microsomal extract. Elution was with a KCl gradient, and 15-ml fractions were collected. For assay of P450 (•), undiluted samples were reduced with dithionite and flushed with carbon monoxide, and the CO-dependent A_{450}–A_{490} difference was determined in a 1-cm light path. NADPH-cytochrome P450 reductase activity (Δ) was estimated spectrally by cytochrome *c* reduction and expressed as the increase in A_{550} per minute per 0.4-ml aliquot of column eluate. The effect of the third component (○) on laurate hydroxylation in the presence of the P450 and reductase components was determined and expressed as mμmol of ω-hydroxylaurate formed per minute per 0.05 ml of column eluate. Taken from Reference 21.

James Gillette, an expert on biochemical pharmacology, and other distinguished scientists, including Allan Conney, George Cosmides, Ronald Estabrook, James Fouts, and Gilbert Mannering, the program included 27 lectures by experts from around the world on microsomal morphology and what was known about drug metabolism. (Posters had not yet been invented.) The properties of the endoplasmic reticulum were described, and evidence was presented that approximately 20 compounds, encompassing several drugs, steroids, and hydrocarbons, as well as fatty acids (34), undergo oxidation in liver microsomes from experimental animals. Hydroxylation, including drug *N*-demethylation, was the only reaction considered. Carbon monoxide and SKF-525A were the inhibitors mentioned, and phenobarbital and 3-methylcholanthrene the two chief inducers. Debate ensued on how many "forms" of P450 exist, with one camp believing in only a single enzyme. The interesting proposal was also made on the basis of the effects of inducers on

the activities and spectra of liver microsomes isolated from the treated animals for two types of CO-binding pigments, or possibly two interconvertible forms of a single cytochrome. With respect to the active oxidant produced by P450, oxene, analogous to compound I of peroxidases, was proposed. All in attendance agreed that the very intriguing field of drug metabolism was on the threshold of major progress.

This prefatory chapter is concerned with my research interests that led our laboratory to study the biochemical aspects of drug metabolism, and no attempt is made to provide a general review of what has become a huge field of endeavor. However, mention should be made of the outstanding contributions of the Gunsalus laboratory with bacterial P450cam, a nonmembranous cytochrome that is specific for camphor oxidation (35, 36) and has served as a model for the versatile but less tractable mammalian P450s. Readers interested in developments in this field over the years are referred to the proceedings of several series of international meetings, all with an emphasis on basic science: Symposia on Microsomes and Drug Oxidations, as already mentioned; Conferences on the Biochemistry and Biophysics of Cytochrome P450 (37), originated in 1976 by Klaus Ruckpaul, who was working at Berlin-Buch to overcome the barriers that had divided eastern and western Europe since the end of World War II, and whose valiant efforts in this endeavor attracted worldwide support as acknowledged by Sinisi Maricic, the organizer of the first conference (38); meetings on Cytochrome P450 Diversity (37), with an emphasis on microbial and plant systems, initiated by Hans-Georg Mueller, a colleague of Ruckpaul's at Berlin-Buch; and meetings of the International Society for the Study of Xenobiotics, started by Bruce Migdaloff in discussions with Fred DiCarlo, John Baer, and Ina Snow at the 1980 Gordon Conference on Drug Metabolism and launched the following year. Perhaps surprisingly, sufficient new results are obtained from laboratories around the world to justify all of these and other related meetings on a regular basis. I had the pleasure of chairing the committees that provided oversight for the Microsomes and Drug Oxidations symposia and P450 conferences for many years. Without a doubt, the collaborations and friendships that grew out of such international meetings were a major stimulus to the rapid development of this broad field, including its application to drug design and development.

MULTIPLICITY OF P450 CYTOCHROMES

In a recent review of the induction of drug-metabolizing enzymes, Allan Conney (39) has summarized the extensive evidence from his laboratory and elsewhere that treatment of animals with different microsomal inducers results in different profiles of catalytic activity for the metabolism of foreign compounds and steroid hormones. Such studies in many laboratories strongly suggested the occurrence of multiple cytochromes P450 with different substrate selectivities and provided a stimulus for biochemical characterization of the proposed catalysts. Furthermore,

enhancement by induction of the levels of individual enzymes in particular species, tissues, and organelles could facilitate purification. It was also widely recognized, however, that the catalytic changes seen in induced microsomes could be explained otherwise, by posttranslational modification by proteolysis or by addition of other chemical groups to a single cytochrome; by alteration of the membrane environment; or even by changes in other components of the system that might alter the rate-limiting steps, such as NADPH-cytochrome P450 reductase, cytochrome b_5, or phospholipids. In addressing this important question, Nebert et al. (40) showed that in genetically "responsive" mice, but not in aromatic hydrocarbon-treated "nonresponsive" mice, the inducible hydroxylase activity is localized exclusively in the P450, or what was then called the P448 fraction. A similar conclusion had been reached by Lu et al. (41) upon reconstitution of P450 and P448 separately with the reductase and phospholipid from microsomes of both phenobarbital- and 3-methylcholanthrene-treated rats.

A sophisticated understanding of drug metabolism, including the complexities of regulation and formation of diverse products that occasionally lead to toxicities, clearly required thorough characterization of the proposed individual P450 enzymes as well as the reductase. Progress toward this goal was made possible by the availability of the detergent-solubilized P450 system from microsomes, but it was difficult because these hydrophobic enzymes displayed a high degree of similarity and a tendency to aggregate (42). Indeed, it took over four years for the first mammalian P450, the phenobarbital-inducible form in rabbit liver microsomes, to be purified and characterized (43–46). The procedures that were developed, including column chromatography, had to be carried out in the presence of detergents. The resulting isolated cytochrome, now designated P450 2B4 according to the nomenclature on the basis of divergent evolution as judged by sequence similarity (47) (originally called LM_2, or liver microsomal form 2), differs from β-naphthoflavone-inducible P450 1A2 (form 4) in its physical and chemical properties (45, 46), including electrophoretic behavior; monomeric molecular weight; immunological reaction with specific antibodies (48, 49); absorption spectra in the oxidized, reduced, and CO-bound states (46); CD spectra (50); and fluorescence properties (51). Such individual P450s, which arise from genetically controlled de novo protein synthesis (52), are called isoforms or isozymes. The latter term was coined years ago to describe multiple forms of an enzyme identical in function but differing in some other property such as maximum activity, substrate affinity, or regulation. A 2B4-like pseudogene was also isolated and characterized; the alterations supported the view that it would not encode a functional cytochrome (53). The individual members of what is now called the P450 superfamily often have numerous functions (sometimes overlapping, as described below) but are still commonly called isozymes. Further evidence for multiple microsomal P450s was obtained from the differences in amino acid composition, COOH- and NH_2-terminal amino acid residues (46, 54), and eventually the complete amino acid sequences (55, 56). P450 2B4 was the first example of a mature protein found to have retained its so-called signal peptide. Research in several laboratories turned to

the question of how many of these unique catalysts occur in different species. The reductase, which fortunately occurs in only a single form, was purified from both rat (57) and rabbit liver microsomes (58), the structural features were established (59), and the crystal structure was subsequently reported (60). The interactions among the various purified components of the enzyme system have been investigated by several techniques (61, 62), including the puzzling effects of cytochrome b_5, which may be stimulatory or inhibitory (63–66).

Among the other rabbit cytochromes identified and then isolated in our laboratory were P450s 2C3 (form 3b) (67), 3A6 (form 3c) (68), and 1A1 (form 6) (69). In addition, the P450 that catalyzes the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one, an intermediate in the conversion of cholesterol to cholic acid, was purified in collaboration with Kyu-Ichiro Okuda and his associates in Hiroshima (70). Thanks to the painstaking work of Dennis Koop, Edward Morgan, and George Tarr (71), ethanol-inducible cytochrome P450 was discovered in rabbit liver microsomes, purified, and characterized as a unique isozyme with unusually interesting properties. This cytochrome, designated 2E1 (form 3a), displayed the highest activity of the rabbit isozymes in the oxidation of ethanol to acetaldehyde and was also found to oxidize other alcohols, aniline, and several drugs (72). The existence of such a "microsomal ethanol-oxidizing system," first proposed by Lieber & DeCarli (73), had previously been the subject of much debate. Although this may not be a major pathway for alcohol oxidation under most circumstances, the increased levels of 2E1 resulting from the diabetic state, fasting, and exposure to ethanol and several other diverse agents, including acetone, imidazole, benzene, and isoniazid, is a cause for concern because of resulting toxicities (74). In particular, acetaminophen, a widely used antipyretic and analgesic drug, is normally nontoxic, but in large doses it produces acute hepatic necrosis when converted to a reactive metabolite. Of a series of P450 isoenzymes examined, 2E1 was one of the most active in producing this metabolite (75). As summarized elsewhere (76), the predominant role of alcohol-inducible P450 in oxidative damage involves activation of carcinogenic nitrosamines (77) and the "leakiness" of this cytochrome in generating hydrogen peroxide and oxygen radicals (78), as well as alkoxy radicals in the cleavage of lipid hydroperoxides (79). The rabbit is apparently unique in having two genes in the alcohol-inducible P450 subfamily, the exon-intron organization of which was determined by restriction mapping and sequence analysis (80). The genes are not coordinately expressed or regulated, and chemical inducers act through changes in the rate of synthesis or degradation of the enzyme, rather than through increased gene transcription (81). The corresponding enzymes are 97% identical in amino acid sequence and have similar substrate selectivity, with 2E2 always somewhat less active. The regulation of 2E1 is particularly complex and includes effects of insulin and thyroid hormone on mRNA turnover (82) as well as of cytokines on the transcriptional regulation of both 2E isoforms (83).

Todd Porter, already an expert on NADPH-cytochrome P450 reductase when he joined the Biological Chemistry Department as a faculty member, contributed greatly to our research progress with his experience in molecular biology and

molecular genetics. As he has recently commented elsewhere (84), although the use of bacteria had become the most common approach to heterologous protein expression, there was skepticism whether this technique would lead to the retention of activity by membrane-bound enzymes. However, Michael Waterman's laboratory, working with 17-α hydroxylase (85), and our laboratory, working with 2E1 (86), were successful with this approach, which often requires modification of the NH_2-terminal signal peptide. Studies by Larson & Porter with 2E1 (86) and by Steve Pernecky et al. with 2B4 (87) contributed to this highly useful method for P450 expression and characterization. Of particular importance to those who crystallized functional truncated microsomal cytochromes, as indicated below, was our surprising finding that the NH_2-terminal segment of these cytochromes is unnecessary for catalytic activity.

It should also be noted that P450 cytochromes occur in a variety of tissues other than liver. For example, Xinxin Ding purified several from rabbit olfactory and respiratory nasal mucosa (88), including 2A10/11 (form NMa) (89) and 2G1 (form NMb) (90), which is active in steroid metabolism and uniquely expressed in the olfactory mucosa of nasal microsomes in animals. Ding & Kaminsky (91) have recently reviewed human extrahepatic P450s. The procedures that led to unequivocal evidence for the multiplicity of microsomal cytochrome P450s in the rabbit were soon applied to other species, including rats and mice, as described in a comprehensive review by Lu & West (92). Expansion of knowledge about cytochrome P450, aided by rapid progress in molecular genetics, has shown that this remarkable catalyst occurs throughout nature, including bacteria, fungi, and plants, as well as animals (93). Multiplicity of isoforms is typical in the various species, and with the availability of techniques for cloning and heterologous expression, the purified enzymes are readily available for characterization with respect to substrate specificity and other properties. Of biomedical importance in understanding the complexities of drug metabolism, the human species is now known to have 59 functional P450 genes (93).

MICROSOMAL P450 CYTOCHROMES CATALYZE NUMEROUS REACTIONS WITH COUNTLESS SUBSTRATES

When the components of the microsomal oxygenating system had been purified and characterized, we turned our attention to the individual steps in the hydroxylation reaction, which has the following overall stoichiometry:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+,$$

where RH represents a drug or some other typical substrate and ROH is the product. Our findings over many years are summarized in the schemes in Figures 2*A*,*B*, which indicate how reducing equivalents are transferred from NADPH via the reductase cycle to the P450 cycle, with one atom of molecular oxygen inserted into the substrate and the other reduced to water. Jan Vermilion and colleagues

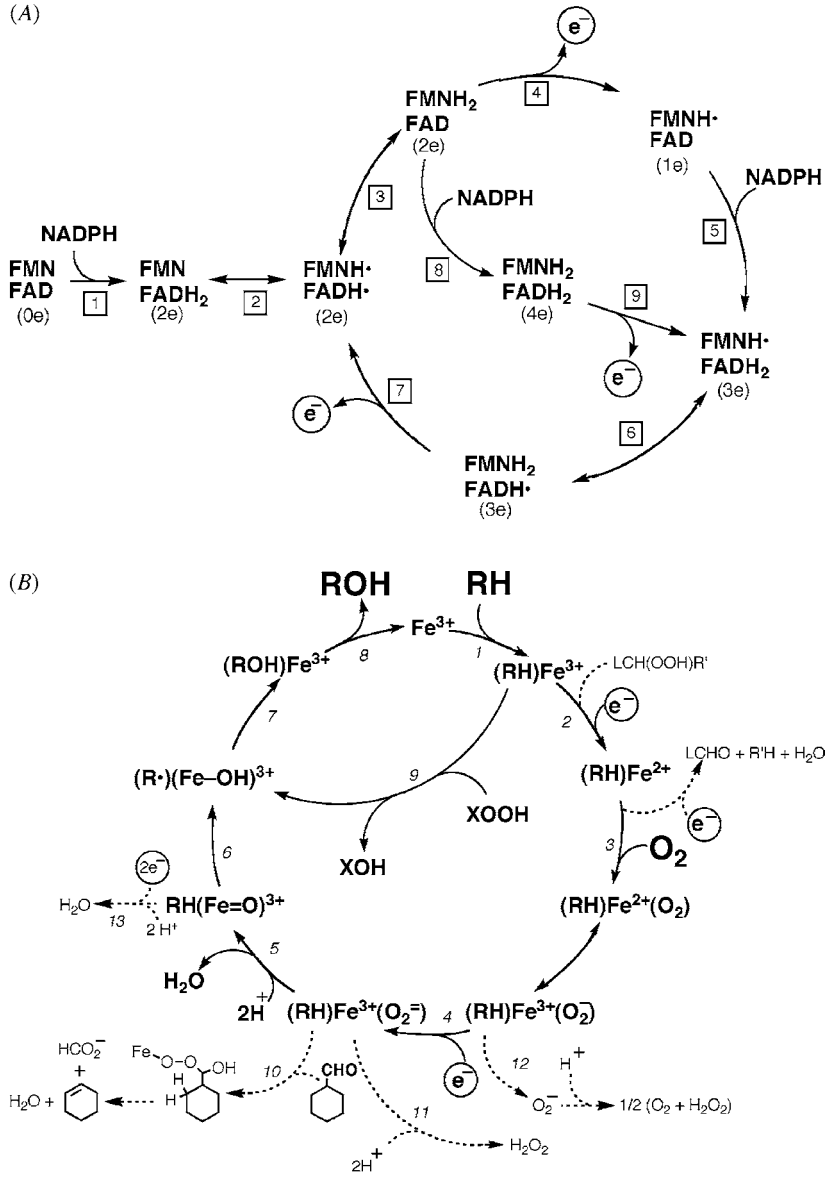

Figure 2 Joint function of P450 and reductase in drug metabolism. The schemes account for the oxygenase, oxidase, and peroxygenase reactions of cytochrome P450 with electron transfer from NADPH via the reductase. (*A*) The reductase cycle is modified from that in Reference 94 with the model for rapid interflavin electron transfer in Reference 95. (*B*) The P450 cycle is based on that in Reference 96. Fe represents the heme iron atom, RH a drug or other substrate, and ROH the corresponding product.

(94) carried out stopped-flow experiments with reductase preparations in which the FMN was replaced by artificial flavins that had a range of redox potentials. Her results supported an electron transfer sequence in the holoreductase, NADPH → FAD → FMN → P450, and led to the conclusion that the flavoprotein cycles mainly between the 1e- and 3e-reduced states during turnover (Figure 2*A*). Electron donation to P450 occurs via the reaction $FMNH_2 \rightarrow FMNH^{\bullet}$, the semiquinone. In addition, Dan Oprian (95), on the basis of a multiwavelength analysis by stopped-flow spectrophotometry, developed a model that successfully predicted the spectral course of each phase of the reaction of NADPH with the reductase under anaerobic conditions. This model was based on the model developed for xanthine oxidase by Olson et al. (97) at Michigan. Oprian's findings corresponded to those predicted for rapid electron transfer between the two flavins in which the distribution of electrons was governed at any time by the reduction potentials for the individual flavins. As indicated by the scheme in Figure 2*A*, the flavoprotein in its fully oxidized state is primed for its function by reduction of FAD by NADPH (Reaction 1). This is followed by electron redistribution (Reaction 2) to give the flavin diradical in equilibrium with $FMNH_2$-FAD (Reaction 3), which can then donate an electron to P450 to yield the Le-reduced flavoprotein (Reaction 4). Reduction of FAD by NADPH (Reaction 5), followed by electron redistribution (Reaction 6), provides $FMNH_2$ as a potential donor to P450 (Reaction 7). Alternatively, $FMNH_2$-FAD may be reduced by NADPH to give $FMNH_2$-$FADH_2$ (Reaction 8) as a donor (Reaction 9). Thus, $FMNH_2$ serves its role in providing reducing equivalents for oxygen activation by P450, regardless of whether the FAD moiety is in the fully reduced, semiquinone, or oxidized state.

The scheme in Figure 2*B* includes the basic reaction cycle for oxygen activation proposed in 1980 by White & Coon (98). The individual steps are based on experimental work in our own and other laboratories, and they are in accord with the expected stoichiometry as follows: substrate (RH) binding to ferric P450 (Reaction 1), first electron transfer from $FMNH_2$ (Reaction 2), dioxygen binding (Reaction 3), second electron transfer from $FMNH_2$ (Reaction 4), uptake of two protons and heterolytic splitting of the oxygen-oxygen bond with generation of the putative iron-oxene species (Reaction 5), proposed formation of a substrate radical as a transient intermediate on the basis of a collaborative investigation with John Groves on norbornane and 2B4 (Reaction 6) (99), oxygen insertion into substrate (Reaction 7), and product dissociation with return of P450 to the resting state (Reaction 8). Figure 2*B* includes additional reactions discovered subsequently (96), such as the ability of ferrous P450 to donate electrons in a stepwise fashion to bring about substrate reduction, as in the cleavage of a lipid hydroperoxide [shown as $LCH(OOH)R'$] to yield a ketone accompanied by hydrocarbon formation (100). For example, 13-hydroperoxy-9,11-octadecadienoic acid was found to give rise to 13-oxo-9,11-tridecadienoic acid and pentane. Lipid peroxidation is generally looked on as a destructive process in membranes of living cells, with formation of pentane and other hydrocarbons by various species, including the human, as a measure of this pathophysiological process. Also shown on the lower left in

the P450 cycle in Reaction 10 is the oxidative deformylation of an aldehyde with loss of the aldehyde carbon as formate (101). This reaction, which we believe to proceed via a peroxyhemiacetal-like adduct as indicated, is a model for the oxidative demethylation that accompanies steroid aromatization (102) and indicates a possible route for modification of drugs that contain a carbonyl function, as in aldehydes and ketones. The diverse chemical transformations carried out by this extremely versatile enzyme system include, in addition to those already mentioned, *N*-oxidation; sulfoxidation; epoxidation; oxidative ester and amide cleavage; *N*-, *S*-, and *O*-dealkylation; peroxidation; *ipso*-substitution (103); dehalogenation; desulfuration; and deamination; as well as reduction of epoxides, *N*-oxides, azo groups, and nitro groups. Additional chemical reactions attributable to P450 continue to be discovered (104), and it is likely that still more will be found, considering the major role of this cytochrome in the plant and microbial worlds, perhaps with counterparts in animals and the human species.

In the early days of P450 research, only a few types of organic compounds were thought to serve as P450 substrates, but this list continues to grow rapidly. Most of the following have been employed in our laboratory as well as by many other investigators: xenobiotics, including drugs, solvents, anesthetics, pesticides, petroleum products, antioxidants, dyes, and plant products such as flavorants and odorants, and compounds of physiological importance, such as steroids, fatty acids, and lipid hydroperoxides, as already mentioned, but also fat-soluble vitamins, amino acids, eicosanoids, and retinoids. The oxygenation and other alterations of such a variety of substrates by microsomal P450s may seem indiscriminate, but in many instances the modification is positionally and even stereochemically specific (105, 106).

Also shown in the scheme in Figure 2*B* is the release of products of O_2 reduction that are not coupled to substrate oxygenation, such as hydrogen peroxide (Reaction 11); superoxide (Reaction 12); and, in the 4-electron NADPH oxidase reaction (Reaction 13), water when the $(FeO)^{3+}$ species is reduced by electrons from NADPH (107). Reaction 9 illustrates the well-known peroxide shunt in which H_2O_2 (108) or an alkyl hydroperoxide (109), peracid, or iodosobenzene donates the oxygen atom for substrate hydroxylation with no requirement for molecular oxygen or for NADPH as an electron donor. Homolytic cleavage of the oxygen-oxygen bond occurs as shown, but heterolytic cleavage is also possible with some hydroperoxides, in which case Fe = O would be formed directly, as observed with iodosobenzene. A large variety of such donors is known from the work of Bob Blake (110, 111).

The availability of purified individual microsomal P450s soon made it clear that they do not conform to the typical textbook definition of an enzyme as a highly specific biological catalyst. For example, 2B4 and 1A2 were both found as early as 1975 to catalyze the oxidation of several substrates, including benzphetamine, ethylmorphine, *p*-nitroanisole, aniline, biphenyl, and testosterone; furthermore, the attack on the latter two substrates occurs in more than one position (45). To comment briefly on the total number of substrates for the hepatic microsomal P450s,

no investigators in the field are surprised by the prediction of a million or more. The present availability of combinatorial techniques in chemical synthesis further inflates this prediction. It is widely recognized that almost all drugs, including those to be produced in future years by the pharmaceutical industry, will serve as P450 substrates. In most instances the metabolic changes lead to drug inactivation and excretion of the more polar products, but some compounds that function as prodrugs become activated and others yield products that inactivate the cytochrome itself, as in the case of phencyclidine, which was first developed as a short-acting dissociative anesthetic. As shown by Yoichi Osawa (112), the mechanism-based inactivation of 2B4 is brought about by this drug and its oxidation product, the iminium compound.

MULTIPLE OXIDANTS AND MULTIPLE MECHANISMS IN P450 CATALYSIS

As already indicated, my interest in cytochrome P450 grew out of intense curiosity as to how an enzyme could accomplish with ease in an aqueous environment at neutral pH and mild temperatures one of the most difficult reactions in nature, hydroxylation of the unfunctionalized alkyl group in hydrocarbons and fatty acids. The details of such reactions have intrigued chemists and biochemists for decades. In studying this problem, my laboratory has been fortunate in having available microsomal P450s that oxidize virtually any organic compound that might be of mechanistic interest. We have also benefited greatly by collaboration with organic chemists, biochemists, and pharmacologists who were attracted to study the enzyme system that my Illinois colleague Steve Sligar calls "Nature's Blowtorch."

An early example was a study by Groves et al. (99) of deuterated norbornane in which mass spectral analysis of the exo- and endo-2-norborneol products indicated a very large isotope effect and significant epimerization in the hydroxylation reaction. The results indicated an initial hydrogen abstraction to give a presumed carbon radical intermediate in what has been called the hydrogen abstraction-oxygen rebound pathway. In another reaction not involving molecular oxygen, the effect of a series of *meta*- and *para*-substituents on cumene hydroperoxide as the oxygen donor and toluene as the oxygen acceptor was determined (110). The results supported a homolytic mechanism of oxygen-oxygen bond cleavage but not the heterolytic formation of a common iron-oxo intermediate from the various peroxides. The surprising range of substrates modified by P450 2B4 is also indicated by the aromatization of a bicyclic steroid analog, 3-oxodecalin-4-ene-10-carboxaldehyde (113). The products were formate and 3-hydroxy-6,7,8,9-tetrahydronaphthalene, thus showing that the artificial substrate is a relevant model for the conversion of androgens to estrogens. Even the number of P450 inhibitors appears to be almost unlimited. For example, the human placental aromatase P450 binds and is inhibited by a variety of substituted pyridines and other nonsteroidal compounds (114).

More recently, we have gained further insight into the details of oxygen activation by site-directed mutagenesis of mammalian 2B4 and 2E1 in which the highly conserved I helix threonine residue was replaced by alanine (115, 116). The impetus for our studies was the finding by the Ishimura (117) and Sligar (118) laboratories that the analogous mutation in P450cam apparently caused disruption of proton delivery, thereby interfering with the conversion of dioxygen to the oxenoid species and, therefore, the oxidation of the substrate, camphor. Replacement of threonine-302 by alanine in 2B4 virtually obliterated benzphetamine demethylation and also caused decreases in cyclohexane hydroxylation and phenylethanol oxidation. In sharp contrast, the deformylation of cyclohexane carboxaldehyde was increased approximately tenfold (115, 119). On the basis of these findings and our previous evidence that P450-dependent aldehyde deformylation is supported by added H_2O_2, we concluded that the iron-peroxo species, not oxenoid-iron, is the direct oxygen donor (115). Furthermore, in a study of olefin epoxidation (with cyclohexene, styrene, and the *cis*- and *trans*-isomers of 2-butene as substrates) by the T302A and T303A mutants of P450s 2B4 and 2E1, respectively, we obtained evidence for hydroperoxo-iron (as well as oxenoid-iron) as an electrophilic oxidant (116). Thus, our results support the involvement of three functional species produced during the reduction of molecular oxygen: peroxo-iron, hydroperoxo-iron (or its protonated version, iron-complexed hydrogen peroxide), and oxo-iron, as shown in Figure 3.

In the past few years, in collaboration with Martin Newcomb and Paul Hollenberg, we have examined hydroxylations of unactivated C-H bonds in hydrocarbons

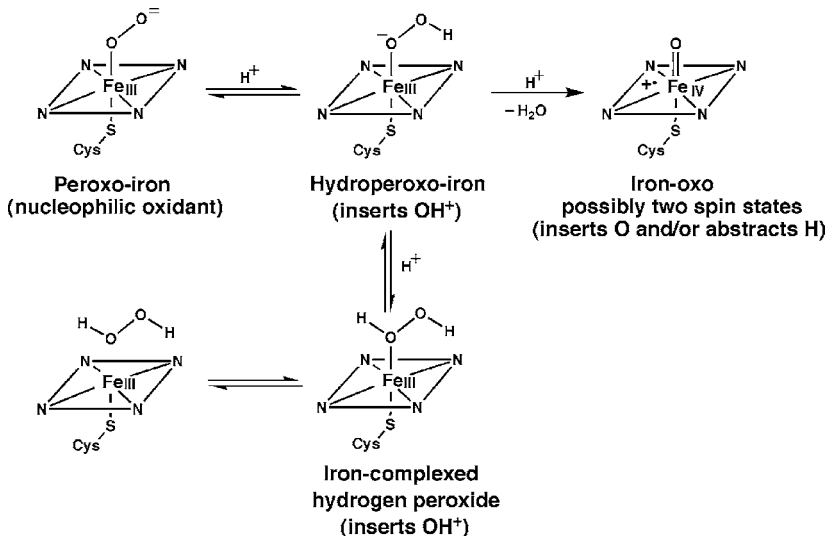

Figure 3 Versatility in P450 oxygenating species. The iron-oxygen intermediates in P450 catalysis and their proposed roles as oxidants. Modified from References 120 and 121.

and related compounds by use of highly reactive "radical clocks." These mechanistic probes, including *trans*-2-methoxy *trans*-3-phenylmethylcyclopropane and methylcubane, were chosen to differentiate between cationic and radical species because for these two kinds of intermediates different structural rearrangements occur. When these probes were used with several P450s, cationic products were found, and the small amounts of radical-derived products indicated that radical-like species were very short lived (subpicosecond) (122). A recent review presents our knowledge of the very complex P450-catalyzed hydroxylation reaction (121). In summary, in addition to the commonly accepted iron-oxo species, a second electrophilic oxidant is believed to exist. This scheme also takes into account computational work by Shaik et al. (123) and Yoshizawa et al. (124) that suggests the iron-oxo species may function in multiple spin states, possibly one that would involve oxygen insertion as envisioned in the early days of P450 mechanistic research (125, 126), and the other that would, by hydrogen abstraction, give a radical intermediate and thus resemble the oxygen-rebound pathway (99). A related long-standing question is whether the thiolate provided by a cysteine residue as the proximal heme ligand contributes to the chemical reactivity of these catalysts. Replacement of the active site cysteine-436 by serine has recently been shown to convert P450 2B4 into an NADPH oxidase with negligible monooxygenase activity (119). Remaining problems of oxygen activation will continue to be solved, but it is now clearly evident that the occurrence of multiple oxidizing species contributes to the remarkable versatility of the P450 family of isozymes in the modification of drugs and other substrates.

Of major importance, some crystal structures of mammalian P450s are now known. The first, reported in 2000 by Cosme & Johnson (127) and Williams et al. (128), was that of isoform 2C5 that had been engineered to delete the single N-terminal transmembrane domain and to mutate a peripheral membrane-binding site. More recently, Scott et al. (129) have reported the structure of 2B4, which revealed a large open cleft that extends from the protein surface directly to the heme iron. Differences between the two structures suggest that defined regions of these xenobiotic-metabolizing cytochromes may assume a substantial range of energetically accessible conformations. This flexibility is likely to facilitate substrate access, metabolic versatility, and product egress. The structural and functional data available suggest that conformational flexibility may be central to the ability of family 2 cytochromes to bind such a diverse array of xenobiotics. Thus, both the structural features of the cytochromes and the generation of multiple oxidants with different properties (120) may contribute to their exceptional diversity in catalysis.

Sixty years have passed since I decided on the branch of science I would pursue. During that time, technological achievements and increasing overlap among disciplines have made advances possible in fields that were poorly understood, and I have been fortunate to share in such progress. In addition to the thrill of research discoveries, I have enjoyed friendships and interactions with students, postdoctoral associates, and other collaborators. These have included biochemists, pharmacologists, toxicologists, chemists, biophysicists, molecular biologists, and occasionally

even clinicians interested in the application of basic science to biomedical problems related to drug metabolism. Regretfully, not all could be adequately recognized in this brief presentation, and readers are referred to the ever-increasing literature in the vast P450 field.

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