0090-9556/03/3112-1461–1473$7.00
DRUG METABOLISM AND DISPOSITION
DMD 31:1461–1473, 2003

Vol. 31, No. 12
1157/1111048
Printed in U.S.A.

A PASSION FOR P450s (REMEMBRANCES OF THE EARLY HISTORY OF RESEARCH ON CYTOCHROME P450)

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(Received April 22, 2003; accepted June 4, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Many members of the superfamily of hemeproteins, known as cytochrome P450 (P450 or CYP), are currently described in the literature (over 2000 at the date of this writing) [see Nelson, 2003 (http://drnelson.utmem.edu/CytochromeP450.html)]. In mammalian tissues, the P450s play central roles in drug and xenobiotic metabolism as well as steroid hormone synthesis, fat-soluble vitamin metabolism, and the conversion of polyunsaturated fatty acids to biologically active molecules. P450s also play a major role in plants by catalyzing the synthesis of a large number of secondary metabolites. Today we appreciate the unique oxygen chemistry catalyzed by the P450 enzymes as well as the dramatic effect of protein structural changes resulting in modifications of substrate specificity. Recent scientific advances have shown the importance of genetic differences (polymorphisms) in altering the physiological response of an animal to endo- and exo-biotic chemicals. In many instances these changes can be directly attributed to small differences in the amino acid sequence of a P450. The present article describes some of the early events associated with the establishment of the biological function of P450s. The 1950s and 1960s showed the transition of P450 from an unknown spectroscopic curiosity to the major player it now occupies in maintaining cellular homeostasis. The P450s are now recognized to occupy a great variety of phylogenetically distributed isoform activities. Much has been learned about the P450s, but much more remains as poorly understood. It has been almost 50 years since this class of unique proteins were discovered and their catalytic functions characterized. The present article describes the background and early history of research leading to our present knowledge of the cytochromes P450. Hopefully we will learn lessons from this history as we venture forward down the path of future scientific discovery.

Preface

This article summarizes the lecture I presented as the recipient of the 1997 Bernard B. Brodie Award in Drug Metabolism sponsored by the Drug Metabolism Division of the American Society for Pharmacology and Experimental Therapeutics. It is a great honor to be included in the list of outstanding scientists who are the recipients of this recognition. I was a great admirer of Dr. Brodie and his many achievements (Fig. 1A); in particular, his training of key players in our understanding of the pharmacology and biochemistry of drug metabolism. Many of these individuals have become my life-long friends—in particular, Jim Gillette, Bert La Du, John Burns, Elliott Vessell, and a host of others who put in place the foundation on which our scientific interests have developed. I thank the Division of Drug Metabolism of ASPET for this honor and I will greatly cherish this moment in my career. Furthermore, I apologize for the long delay in committing this lecture to a manuscript. There never seem to be enough hours in the day.

On this special occasion I want to add a few words in tribute to Jim Gillette. This special issue of *Drug Metabolism and Disposition* is dedicated to the memory of Jim Gillette. Jim was a colleague, collaborator, and special personal friend. I was pleased to serve as co-editor of the first volume of this journal with Jim Gillette and Ken Leibman. The first volume of DMD presented the Proceedings of the 2nd Microsomes and Drug Oxidation Meeting held in Palo Alta, CA on the campus of Stanford University from July 29 to 31, 1972. It was during this meeting and many subsequent international meetings that I formed a special bond with Jim Gillette. I learned a great deal about microsomal drug metabolism reactions from Jim, and he was a critical collaborator during the early phases of my introduction to the field of drug metabolism. In later years we shared a common interest in family genealogy, and I recall Jim's enthusiasm when he found we had a common relative born in the early 1800s. Jim is greatly missed and his seminal contributions to our understanding of the reactions of drug metabolism remain as a monument to his excellence as a scientist.

Introduction

Today we recognize members of the superfamily of hemeproteins, called cytochromes P450 (CYP, P450[1]) (see Nelson, 2003), as essential protein catalysts for the oxidative metabolism of many xeno- and endobiotics. These P450s play key roles in steroid hormone biosynthesis, the activation and detoxication of many drugs and environmentally contaminating chemicals, the metabolism of polyunsaturated fatty acids (such as arachidonic acid and prostaglandins), activation of vitamins A and D_3 to biologically active hormones, the synthesis of a vast array of secondary metabolites in plants and insects, the metab-

[1] Abbreviations used are: P450, cytochrome P450; TPNH, triphosphopyridine nucleotide.

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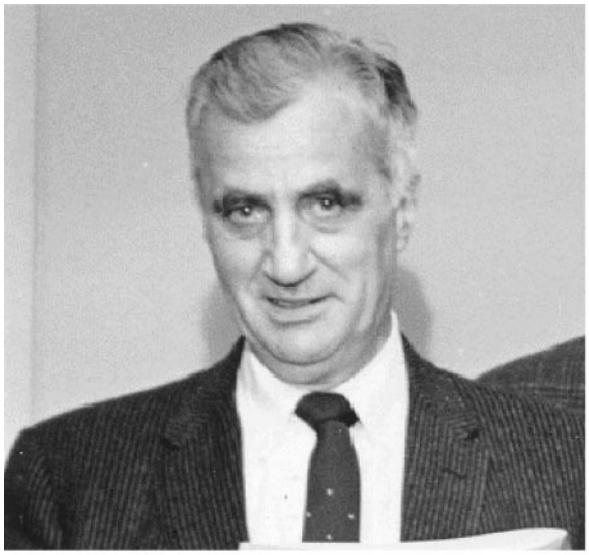


Bernard B. Brodie (1A)

Otto Warburg (1B)

R.T. Williams (1C)

Betty and Jim Miller (1D)

Howard Mason (1E)

Osamu Hayaishi (1F)

FIG. 1. *Outstanding scientists who established the foundation of science leading to the discovery of cytochrome P450 (pictures from the personal collection of Ronald Estabrook).*

olism of contaminating environmental chemicals to toxic and carcinogenic agents—and the list goes on and on. The inventory of P450 gene products now surpasses 2000, representing the largest family of proteins currently listed in gene data banks (Nelson, 2003).

However, it was not always this way. I have been fortunate to have been present and participated in the founding and characterization of P450, and I have served for almost 50 years as an interested and active research scientist contributing to our knowledge of these fascinating

colored proteins. It is a unique experience to watch a field of science grow from its origin to become a major force in the medical sciences.

It is difficult to know what to say in a historical review article of this type. First, one's memory becomes selective with age and the interpretation of events is seen differently by different individuals. Second, the priority of importance of one event over another is my selection, and my ranking may be argued. Third, I was not trained as a pharmacologist or steroid chemist and my knowledge of the physiological effects of many of the reactions catalyzed by P450s may be naive. All these factors may contribute to an erroneous impression of why certain experiments were done at a given time and the conclusions derived from such experiments.

I start by apologizing to the many contributors to our current knowledge of the P450s that I have neglected to mention. Please understand that the omissions are not intentional but merely the consequence of time and space.

As It Was In The Beginning

Where to start? The 1940s and 1950s were an exciting time for the biomedical sciences. World War II and the G.I. Bill's payment for a college education for a large number of veterans provided the opportunity for many individuals to enter science who had not considered science as a career option. The establishment of the National Institutes of Health and the commitment of the federal government to provide funds for research at universities opened many new avenues of training and research in poorly explored areas of science. The success of physics and the construction of atomic bombs and the associated development of sophisticated instrumentation used in the war served as a valuable impetus for the design and production of new equipment applicable to studies carried out in biomedical research.

As I reflect back to my introduction to the biochemistry of cellular respiration, I recognize that a significant literature had already developed by the early 1950s describing the physiological and pharmacological effects of drugs and steroids. But the vast majority of these studies were based on in vivo observations. It seems unbelievable today to realize that it was the development of the high speed centrifuge which opened many doors for biochemical studies related to cellular functions and for the discovery of the mechanisms of drug metabolism.

This review describing the discovery and early research on the cytochromes P450 will be largely based on in vitro biochemical studies and, therefore, the results described will date only from the late 1940s. However, a historical summary of this nature would be remiss if the contributions of two giants in the field of biochemical respiration and xenobiotic metabolism were not mentioned. Otto Warburg (Fig. 1B) stands as a pillar of scientific contributions to our knowledge of cellular respiration. Likewise, Professor R. T. Williams (Fig. 1C) changed our understanding of the role of metabolic chemistry by his numerous contributions to the metabolism of a large variety of foreign chemicals by mammalian tissues. Each of these individuals in his own way is responsible for the discovery of the P450s and both would have enjoyed the opportunity to see how this area of science has blossomed.

In my estimation, the seminal observations that led to the discovery of the P450s stem largely from three sources. These are the works in the late 1940s originating in the laboratories of a) Jim and Elizabeth (Betty) Miller (Fig. 1D) at the McArdle Memorial Laboratory at the University of Wisconsin; b) the Laboratory of Chemical Pharmacology of the National Heart Institute of the NIH, Bethesda, MD, headed by Bernard B. Brodie (Steve or BBB) in which Jim Gillette, Julius Axelrod, Bert La Du, and many others worked and were trained; and c) the many laboratories studying the conversion of cholesterol to active steroid hormones, in particular, work at the Medical Laboratories of Massachusetts General Hospital and Harvard University where Lewis Engel and Ken Ryan made their seminal discoveries on steroid hormone metabolism.

One must attribute to the Millers (Fig. 1D) the credit for discovering that the key enzymes for xenobiotic metabolism were located in the "particulate fraction" of liver cells. In the late 1940s the Millers and their colleagues (for a review of these discoveries see Miller, 1998) were studying the metabolism of the aminoazo dye *N-N*-dimethyl-4-aminoazobenzene (butter yellow) and its covalent binding to cellular proteins as it relates to tumor formation (the beginning of our understanding of chemical carcinogenesis). In two papers (Mueller and Miller, 1949 and 1953), they described the role of NADPH and the need for oxygen, together with a particulate fraction separated by centrifugation of a homogenate of rat liver that was capable of catalyzing the metabolism of some aminoazo dyes. At about this time a series of similar experiments was being carried out in Bethesda, MD in the laboratory directed by Brodie (see the reviews by Brodie et al., 1958, and Axelrod, 1982). There were two key players in these developments at NIH. Axelrod (Fig. 2A) was a master at developing methods for measuring metabolites of drugs, and he applied this skill to the in vitro measurement of the metabolism of psychoactive drugs, such as amphetamines and ephedrine. In these studies he showed that rabbit liver microsomes required the presence of reduced triphosphopyridine nucleotide and oxygen to support metabolism (Axelrod, 1955). For this work Julius Axelrod was awarded the Nobel Prize in 1970. Many others in the Brodie laboratory were also seeking to understand the enzymes of the liver microsomal drug-metabolizing enzyme system. Most notable was Jim Gillette (Fig. 2B). The critical contributions of Jim Gillette to our early knowledge of drug metabolism are highlighted in a review he wrote just prior to his terminal illness (Gillette, 2000). Many of the key questions concerning the mechanism(s) of enzymatic function in drug metabolism were formulated in the 1950s by Jim Gillette and serve as the basis for his life-long contributions to our knowledge of drug metabolism and toxicology.

The third major contribution that served as the basic structure for the discovery of P450s was in the area of steroid hormone metabolism. The 1950s saw a flurry of activities by a number of laboratories to understand the complex role of steroid hormones in humans. These studies eventually led to the discovery of modified steroids as birth control agents. A few of the outstanding leaders in steroid hormone research at that time were Leo Samuels' laboratory in Salt Lake City, Utah; Hyano and Dorfman as well as Hechter and Pincus at the Worcester Foundation, Shrewsbury, Massachusetts; and many, many more (including Alexandro Zaffaroni, who was a postdoctoral fellow in the Department of Biochemistry at the University of Rochester while I was a graduate student. Zaffaroni developed the paper chromatography method for detecting different steroids). Most important to future work revealing a role for P450 in steroid metabolism was the landmark paper by Ken Ryan and Lewis Engel (Ryan and Engel, 1957). This paper served as the pattern for our own future studies leading to the discovery of a function for P450s (Estabrook et al., 1963; Cooper et al., 1965). The paper by Ryan and Engel described experiments showing the requirement for TPNH and atmospheric oxygen together with a microsomal fraction from bovine adrenal cortex for the C-21 hydroxylation of progesterone. In this paper they also showed the carbon monoxide inhibition of the reaction and its reversal by light. One must admire the excellence of this study and its prediction of events to follow. However, they were not able to link this study to a role for cytochrome P450 as the key catalyst of the

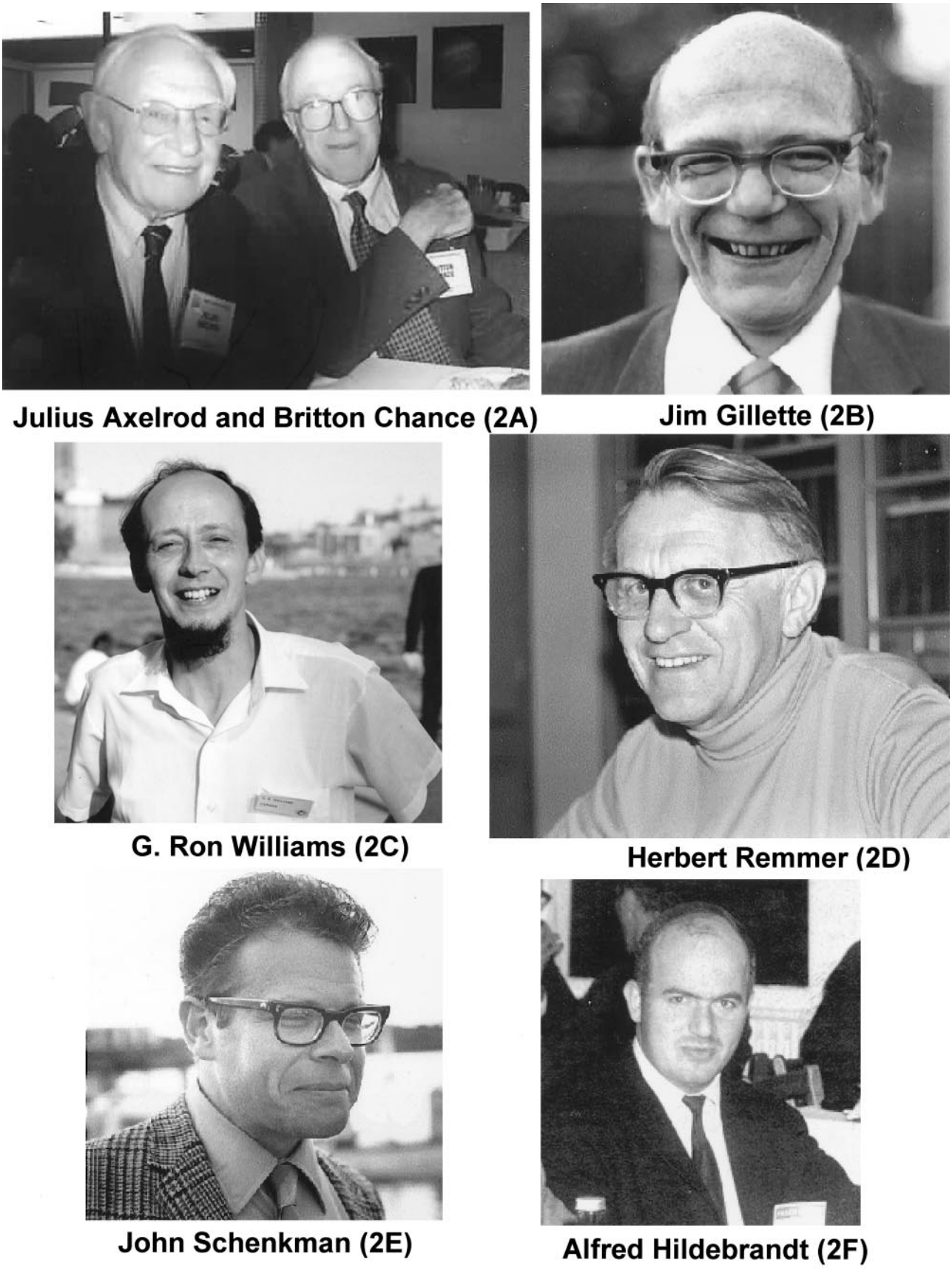


FIG. 2. *Scientists whose contributions in the 1950s and 60s were essential components for the discovery of the cytochromes P450 (pictures from the personal collection of Ronald Estabrook).*

reaction since P450 had not yet been observed and the spectrophotometric method they used was not appropriate (see below).

The Key Role of New Instruments

As mentioned above, the development of new instruments played a key role in the discovery of P450s. In particular, three new techniques became available in the 1950s and were applied to studies of cellular respiration and oxygen metabolism which influenced the course of research leading to the P450s.

First was the construction of mass spectrometers and the application of this technique to the study of oxygen metabolism using the heavy isotope of oxygen ($^{18}O_2$). Almost simultaneously, Mason et al. (1955) (Fig. 1E) and Hayaishi et al. (1955) (Fig. 1F) published papers demonstrating the incorporation of atmospheric oxygen into an organic substrate molecule during metabolism catalyzed by metalloprotein enzymes. This was the birth of mammalian oxygen fixation reactions catalyzed by enzymes we know today as oxygenases (Hayaishi, 1962) or mixed-function oxidases. These revolutionary observations and results focused further attention on the many roles of oxygen metabolism in biological systems (Mason, 1957). We know today that the best known of such reactions are those catalyzed by P450s.

Second was the development of a new class of spectrophotometers, in particular, the sensitive double beam rapid scanning machines designed and constructed by Britton Chance (Fig. 3) and demonstrated to be applicable to the study of respiratory pigments in turbid preparations of cellular particulates, such as mitochondria and microsomes (Chance, 1951, 1952, 1957). The compensating "double beam" machine obviated the need to add detergents to clarify samples undergoing spectrophotometric analysis (Fig. 3C). The ability to do such experiments in the absence of denaturing detergents was a key factor in determining the presence of P450 in the microsomal fraction of liver and adrenal cortex (see results described above by Ryan and Engel, 1957).

Third was the "oxygen electrode" (Fig. 3B). This simple polarographic method for measuring rates of oxygen utilization during respiratory metabolism was useful for determining the stoichiometry of oxygen consumed, substrate metabolized, and reducing equivalents utilized by P50 reactions (Estabrook, 1967). All three of these methods proved to be essential for the discovery of the role of P450s in substrate hydroxylation and drug metabolism reactions.

The Introduction to P450s and Preliminary Studies

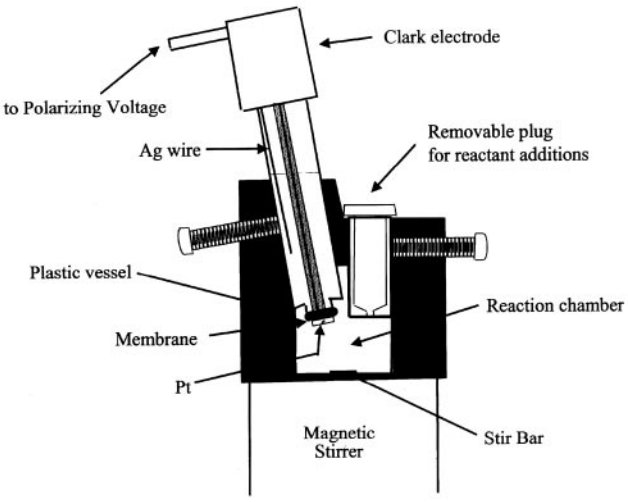
Life at the Johnson Research Foundation of the University of Pennsylvania School of Medicine in the 1950s and working under the direction of Britton Chance was everything a young scientist could desire. I soon learned to master the idiosyncrasies of the fragile double beam and wavelength scanning spectrophotometers and had ventured off on my own project of low temperature spectrophotometry (i.e., measuring optical changes of cytochromes cooled to the temperature of liquid nitrogen). At about the same time, I developed an expertise in building oxygen electrodes and made a short venture into business (with David Fitzpatrick) for the construction and sale of oxygen measuring instruments. Also, I became fascinated with the use of front surface reflectance fluorometry for the measurement of reduced pyridine nucleotide oxidation in turbid mitochondria and for the coupled analysis of intermediates of carbohydrate metabolism. As a result, I became a resource at the Johnson Foundation (JF) for teaching new people visiting the laboratory how to use these machines and interpret the results.

In 1954 a young German physical chemist named Martin Klingenberg joined the JF. He was interested in learning how to use the instruments developed by Britton Chance with a goal of applying these instruments for the measurements of rapid kinetics of biological electron transport reactions. At that time the main activities and interest of Chance's laboratory were directed toward unraveling the complex reactions of oxidative phosphorylation as catalyzed by liver and heart mitochondria. The classic series of papers authored by Britton Chance and G. R. (Ron) Williams (Chance and Williams, 1955a,b, 1956) stands as testament to the success of this work and remains today as the cornerstone of knowledge of respiratory control and the coupling of energy for ATP formation. As a result, rat liver mitochondria were prepared almost daily by Ron Williams (Fig. 2C), leaving a large amount of turbid supernatant fluid containing small particles of the endoplasmic reticulum (what we know today as the "microsomal fraction" or S9 fraction) to be discarded. Earlier, Chance and Williams (1954) had isolated the microsomal fraction from this postmitochondrial supernatant by use of a high speed centrifuge, and they showed by spectrophotometric studies the presence of the hemeprotein now known as cytochrome b_5 (although named cytochrome m in those days). Their studies also revealed an unexplained discrepancy in electron balance when the reduction of microsomal cytochrome b_5 was measured spectrophotometrically by titration with NADH. My memory indicates that the first observation of P450 occurred at this time, when carbon monoxide was gassed into the spectrophotometer cuvette containing liver microsomes in the presence of a reducing agent (NADH or sodium dithionite). I often attribute the discovery of P450 to these early studies [see Fig. 4; Martin Klingenberg in a footnote in his initial paper (Klingenberg, 1958) credits G. R. Williams for this initial discovery, although Ron Williams has only a vague recollection of such experiments (personal communication)].

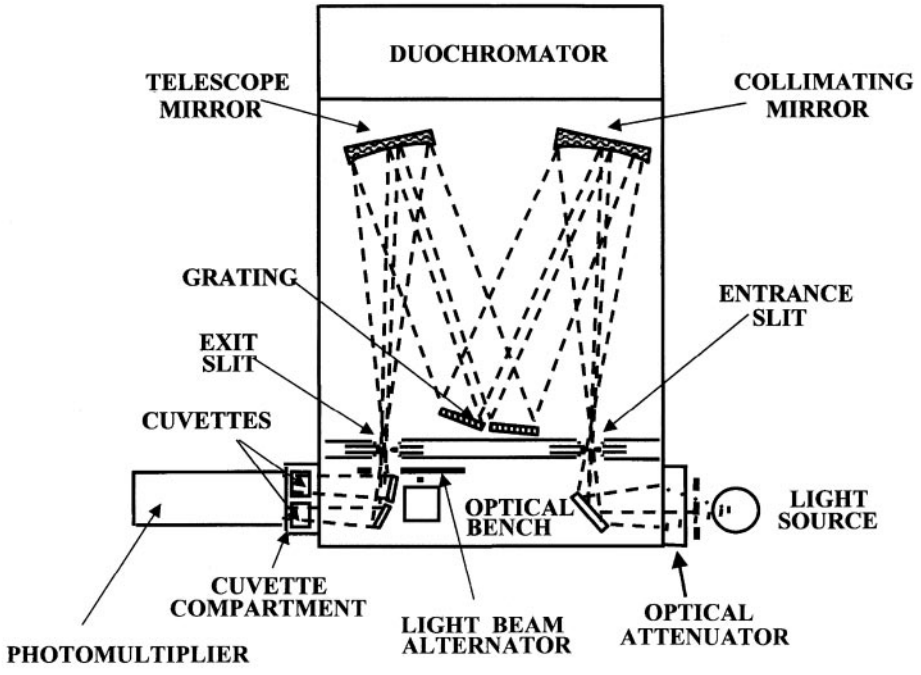
Martin Klingenberg undertook a series of studies to repeat and expand these earlier studies of Chance and Williams to determine the stoichiometry of electron reduction of liver microsomal b_5. At about this same time a graduate student of Britton Chance named LeRoy Castor, and a postdoctoral Research Fellow, Lucille Smith, were busy discovering and characterizing in tissue culture cells and different types of bacteria new cellular oxidases that formed complexes with carbon monoxide (Smith, 1954; Castor and Chance, 1955). It was from their studies that I learned about the "photochemical action spectrum" method of Warburg for characterizing CO-binding hemeprotein oxidases. As a result of these studies by Lucille Smith and Roy Castor, it became routine in the Chance laboratory to always end spectrophotometric experiments by adding an excess of reducing agent, such as sodium dithionite or NADH, followed by gassing the contents of the spectrophotometer cuvette with carbon monoxide.

Martin Klingenberg followed the path of a well trained German chemist: he was meticulous in his techniques, rigorously accurate in his experimentation, and not afraid to repeat an experiment. During his studies for measuring the reduction of cytochrome b_5, Martin observed the presence of a carbon monoxide-binding pigment with a rather large and broad absorbance band with a maximum at about 450 nm (Fig. 4). I do not remember how the name P450 was assigned to this material, but it seems obvious—a pigment (*P*) with a spectral absorbance maximum at *450 nm* seen in the presence of a reducing agent and carbon monoxide. I do recall many discussions in the laboratory trying to understand the nature of this CO-binding pigment. Klingenberg's studies showed the presence in liver microsomes of an unknown electron acceptor that was in significant concentration (about twice the electron-accepting capacity of b_5). Was this electron acceptor the same as the pigment that binds carbon monoxide? Was it a hemeprotein? Klingenberg measured the content of heme in liver microsomes and found there was a significant excess of heme over

(3A) Britton Chance with Hugo Theorell (about 1955)



(3B) The Oxygen Electrode

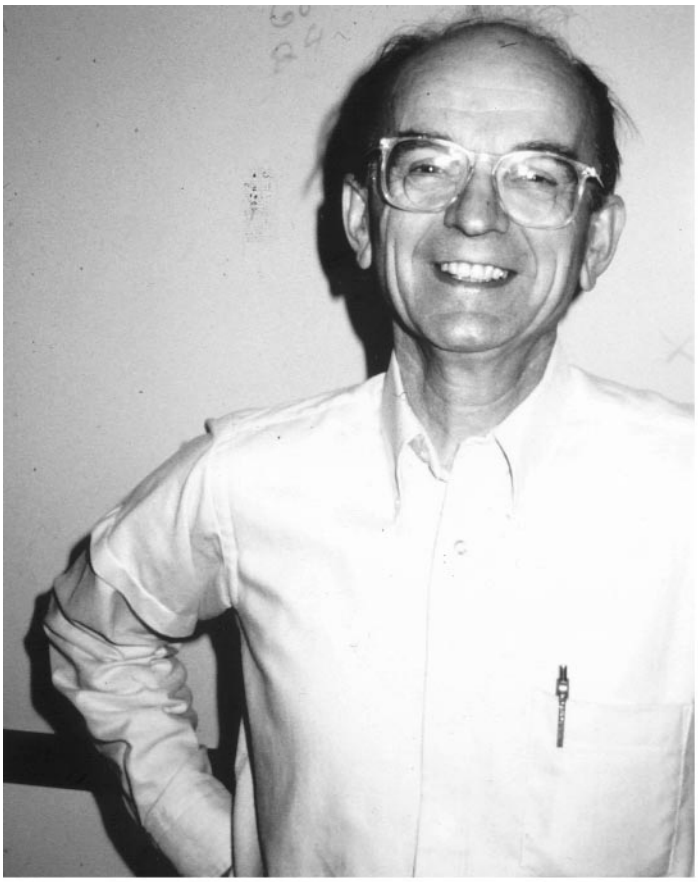


(3C) The Double-beam Rapid Scanning Spectrophotometer

FIG. 3. *Britton Chance (pictured with Hugo Theorell) whose development of the double-beam and dual-wavelength spectrophotometers and whose application of the "oxygen electrode" to the study of respiratory enzymes provided the essential methodologies for the discovery of the cytochrome P450s.*

A, from the personal collection of Ronald Estabrook; B, reprinted from *Methods in Enzymology*, Vol. 10, Estabrook RW, "Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios", Fig. 1, p 43, ©1967 with permission from Elsevier; C, reprinted from *Methods in Pharmacology*, Vol. 2, Estabrook RW, Perterson JA, Baron J, and Hildebrandt AG, "The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism", Fig. 3, p 381, ©1958 with permission from Elsevier; C, reprinted from "The spectrophotometric measurement of cytochromes", Estabrook RW, Peterson JA, Baron J, and Hildebrandt AG, in *Methods in Pharmacology, Vol. 2, Physical Methods* (Chignell C ed.), ©1972, Appleton-Century-Crofts.

that which could be accounted for by the content of cytochrome b_5. But this CO-binding pigment appeared to be different. At that time we believed that all reduced hemeproteins that bound CO underwent a hypsochromic shift (i.e., a blue shift) during the binding of CO. However, the pigment P450 showed a bathochromic shift (i.e., a red shift) when the reduced hemeprotein bound carbon monoxide. Fur-



Martin Klingenberg

Pigments of Rat Liver Microsomes[1]

Martin Klingenberg[2]

From the Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania

Received August 13, 1957

The Effect of Carbon Monoxide on the Spectra

Upon addition of carbon monoxide to microsomes reduced with either DPNH or dithionite, a rather broad absorption band with a maximum at 450 mμ appears[4] (Fig. 3, curves *A* and *B*). This CO band forms slowly ($t_{1/2ox}$ = 10 sec.). In microsomes reduced by DPNH the absorption at 450 mμ (curve *A*) is about 1.5 times as intense as the α-band of reduced cytochrome b_5. The addition of dithionite increases this two to three times. The intensity relationships are not the same for microsomes isolated from other mammalian livers. The trough at 404 mμ indicates the

[1] B. Chance and E. Boeri, unpublished experiments.
[4] Unpublished observations of Dr. G. R. Williams.

PIGMENTS OF RAT LIVER MICROSOMES 381

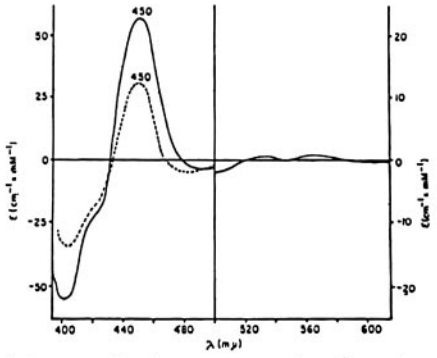


FIG. 3. Carbon monoxide difference spectra of rat liver microsomes. The millimolar extinction coefficients refer to the cytochrome b_5 present in the microsomes. ------ Curve *A*: Carbon monoxide with DPNH reduction. ——— Curve *B*: Carbon monoxide with dithionite reduction.

FIG. 4. *Martin Klingenberg and the initial report of his observation of a carbon-monoxide binding pigment present in liver microsomes.*

Left panel, from the personal collection of Ronald Estabrook; right panel, reprinted from the *Archives of Biochemistry and Biophysics*, Vol. 75, Klingenberg M, "Pigments of rat liver microsomes", Fig. 3, p 381, ©1958 with permission from Elsevier.

thermore, the P450 pigment had very weak absorbance bands in the visible region of the spectrum, which was certainly uncharacteristic of most protoporphyrin IX hemeproteins known at the time. So, P450 was relegated to a role as an interesting but unexplained spectrophotometric curiosity present in liver microsomes. The next year David Garfinkel joined Martin Klingenberg in an unsuccessful attempt to solubilize and purify b_5 and P450 from liver microsomes (Garfinkel, 1957, 1958). Martin Klingenberg subsequently returned to Germany to study the electron transport properties of mitochondria and the role of ion and ADP transporters of mitochondria, while David Garfinkel became an expert in computer simulation of enzymatic reactions. Thus, the original observation of P450 lay abandoned for over 5 years.

The Start of a Unique Collaboration: From Cytochrome b_5 to P450

The University of Pennsylvania School of Medicine was a special place to do research in the 1950s and 1960s. There was a strong spirit of comradeship throughout the faculty, and this was fostered by a special evening dining club called The John Morgan Society. The John Morgan Society met four times a year in the University Faculty Club to hear research presentations by both clinical and basic science faculty. It was at such a dinner that I met David Cooper, a surgeon in the Harrison Department of Surgical Research of the University of Pennsylvania School of Medicine. David described to me his work studying steroid metabolism using fractions from the bovine adrenal cortex. We soon developed a collaboration to study the pigments present in various fractions of the adrenal cortex, since David was puzzled by a recent report from Spiro and Ball (1961) that concluded "a microsomal cytochrome of the type so abundantly present in the medulla (of the adrenal gland) could not be demonstrated in the cortex." Was this the consequence of the detergent used by the Boston group when doing spectrophotometric studies? Since I had access to the Chance spectrophotometers, where one did not need detergent to examine turbid preparations of microsomes, we agreed to do some collaborative experiments. We soon showed (Cooper et al., 1963b) that adrenalcortical microsomes did have b_5 and that it was reducible by NADH or NADPH in a manner similar to that described for liver microsomes. Furthermore, we reported (Cooper et al., 1963b) that the microsomal fraction from the adrenal also contained the CO-combining pigment described by Martin Klingenberg (Klingenberg, 1958) with an absorbance maximum at 450 nm. This was the start of a magnificent collaborative adventure.

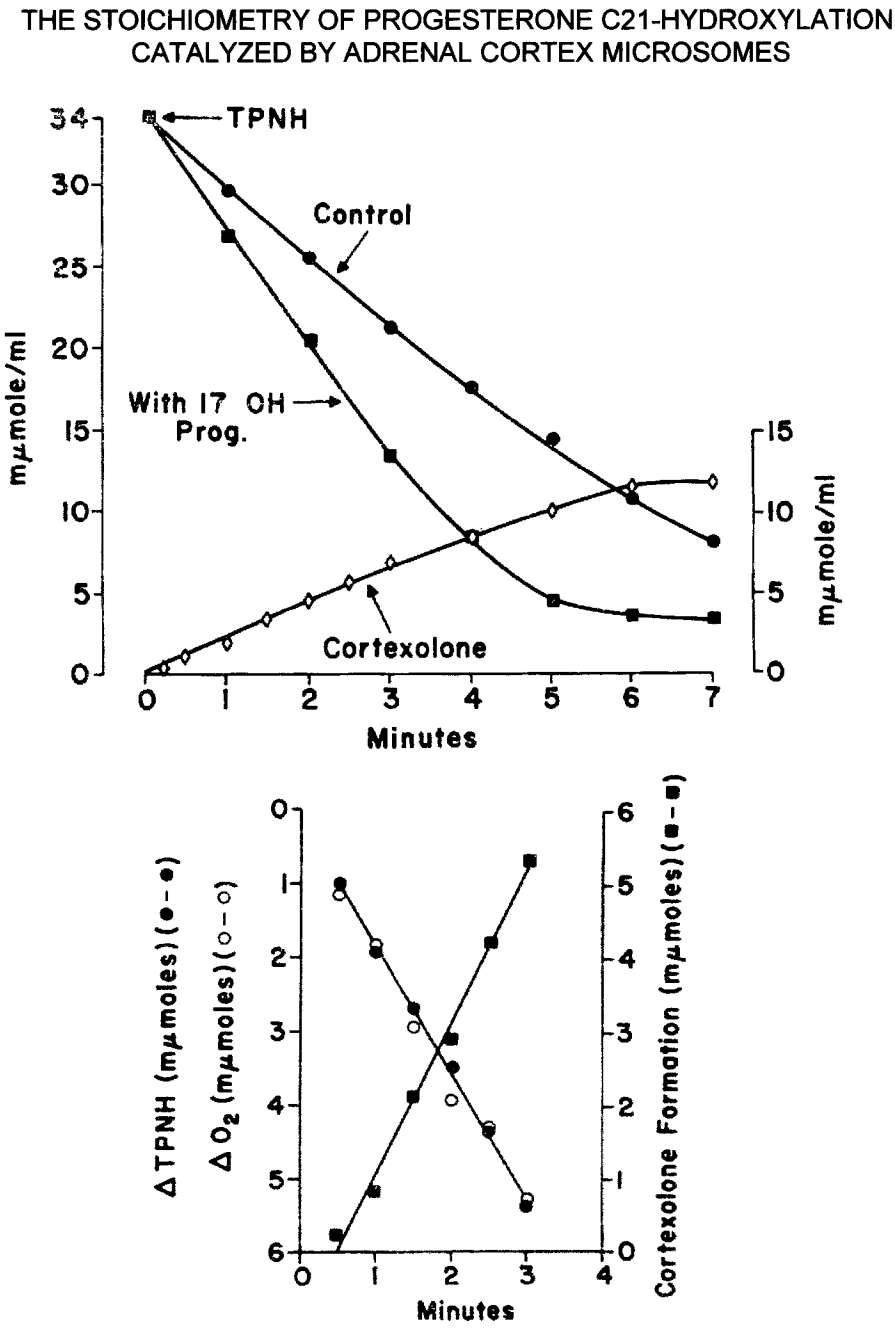


FIG. 5. *Results showing the determination of oxygen uptake, 17-OH progesterone metabolism, and NADPH (TPNH) oxidation as catalyzed by bovine adrenal cortex microsomes.*

Reprinted from the *Journal of Biological Chemistry*, Vol. 238, Cooper DY, Estabrook RW, and Rosenthal O, "The stoichiometry of C21 hydroxylation of steroids by adrenocortical microsomes", Figs. 2 and 3, pp 1321 and 1322, ©1963 with permission from the Journal of Biological Chemistry.

Our next collaboration turned to understanding the mechanism(s) of steroid hydroxylation reactions catalyzed by adrenal cortex microsomes. We were aware of the pioneering work of Howard Mason and his review on mixed-function oxidases (Mason, 1957). Is it possible that the reactions of steroid hormone synthesis under study by David Cooper and Otto Rosenthal were members of this new class of enzymes called oxygenases? How could we prove it?

The Importance of Numbers: Stoichiometric Considerations

As mentioned above, I had become proficient in using the oxygen electrode to study respiratory enzymes as well as a fluorometric method for measuring the oxidation of reduced pyridine nucleotides. Combining this knowledge with David Cooper's expertise in measuring steroid metabolites and producing adrenal cortex microsomes that could catalyze the hydroxylation of progesterone to 21-hydroxyprogesterone (cortexolone), we set out to measure the stoichiometry of the reaction to see if it conformed to Mason's mixed-function oxidases class of respiratory enzymes. Using an adaptation of available instruments in my laboratory, we soon were able to establish that the stoichiometry of the reaction was approximately 1 mol of oxygen utilized for 1 mol of NADPH oxidized for each mole of product formed (Fig. 5) (Cooper et al., 1963a). Thus, we knew we were

A New Cytochrome in Liver Microsomes

TSUNEO OMURA AND RYO SATO

From the Institute for Protein Research, Osaka University, Osaka, Japan

(Received for publication, January 12, 1962)

RYO SATO

TSUNEO OMURA

FIG. 6. *Pictures of Ryo Sato and Tsuneo Omura taken in the early 1960s (from the personal collection of Ronald Estabrook).*

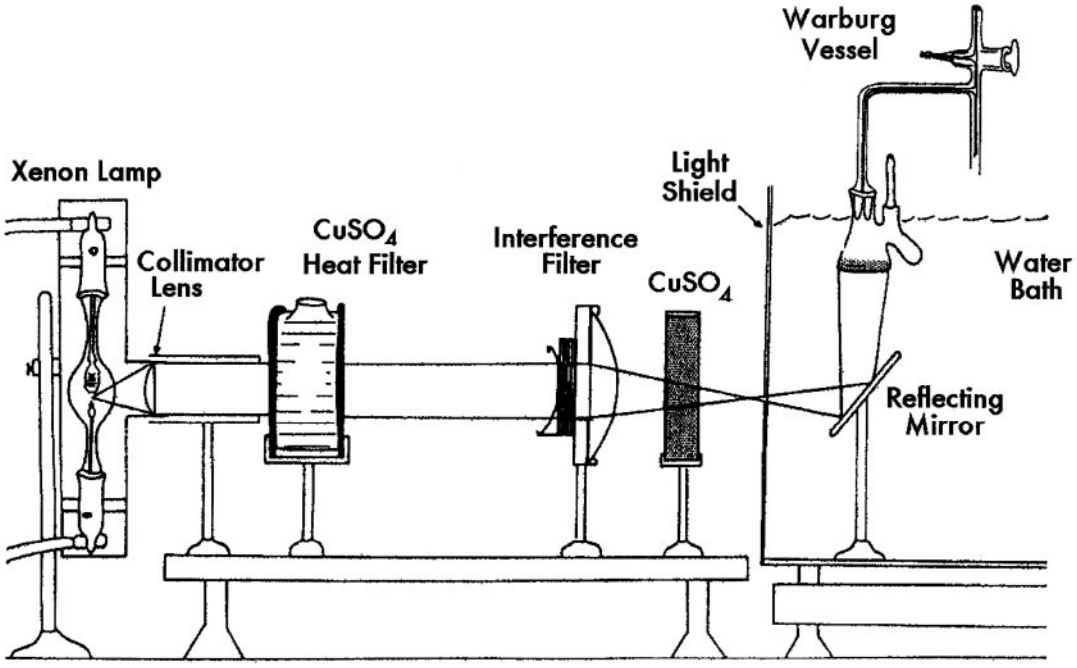
Their report on the hemeprotein properties of the CO-binding pigment in liver microsomes served as a key for unlocking the role of P450 in hydroxylation reactions.

working in the new area of respiratory enzymes called oxygenases. But, was there a link between the CO-binding pigment P450 of microsomes and the mixed-function oxidase of steroid metabolism present in adrenal cortex microsomes?

The Photochemical Action Spectrum

At about this time, a short communication appeared in the *Journal of Biological Chemistry* in which Ryo Sato and Tsuneo Omura reported that the pigment of liver microsomes that binds carbon monoxide to form an absorbance maximum at 450 nm, as first described by Martin Klingenberg (Klingenberg, 1958) was a hemeprotein (Omura and Sato, 1962) (Fig. 6). They christened this pigment a cytochrome. Recalling my conversations with LeRoy Castor and Lucille Smith, it was obvious that we had to determine the action spectrum for the photo-reversal of the carbon monoxide inhibition of the steroid hydroxylation reaction catalyzed by microsomes from the adrenal cortex. Of course, we were also aware that Ryan and Engel (Ryan et al., 1957) had already successfully done this experiment but had failed to characterize the pigment involved. The next 6 months saw a flurry of activity with many failures and false starts. The burden of experimentation fell on the shoulders of David Cooper, with the advice of Otto Rosenthal (Fig. 7). My role was that of an interested participant who wandered over daily to David Cooper's laboratory in the Surgery Department to give advice and follow the course of the experiments. David Cooper has written a colorful accounting of those days in a reflective review (Cooper, 1973). The equipment used to set up the apparatus to carry out a photochemical action spectrum experiment was rudimentary. We had selected a laboratory in the old Malone Building to do the experiments since it was possible to vent the excess carbon monoxide through an open window. David found an old fish tank that was used as the bath for controlling temperature; there was an old Warburg manometer shaker flask brought to the United States by Otto Rosenthal, and this was recruited to serve as the reaction vessel in which samples could be incubated under various mixtures of oxygen and carbon monoxide; a 2500 W xenon lamp of the type used to illuminate athletic fields served as the light source; filters transmitting various specific wavelengths of light were borrowed from a colleague in the Biology Department where he was studying photosynthesis; and the heat of the transmitted light was reduced by a solution of copper sulfate dissolved in concentrated sulfuric acid which came to a boil if the experiment proceeded for too long, and many more "horror stories." But it worked, and the first photochemical action spectrum was produced (Fig. 8).

In early April of 1963, a manuscript titled "The Action Spectrum for the CO Complex of the Oxygen Activating Enzyme for the C-21 Hydroxylation of Steroids" was submitted for publication as a Preliminary Communication to the *Journal of Biological Chemistry*. The Editor's response to this manuscript was discouraging. The reviewers



THE PHOTOCHEMICAL ACTION SPECTRUM EQUIPMENT

FIG. 7. *David Cooper and Otto Rosenthal in their laboratory in the Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia, PA where the first photochemical action spectrum establishing the role of the adrenal cortex microsomal cytochrome P450 in the C-21 hydroxylation of steroids was determined.*

Top panel, from the personal collection of Ronald Estabrook; bottom panel, reprinted from *Science*, Vol. 147, Cooper DY, Levin S, Natashimula S, Rosenthal O, and Estabrook RW, "Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems", Fig. 1, p 401, ©1965 with permission from Science.

wanted more evidence and more experiments. In the meantime, I had received a letter from Feodor Lynen inviting me to submit a manuscript to *Biochemische Zeitschrift* to be included in the Festschrift volume honoring Otto Warburg's 80th birthday. I felt this was a unique opportunity to bring visibility to our work. The revisions of the *Journal of Biological Chemistry* note languished, and I became concerned about the

Biochemische Zeitschrift 338, 741—755 (1963)

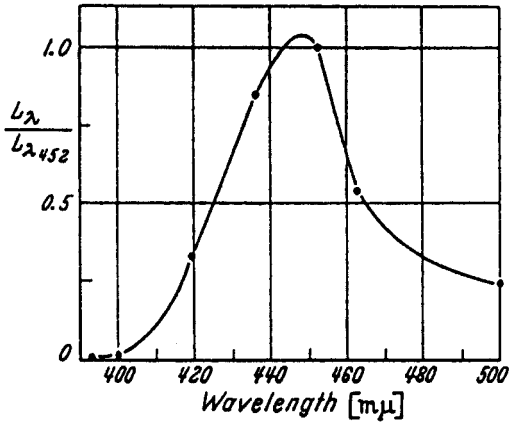
The Light Reversible Carbon Monoxide Inhibition of the Steroid C21-Hydroxylase System of the Adrenal Cortex* **

By
RONALD W. ESTABROOK***, DAVID Y. COOPER+ and OTTO ROSENTHAL++

Johnson Foundation for Medical Physics
and the Harrison Department of Surgical Research, University of Pennsylvania

With 10 Figures in the Text

(Received June 10, 1963)



Photochemical Action Spectrum of the Terminal Oxidase of Mixed Function Oxidase Systems

DAVID Y. COOPER, SIDNEY LEVIN
SHAKUNTHALA NARASIMHULU
OTTO ROSENTHAL
RONALD W. ESTABROOK

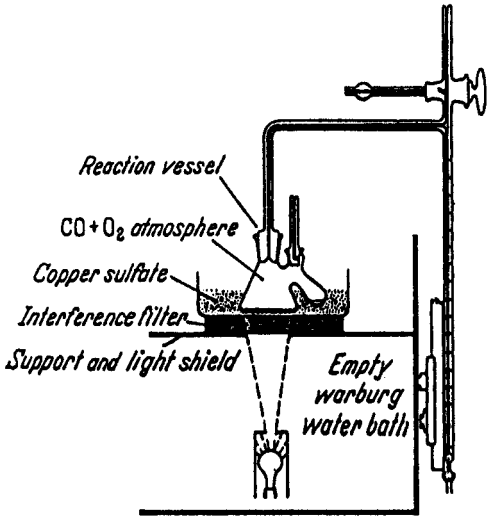


FIG. 8. *The two initial publications describing experiments establishing the role of cytochrome P450 in the hydroxylation of steroids and the metabolism of drugs.*

Reprinted from *Biochemische Zeitschrift*, Vol. 338, Estabrook RW, Cooper DY, and Rosenthal O, "The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex", Fig. 7, p 750 (top right), Fig. 10, p 754 (bottom right), ©1963 with permission from Springer-Verlag.

timely publication of our results. Benno Hess, the editor of Biochemische Zeitschrift at that time, was sending me notes that we had passed the deadline for submission of a manuscript to the Warburg Festschrift. In a moment of haste (which caused a long-term rift in my relationship with David Cooper) I reworked the data in the *Journal of Biological Chemistry* Preliminary Communication and submitted a paper to Feodor Lynen for inclusion in the Warburg Festschrift (Fig. 8). As we know today, this was a seminal paper, for it clearly established a role for P450 as the terminal oxygenase of steroid hydroxylation reactions.

P450 and Drug Metabolism

Soon after the paper appeared in *Biochemische Zeitschrift* (Estabrook et al., 1963), I received a letter from the German pharmacologist, Herbert Remmer (Fig. 2D). Remmer described his studies (Remmer et al., 1965) of induction of drug-metabolizing enzymes present in the endoplasmic reticulum of liver and his observation of an increase in the concentration of the CO-binding pigment of liver microsomes during induction. Herbert Remmer said that he planned to attend a meeting of the New York Academy of Sciences to present these findings and he would like to stop in Philadelphia to discuss his work with us. This was a magnificent stroke of luck. Remmer proved to be an excellent colleague and a fountainhead of knowledge of drug metabolism. He arranged for Alfred Hildebrandt (Fig. 2F) to join my laboratory for collaborative studies. In the meantime we had arranged with Jim Gillette and Henry Sasame to visit Philadelphia to teach us how to measure reactions of drug metabolism. This was an exciting time. Every experiment we did seemed to produce new and important information. Dave Cooper and his colleagues turned their attention to studying the reactions of drug metabolism catalyzed by rat liver microsomes using the technique of the photochemical action spectrum (Cooper et al., 1965). These results using rat liver microsomes and the drugs codeine, aminopyrene, and acetanilide as substrates confirmed our earlier studies carried out by measuring steroid hydroxylation reactions catalyzed by microsomes prepared from the adrenal cortex. These studies represent the birth of P450 as the key component for many reactions of drug and xenobiotic metabolism. The rest is history.

Final Thoughts. There is so much more that could be said. But time has run out and this manuscript is already overdue. Perhaps I will continue with the next chapter in the near future. In this article I have left out a description of experiments that led to our understanding of drugs as ligands for the heme of P450; I have omitted experiments that led to the recognition of the cyclic function of P450 during metabolism and what our speculations were on the mechanism of oxygen activation; I have not mentioned the characterization of the adrenal

FIG. 9. *Pictures taken at the First International Symposium on Microsomes and Drug Oxidations, held in Bethesda, MD, February 1968 (from the personal collection of Ronald Estabrook).*

mitochondrial P450s and David Cooper's ability to reconstitute enzymatic functions with such a system; the role of Tsuneo Omura in the isolation, purification, and characterization of adrenodoxin as an iron-sulfur protein functional in mitochondrial P450 reactions; and many more exciting studies.

I have a great debt of gratitude to so many, many colleagues—in particular, David Cooper and Otto Rosenthal, who tolerated me at the beginning of this story. Many of the young workers who participated in the early days of P450 research, such as John Schenkman (Fig. 2E), were witness to these historic events. Acceptance of the P450s as a catalyst for drug metabolism received a major boost in its momentum from the first meeting on Microsomes and Drug Oxidations (Gillette, 1969) (Fig. 9). Jim Gillette played a key role in organizing and arranging for that meeting. This is another example of the debt of gratitude we owe Jim Gillette.

Each of us entered science with the ambition of contributing knowledge that would influence the well being of mankind. Cytochrome P450 has taken a central role in many areas of the biomedical sciences. I have been fortunate to play a role in this research and greatly appreciate the many friends and colleagues I have met along this path of research.

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Ronald W. Estabrook was born at Albany, New York on January 3, 1926. He was educated in the public school system of Albany before entering the Navy in 1943, where he was in the Navy V-12 program at Princeton University and Officer Training School at the University of Notre Dame. He served as an officer on a subchaser and a mine sweeper in Okinawa and Japan. Upon discharge from the Navy, he continued his undergraduate education at Rensselaer Polytechnic Institute, Troy, New York, where he graduated with a B.S. (Biology) in 1950; Estabrook did his graduate training in biochemistry at the University of Rochester, Rochester, New York (1954); and postdoctoral training in biophysics with Britton Chance at the Johnson Research Foundation, University of Pennsylvania. He studied at the Molteno Institute, Cambridge University, in England, with David Kielin. In 1959 Dr. Estabrook joined the faculty of the School of Medicine of the University of Pennsylvania where he advanced to the rank of Professor of Physical Biochemistry. In 1962 he published with David Cooper and Otto Rosenthal, of the Department of Surgery of the University of Pennsylvania, seminal studies describing the enzymatic (functional) properties of the hemoprotein now known as cytochrome P450. In 1968 Estabrook moved to Dallas, Texas to serve as Virginia Lazenby O'Hara Professor of Biochemistry and Chairman of the Department of Biochemistry at the University of Texas Southwestern Medical School. In 1982 Estabrook returned to the laboratory bench where he is now applying the techniques of molecular biology to the study of the enzymatic properties of different P450s expressed in different types of cells.

Estabrook has coauthored over 280 publications including the editing of 15 books. He was elected to the National Academy of Sciences in 1979 and awarded an honorary Doctor of Medicine from the Karolinska Institut in Stockholm, Sweden in 1981 and a Doctor of Science from the University of Rochester (1981). He has served on numerous national and international advisory committees including the Governing Board of the National Research Council of the National Academy of Sciences and the Council of the Institute of Medicine of the National Academy of Sciences.