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# Hepatic Cytochrome P-450 Monooxygenase System

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and
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# HEPATIC CYTOCHROME P-450 MONOOXYGENASE SYSTEM

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Hepatic Cytochrome P-450 Monooxygenase system

# PREFACE

SINCE its discovery in 1958 as a carbon monoxide-binding pigment in liver microsomes, the number of scientific papers published yearly on studies directly related to this hemoprotein have increased almost logarithmically. This great rate of interest has been, in part, due to the excitement generated by the opening of a new field as well as the great energy of the fairly young group of investigators involved.

From its detection in 1958, recognition as a hemoprotein in 1962, and determination of function in 1963, to its solubilization and reconstitution in 1968 was but a short ten years. Even so, the pace has increased to the point where attempts to compile a book on the enzyme system have been thwarted by the speed of obsolescence of reviews.

It has been our purpose to provide both a historical and prospective view of selected areas of the hepatic microsomal enzyme system, picking the liver as a tissue with which we are both familiar and since much of the advances in the field have been with this enzyme system. Enough differences exist between the cytochrome P-450 enzyme systems of microorganisms, mitochondria and other tissue and species as to make an all-embracing text an impossible task. Perhaps our omissions will encourage other would-be authors to fill the void.

Our goal was to develop an in-depth coverage of each component of the enzyme system and of its functions, but also to provide enough background and history to be an aid to the student or investigator newly entering the field. In soliciting material every attempt was made to obtain leading investigators in the field to provide chapters on their areas of expertise. Considerable efforts were also made to include contributions from younger investigators whose studies have begun to make impact on the field.

Today studies on the mixed function oxidase are carried out by investigators in many fields, including anatomy (histology), bacteriology, biochemistry, biology, biophysics, chemistry, environmental sciences, medicine, pharmacy, pharmacology, therapeutics and toxicology. Alphabetically, with careful searching we could probably find other fields in which cytochrome P-450 has generated excitement. Hopefully, our efforts will prove a benefit in these fields, by providing a ready source of information.

JOHN B. SCHENKMAN
and
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# CONTENTS

## Section G—METHODS OF INTEREST

# INTRODUCTION

# A BRIEF HISTORY OF CYTOCHROME P-450

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Hemoproteins seem to hold some fascination for scientists. As a group hemoproteins are one of the more heavily studied biological compounds. Perhaps this is because they are colored and readily observed. Certainly, interest was strengthened by the early observations that cellular pigments hold a key role in respiratory activity and thus in the process of life itself.

Studies on hemoglobins were carried out in the early part of 1800 while studies on tissue pigments (histohaematin) date back to early spectrophotometric analyses of MacMunn in the 1880's up through the early 1900's, when the individuality of the mitochondrial cytochromes was recognized (Keilin, 1966). The microsomes (small bodies), seen as membranous vesicles after disruption of the liver, were isolated by differential centrifugation and were named by Albert Claude (Claude, 1940). Many years passed, however, before these vesicles were shown to be derived from the endoplasmic reticulum (Palade and Siekevitz, 1956).

Early studies on the microsomal fraction revealed the presence of a protoporphyrin containing hemoprotein (Strittmatter and Ball, 1952; Yoshikawa, 1951). The properties of this protein were described and although variously named, cytochrome m (Strittmatter and Ball, 1954) and cytochrome b′ (Yoshikawa, 1951), it came to bear the name cytochrome $b_5$ (Chance and Williams, 1954).

It is of interest to follow some of the threads in the microsomal electron transfer enzyme story. Britton Chance was Professor and Director of the Johnson Foundation at the University of Pennsylvania School of Medicine, when joined by A. M. Pappenheimer, a visitor from New York University. Pappenheimer had described the presence and involvement of cytochrome $b_5$ in NADH oxidase activity in Cecropia silk worm (Pappenheimer and Williams, 1954) while at Harvard. At the Johnson Foundation the kinetics and spectroscopy of cytochrome $b_5$ in the midgut of Cecropia were described (Chance and Pappenheimer, 1954). Chance observed that the hemoprotein was the same as that found in rat liver microsomes and proceeded to study the kinetics of cytochrome $b_5$ in rat liver microsomes (Chance and Williams, 1954). Shortly thereafter David Garfinkel, then a postdoctoral fellow at the Johnson Foundation, reported on the properties of cytochrome $b_5$ isolated from rabbit liver microsomes. In his note (Garfinkel, 1956), he stated that although 50–90% of the cytochrome $b_5$ was solubilized from the microsomes, the sedimented microsomal pellet contained much of the red color of the original microsomes. Contemporary with Garfinkel at the Johnson Foundation at that time were Martin Klingenberg, a Visiting Scientist from the University of Marburg, Germany, Ryo Sato, a Visiting Professor from Osaka University Institute for Protein Research, and Ronald W. Estabrook a Research Associate.

In the initial studies on solubilization of cytochrome $b_5$, by Strittmatter and Ball (1952), 1% deoxycholate was used. Microsomal pellets after this treatment were colorless, and from the pyridine hemochrome yield of 1.45 nmoles hemes per milligram microsomal proteins, both cytochrome P-450 (P-420) and cytochrome $b_5$ must have been solubilized. Of interest was their observation that "equilibrium of a reduced solution with carbon monoxide did not cause a shift of the absorption peaks". Had such a shift been observed then, cytochrome P-450 would have been reported six years earlier than it was. From the elevated pyridine hemochrome levels, it is clear that both hemoproteins were removed

from the microsomes and were present in the extract. Similarly the lack of follow-up of the red pigment remaining in microsomes after up to 90% of the cytochrome $b_5$ was removed by lipase (Garfinkel, 1956) delayed discovery of cytochrome P-450.

Recognition of the presence of the carbon monoxide binding pigment was attributed to G. R. Williams by Klingenberg (1958) who reported that when the ratio of pyridine hemochromogen to cytochrome $b_5$ was determined for liver microsomes, a value of 2.15 was obtained, i.e., there was 1.15 nanomoles of excess protohemin. In this paper is the first published spectra and report of a carbon monoxide binding pigment in the microsomes. It was obtained on addition of carbon monoxide to microsomes reduced with either NADH or dithionite ($Na_2S_2O_4$).

In his study, Klingenberg (1958) pointed out that carbon monoxide addition compound is not stable to 2% cholate or 1% deoxycholate, which would explain why Strittmatter and Ball (1952) did not observe the pigment when they gassed the reduced deoxycholate extracts of microsomes with carbon monoxide. As was shown later (Omura and Sato, 1962; 1964b) reduced cytochrome P-420 is destroyed by oxygen, i.e., by aeration when bubbling with carbon monoxide after reduction. If the solution had been saturated with carbon monoxide first and then reduced with diethionite, perhaps cytochrome P-420 would have been seen. Less than half a year later, Garfinkel (1958) also reported on the pigment, suggesting on the basis of the binding of carbon monoxide and cyanide, that it contained a metal ion, but because the pigment had no alpha or beta peaks and showed no photodissociation of the CO complex, that it was probably not an iron hemoprotein.

Ryo Sato returned to Japan where he was studying, among other things, solubilization of the liver microsomal-xenobiotic hydroxylase. With Tsuneo Omura, Sato began investigating the carbon monoxide binding pigment in 1960 (Sato and Omura, 1978), and by 1962 had obtained evidence that the pigment was a protoheme cytochrome (Omura and Sato, 1962). In that report the pigment was, "provisionally called cytochrome P-450, a new cytochrome of unusual properties". Omura and Sato began attempts to fractionate and solubilize the microsomal hemoproteins, and concluded cytochrome $b_5$ and cytochrome P-450 together account for the total microsomal heme content (Omura and Sato, 1963). However, it wasn't until 1968, that successful separation and solubilization of cytochrome P-450 was achieved (Lu *et al.*, 1968).

Reports from several laboratories rapidly confirmed earlier observations by Sladek and Mannering (1966) that 3-methylcholanthrene induces a new form of cytochrome P-450. Suggestions soon appeared that many isozymes of the hemoprotein exist (Welton and Aust, 1974; Welton *et al.*, 1975; Haugen *et al.*, 1975; Thomas *et al.*, 1976).

Like Klingenberg (1958), Omura and Sato (1964a) were unable to obtain photodissociation of the CO complex of cytochrome P-450. The earliest measurement of photodissociation was of the dithionite reduced CO complex of clarified cytochrome P-450 (Omura *et al.*, 1965), in a collaboration with Q. Gibson at the Johnson Foundation, where photodissociation was obtained. It was not clear whether this was with the liver microsomal or the adrenal cortical mitochondrial cytochrome P-450 (11-$\beta$-hydroxylase), since in that report several systems were studied.

In 1964 Omura joined Estabrook, Rosenthal and Cooper at the Johnson Foundation and the Harrison Department of Surgical Research of the University of Pennsylvania (Cooper, 1973). Cooper had been studying the adrenal cortical microsomal steroid hydroxylase and was having difficulty in obtaining a good stoichiometry for the reaction. He joined forces with Ronald W. Estabrook then an Assistant Professor at the Johnson Foundation for Medical Physics, who had just developed a new sensitive fluorimetric assay for pyridine nucleotides. This was the first step of a successful collaboration, and answered the question of the stoichiometry of the $C_{21}$ steroid hydroxylase (Cooper *et al.*, 1963). By 1963 their collaboration led to testing the light reversibility of the carbon monoxide inhibition of $C_{21}$-hydroxylation of 17-hydroxy progesterone by adrenocortical microsomes (Estabrook *et al.*, 1963). Together they showed a maximal degree of reversibility by light of 450 nm of the carbon monoxide inhibition of cortexolone formation,

and concluded, "these results support the hypothesis that the 450 mu CO compound observed spectrophotometrically functions in oxygen activation for the hydroxylase reaction". The photochemical action spectrum first generated by Estabrook *et al.* (1963) was obtained from carbon monoxide inhibited adrenal cortex microsomes. Later, similar photochemical action spectra were obtained with liver microsomes for codeine metabolism (Cooper *et al.*, 1965a).

In December of 1964 I joined the laboratory of Ronald Estabrook at the Johnson Foundation, then the Department of Biophysics and Physical Biochemistry of the University of Pennsylvania School of Medicine. My stay there overlapped by about a year that of Omura, who subsequently left to work with Philip Siekevitz at the Rockefeller Institute. Estabrook and I examined the effect of drug substrates on the EPR and UV-visible spectrophotometric properties of the mixed function oxidase. Shakunthala Narasimhulu, a Research Associate with David Y. Cooper for a few years, had been studying the properties of the adrenal cortical microsomal mixed function oxidase. She observed (Narasimhulu *et al.*, 1965; Cooper *et al.*, 1965b) that steroid substrate addition caused a change in the microsomal difference spectrum which was substrate dependent. These spectral changes were characterized by a drop in absorbance at 420 nm and a peak at 390 nm. An important observation was also made that the substrate, when added, enhances electron flow from TPNH to cytochrome P-450 in the adrenal microsomes (Narasimhulu *et al.*, 1965). While examining the effect of drug substrates of the monooxygenase in liver microsomes, we observed similar spectral changes. At Estabrook's invitation, Henry Sasame and James R. Gillette visited the Johnson Foundation for a brain session of the meaning of the spectral changes. Also present was Herbert Remmer, then a visiting Professor from Tübingen, Germany. Working late into the night a consensus conclusion was reached that the substrate induced spectral changes were due to formation of an enzyme-substrate complex; a report was sent to Molecular Pharmacology. In that short communication, (Remmer *et al.*, 1966) all bases were touched: Substrates were suggested as substituting for a ligand of the heme, and the two types of spectral changes seen were considered as two types of conformational change, perhaps by ligand binding to opposite sides of the heme. An alternative suggestion similar to that of Narasimhulu *et al.*, 1965 was also considered, involving conversion of an oxygenated form of cytochrome P-450 to an oxidized form (an analogy with peroxidases). Similar observations were made by Imai and Sato (1966) of spectral changes on addition of substrates to rabbit liver microsomes. The spectral changes were later named Type I and Type II spectral changes (Schenkman *et al.*, 1967).

At about the same time cytochrome P-450 was also generating excitement on the international scene. In Germany, Remmer had shown barbiturates to induce the microsomal drug oxidase (Remmer, 1959). In Sweden, Ernster's group showed induction to be related to elevation of the microsomal content of cytochrome P-450 (Orrenius *et al.*, 1965). In Australia, Appleby had isolated a soluble form of cytochrome P-450 from Rhysobium japonicum (Appleby, 1967). In the U.S., cytochrome P-450 was shown to resemble a low spin hemoprotein by electron spin resonance (ESR) spectroscopy (Hashimoto *et al.*, 1962; Mason *et al.*, 1965). With pseudomonas cytochrome P-450 a Type I substrate (camphor) was shown to decrease the amount of low spin cytochrome ESR signal (Gunsalus, 1968). In Japan (Mitani and Horie, 1969) and in the U.S. (Whysner *et al.*, 1969) investigators showed the substrate induced type I spectral change was related to spin state changes of the cytochrome.

Today the excitement in research on cytochrome P-450 shows no signs of abating. Many more researchers in many other countries, too numerous to mention here, have joined in the study on the properties of the cytochrome. From early pharmacological studies on drug metabolism *in vivo*, the field moved to biochemical studies on the enzyme kinetics, through enzyme purification and system reconstitution to current investigations on the thermodynamics of P-450. One can easily see there are many fruitful years of research left on this hemoprotein. As newer techniques are applied to it an even greater understanding of its properties and functions will be obtained.