Microsomal Drug Oxidation

from a black box to a well understood multi-enzyme system

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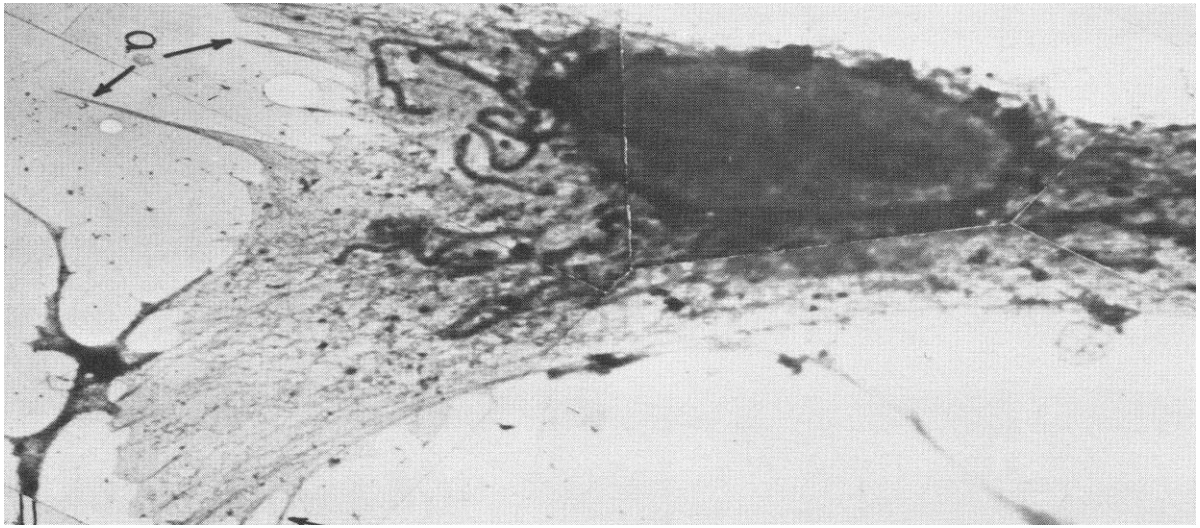
Discovery of "Microsomes"

Fractionation of liver homogenates with an ultracentrifuge yielded a novel fraction consisting of very small particles. It was named "Microsomes" by Claude (1943).

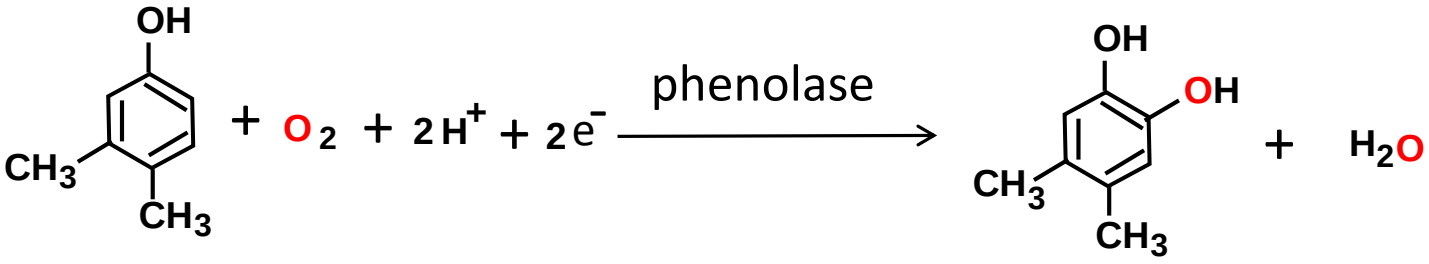
Electron microscopic observation of cultured animal cells revealed an extensive network of membrane in the cytoplasm (Porter, Claude, & Fullam: 1945).
It was named "Endoplasmic reticulum".



Biochemical and morphological studies of the microsomal fraction prepared from animal livers confirmed that the fraction consisted of the fragmented vesicles of endoplasmic reticulum (Palade & Siekevitz: 1956).

Discovery of "Oxygen transferase" reaction

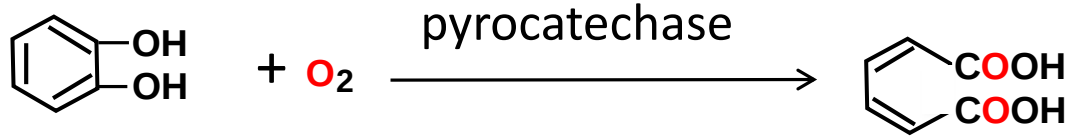
Mason, Fowlks, & Peterson (1955) discovered the incorporation of oxygen atom from atmospheric molecular oxygen into the substrate molecule by the catalysis of the phenolase of a fungus, and concluded it is an oxygen transnsferase reaction.



3,4-dimethyl phenol

4,5-dimethyl catechol

Hayaishi, Katagiri, & Rothberg (1955) also discovered the incorporation of oxygen atoms from atmospheric oxygen into the substrate molecule by the catalysis of the pyrocatechase of a bacterium. It was also an oxygen transferase reaction.

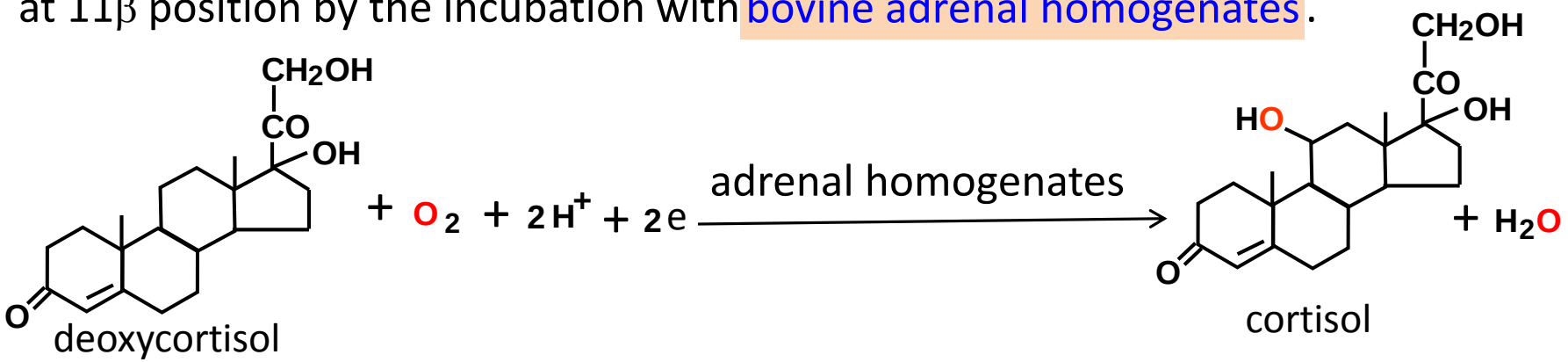


catechol

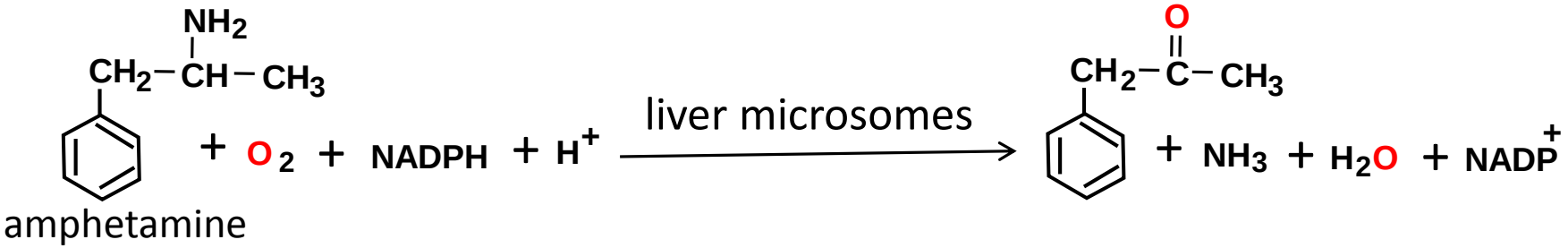
cis-cis-muconic acid

Oxygen transferase reactions in steroid hormone biosynthesis and drug metabolism in animal tissues

Hayano, Lindberg, Dorfman, Hancock, & Doering (1955) found the incorporation of oxygen atom from atmospheric oxygen into deoxycortisol when it was hydroxylated at 11β position by the incubation with bovine adrenal homogenates.



Axelrod (1955) examined the oxidative deamination of amphetamine by rabbit liver microsomes, and confirmed the need for molecular oxygen and NADPH. A few other drugs were also oxidized by liver microsomes in the same way.



Soluble and membrane-bound Oxygen transferases

Many oxygen transferases were found in 1955 and in the following years. The fungal phenolase and the bacterial pyrocatechase were soluble, and were purified easily. On the other hand, the steroid hydroxylating activities of adrenal homogenates were in microsomes and mitochondria. The drug oxidation activity was in microsomes. They were membrane-bound, and resisted to various solubilization treatments.

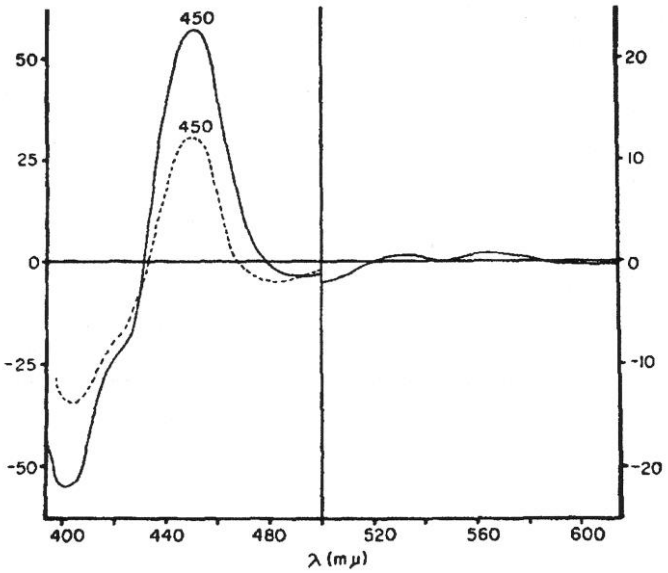
Since the steroid hormone biosynthesis in gonads and the drug oxidation in liver are highly important metabolic activities, many biochemists tried hard in the latter half of the 1950s to purify the enzymes involved in these metabolic activities. However, all attempts to solubilize and purify these metabolic activities failed. The activities were completely lost when detergents were added to the mitochondria or microsomes obtained from animal tissues.

Inhibition of steroid hydroxylation and drug oxidation activities of microsomes and mitochodria by several chemical reagents (sodium azide, carbon monoxide, ...) suggested the role of a heavy metal in these oxygen transferase reactions, but the identification of the metal was not possible without the purification of the enzymes.

Discovery of "CO-binding Pigment" in liver microsomes

Many enzyme activities were found in the microsomes prepared from animal livers in the 1950s. Presence of a few electron-transport enzymes was found : NADPH-cytochrome c reductase activity (Horecker : 1950), cytochrome b5 (Strittmatter & Ball : 1951), NADH-cytochrome c reductase activity (Strittmatter & Velick : 1956). However, the physiological functions of these electron-transport enzymes were unknown.

Klingenberg (1958) found a curious novel pigment in rat liver microsomes. When carbon monoxide was added to NADH- or NADPH-reduced microsomes a big optical absorption peak appeared at 450 nm. The absorption peak disappeared when detergents were added to the suspension of the microsomes. The molecular nature of this novel "CO-binding Pigment" was not elucidated.

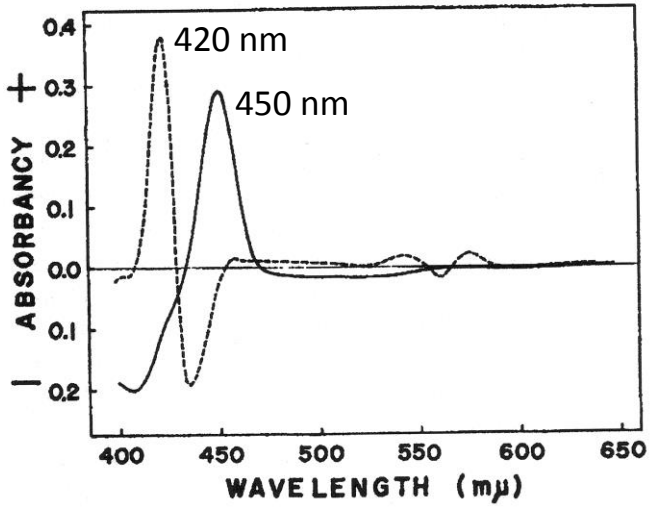


CO-difference spectra of NADH (----)- and NADPH (—)-reduced rat liver microsomes. (Klingenberg)

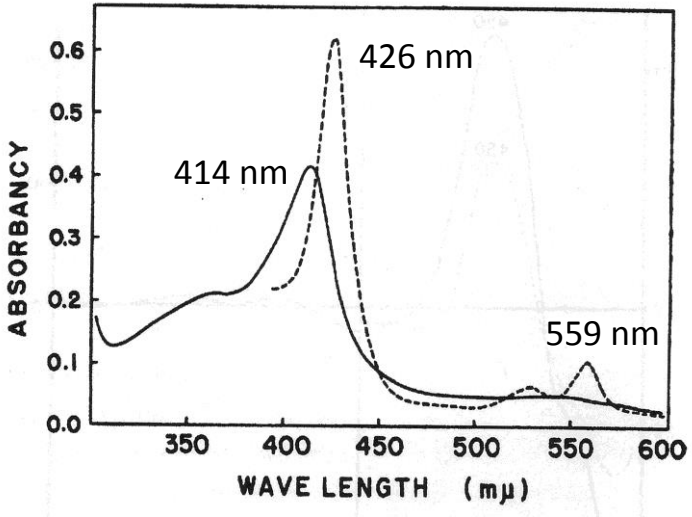
Cytochrome P450

Omura & Sato (1962) examined the properties of "CO-binding pigment" of rabbit liver microsomes, and confirmed the hemoprotein nature of the pigment.

When deoxycholate was added to the suspension of microsomes, the 450 nm peak of CO-binding pigment disappeared, but a new prominent peak appeared at 420 nm. The conversion of the 450 nm form (P450) of CO-binding pigment to the 420 nm form (P420) was confirmed by the time course of the conversion. The solubilized P420 was purified, and its absorption spectra looked like those of a *b*-type cytochrome.



CO-difference spectra of microsomes (——) and deoxycholate-solubilized microsomes (-----).



Absorption spectra of oxidized (——) and reduced (-----) forms of P420.

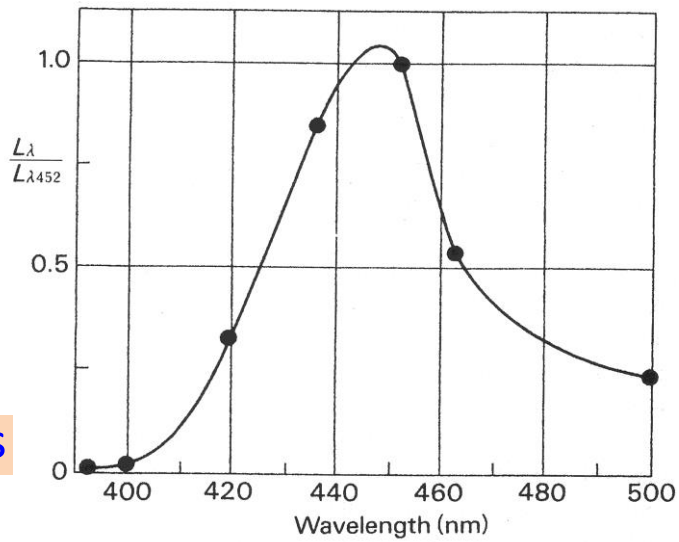
Discovery of the enzymatic activity of Cytochrome P450

The function of cytochrome P450 was discovered by Estabrook, Cooper & Rosenthal In 1963.

Inhibition of the steroid hydroxylase activity of adrenal cortex microsomes by carbon monoxide, and its reversal by the irradiation with white light had been reported by Ryan and Engel in 1957. Estabrook, Cooper & Rosenthal examined the wave length dependency of the photoreversal of the carbon monoxide inhibition of C-21 hydroxylation of 17-hydroxyprogesterone. The photochemical action spectrum of the light-reversal of the carbon monoxide inhibition of the steroid hydroxylase was very similar to the carbon monoxide-difference spectrum of P450.

P450 is steroid hydroxlyase.

Cooper, Levin, Narasimhulu, Rosenthal & Estabrook confirmed the role of P450 in the oxidation of drugs (acetanilide, codeine,….) by liver microsomes using the same photochemical action spectrum method in 1965.



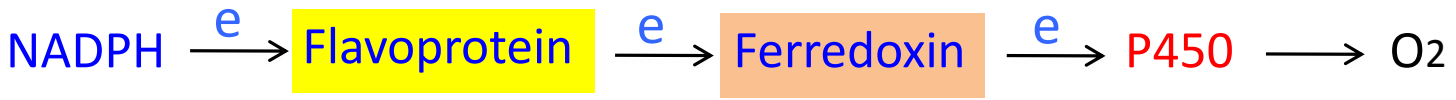
Photochemical action spectrum for the light reversal of CO-inhibition of steroid C-21 hydroxylation.

Analysis of the P450-dependent Steroid hydroxylation system

P450-dependent steroid hydroxylation in adrenal gland requires NADPH and O_2. Since the oxygen molecule must bind to the reduced iron atom of the heme of P450, supply of electrons from NADPH to P450 was the problem to be clarified.

Omura, Sanders, Estabrook, Cooper & Rosenthal (1966) sonicated adrenal cortex mitochondria, and found that the P450 in the sonicated sample was reduced with NADPH, and catalyzed the steroids hydroxylation. When the sample was centrifuged, neither the sedimented pellet containing P450 nor the supernatant catalyzed the hydroxylation reaction, and the P450 in the pellet was no longer reduced by NADPH. Addition of the supernatant to the pellet restored steroid hydroxylation activity and the reduction of P450 by NADPH. The supernatant contained the activity to transfer electrons from NADPH to P450.

The supernatant was analyzed, and a ferredoxin-type iron-sulfur protein and an FAD-flavoprotein were purified. The addition of these two proteins to the P450-containing membrane fraction restored the reduction of P450 by NADPH concomitant with the steroid hydroxylation activity. This was the first elucidation of a P450 enzyme system.



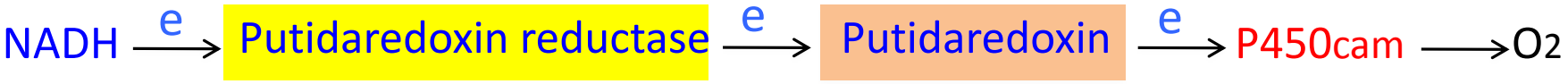
Suzuki & Kimura (1965) also purified a ferredoxin from adrenal cortex mitochondria, and named it "Adrenodoxin"

Wide distribution of Cytochrome P450 among eukaryotes and prokaryotes

P450 was first found in the tissues of animals, but wide distribution of P450 among various eukaryotes and prokaryotes was soon discovered.

Presence of P450 in the microsomes of the yeast *Saccharomyces cerevisiae* was discovered by Lindenmayer & Smith in 1964. A soluble P450 was found in the bacteroids of nitrogen-fixing *Rhizobium*, and partially purified by Appleby in 1967. Another soluble P450 was found in the bacterium *Pseudomonas putida* by Katagiri, Ganguli & Gunsalus in 1968.

The soluble P450 of *Pseudomonas putida* was purified, and was named P450cam. It catalyzed NADH-dependent hydroxylation of camphor. Katagiri, Ganguli & Gunsalus analyzed the hydroxylation system, and found a flavoprotein and a ferredoxin transfer electrons from NADH to P450cam in the catalysis of the hydroxylation reaction. The ferredoxin was named "putidaredoxin". This enzyme system was remarkably similar to the steroid hydroxylation system of animal adrenal cortex mitochondria.



Induction of drug oxidation activities by drugs and chemicals

Induction of the drug oxidation activities of the liver of animals by the administration of some drugs or chemical compounds was discovered in the middle of the 1950s.

Conney, Miller & Miller (1956) : Stimulation of the oxidative N-demethylation of 3-methy-4-methylaminoazobennzene by rat liver homogenates by intraperitoneal injection of 3-methylcholanthrene

Conney, Miller & Miller (1957) : Induction of liver microsomal NADPH-dependent benzopyrene hydroxylase by intraperitoneal injection of benzopyrene and some other polycyclic aromatic hydrocarbons.

Barbitrates were found to increase the microsomal drug-metabolizing activity in the liver. Remmer (1959), Conney, Davidson, Gastel & Burns (1960), Kato (1960).

Orrenius, Ericsson & Ernster (1965) found that the phenobarbital-induced increase of oxidative aminopyrene N-demethylation activity in rat liver was accompanied by parallel increases of microsomal P450 and NADPH-cytochrome c reductase activity.

Symposium "Electron Transport Systems in Microsomes"

A symposium titled "Electron Transport Systems in Microsomes" was held at the "Annual Meeting of the Federation for American Societies of Experimental Biology" in 1965. This was the first meeting on the electron-transport enzymes of microsomes. The organizer of the symposium was Phillip Siekevitz. The lectures given at the symposium were as follows.

P. Siekevitz: Origin and functional nature of microsomes.

T. Omura and R.W.Estabrook: Function of cytochrome P-450 of microsomes.

P. Strittmatter: Microsomal cytochrome b_5 and NADH-cytochrome b_5 reductase.

H. Kamin: Microsomal NADPH-cytochrome c reductase.

H.S. Mason: Metabolism of "Xenobiotics" by microsomal mixed-function oxidase.

L. Ernster: Phenobarbital-induced increase of microsomal drug oxidation activity in rat liver.

The terms "Xenobiotics" and "Mixed-function oxidase" were proposed by Mason.

First meeting on "Microsomes and Drug Oxidations"

An international symposium "Microsomes and Drug Oxidations" was held in February, 1968, in Bethesda, USA.

Organizers of the Symposium:

Allan H. Conney, George J. Cosmides, Ronald W. Estabrook, James R. Fouts, James R. Gillette, Gilbert J. Mannering

Sessions of the Symposium

Morphological and biochemical characteristics of microsomes.
Electron transfer components
Alterations of microsomal enzymes
Drugs and protein synthesis

Participants

49 participants from five countries.
Belgium 3, Germany 3, Japan 2, Sweden 1, USA 40,

Substrate-induced spectral changes of Cytochrome P450

P450-catalyzed oxidation of a substrate requires one mole each of NADPH and O_2. Substrate (SH) binds to P450. Two electrons are supplied from NADPH to P450. Molecular oxygen must bind to the reduced heme of P450 to be activated.

$$SH + NADPH + O_2 + H^+ \xrightarrow{P450} S\text{-}OH + NADP^+ + H_2O$$

Binding of a substrate molecule to the oxidized form of P450 was suggested by an observation of a substrate-induced spectral change of the P450 of adrenal cortex mitochondria in 1965 (Cooper, Narasimhulu, Raich, Foroff, & Rosenthal).

Drugs also induced the spectral changes of oxidized microsomal P450 (Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper, & Rosenthal : 1966), (Imai & Sato :1966) suggesting the binding of substrate drugs to the oxidized form of P450. Two types of spectral changes, type 1 and type 2, were confirmed (Schenkman, Remmer, & Estabrook : 1967)

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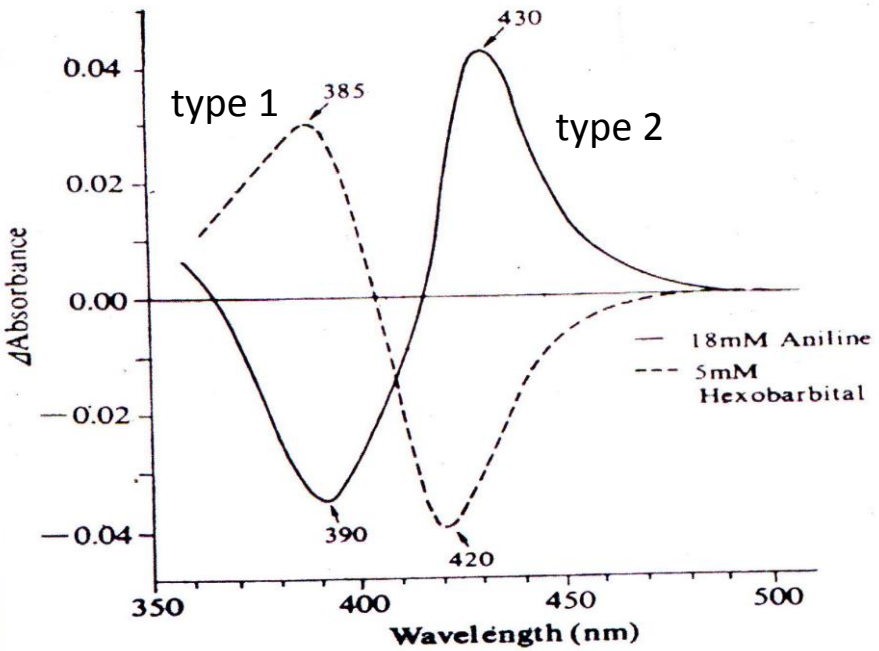


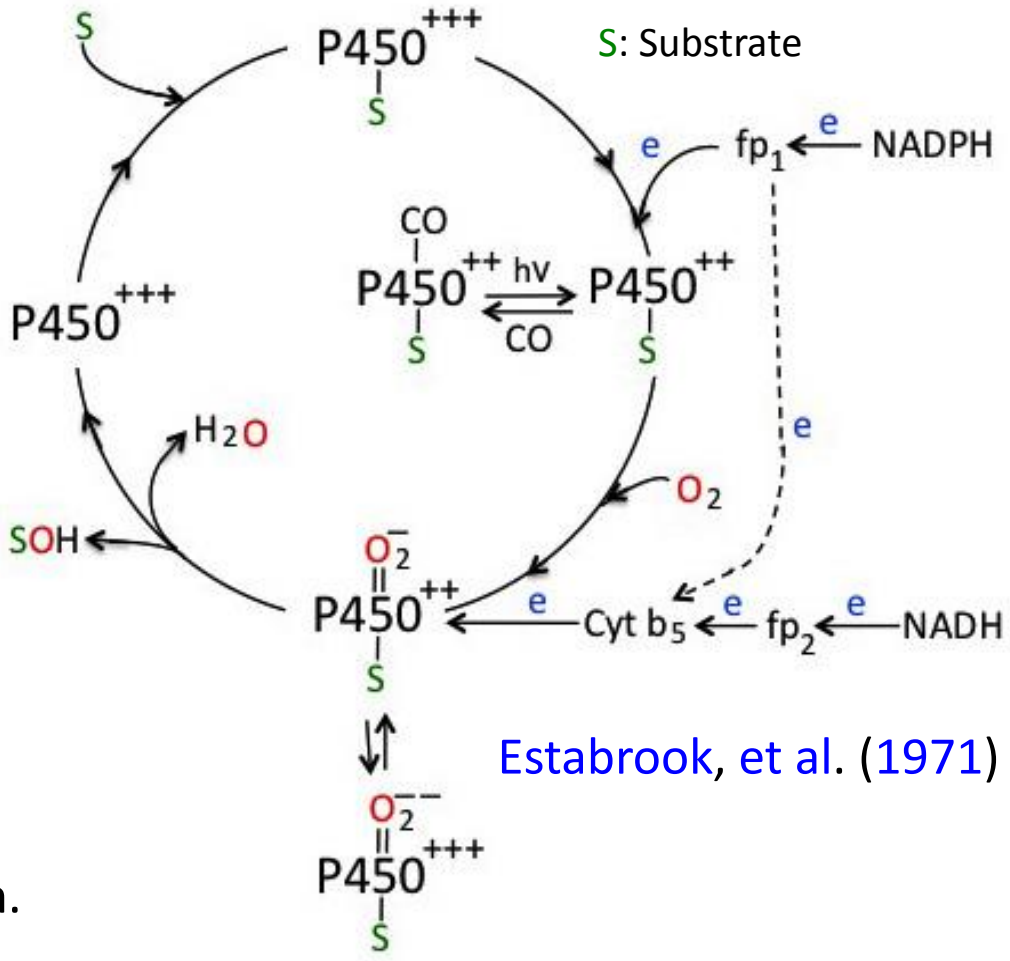
Substrate-induced difference spectra of microsomal P450 (Schenkman, et al.)

Proposal of a cyclic reaction mechanism for P450-catalyzed reaction

Spectral evidence for the oxygenated form of reduced P450 was reported for liver microsomal P450 by Estabrook, Hildebrandt, Baron, Netter, & Leibman (1971) and for a bacterial P450, P450cam, by Ishimura, Ullrich, & Peterson (1971).

Based on this information, a cyclic reaction mechanism for the P450-catalyzed oxygen atom insertion into a substrate molecule was proposed by Estabrook, et al. in 1971.

Supply of two electrons to P450 from NAD(P)H at different points in a cycle of the reaction was assumed. The role of cytochrome b5 in the supply of the second electron was inferred from a change in the reduction level of cytochrome b5 during P450-catalyzed drug oxidation.

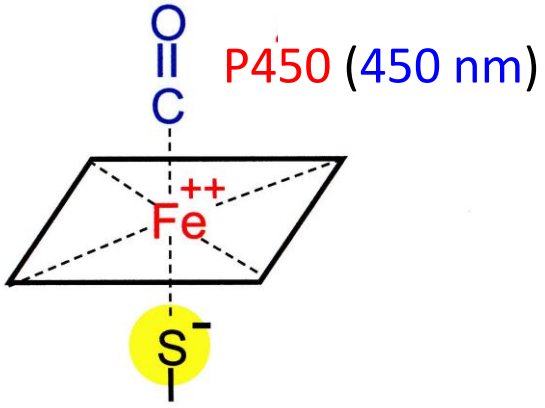


Estabrook, et al. (1971)

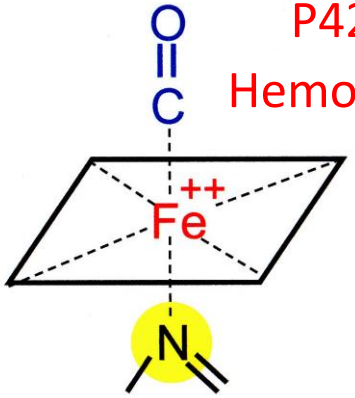
Unique ligand of the heme of Cytochrome P450

The carbon monoxide compound of cytochrome P450 showed a Soret peak at 450 nm, whereas the CO-compounds of other known protoheme hemoproteins that had an imidazole group of histidine at 5th co-ordination position of the heme showed their Soret peaks at around 420 nm. The big red shift of the Soret peak of P450 suggested a unique co-ordination state of the heme of P450.

Stern & Peisach (1974) presented strong evidence for the thiolate anion donated by a cysteine residue of the P450 protein as the ligand trans to carbon monoxide in the CO-compound of reduced P450 by a model compound study. Addition of mercato-ethanol to an alkaline solution of hemin produced 450 nm optical absorption peak when carbon monoxide was added. The axial 5 th ligand of P450 is thiolate anion.



P450 (450 nm)



P420 (420 nm)

Hemoglobin (420 nm)

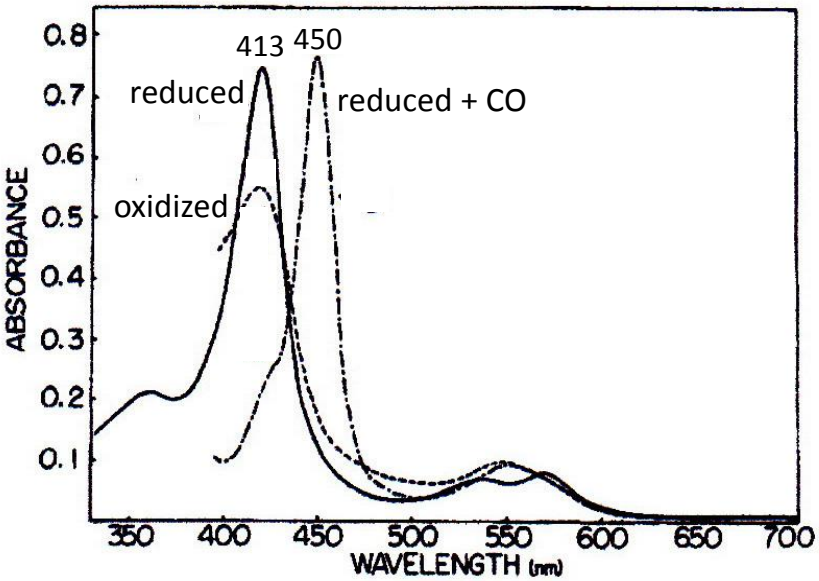
P450 was the first heme-thiolate protein found in nature. But later studies found many hemoproteins with thiolate anion ligand including nitric oxide synthase.

Purification of microsomal and mitochonrial Cytochrome P450

Although soluble P450s of bacteria were easily purified, solubilization and purification of membrane-bound P450s of microsomes and mitochondria were very difficult. The problem was the denaturation of P450 to P420 upon solubilization treatments.

Ichikawa and Yamano (1967) made a breakthrough discovery that high concentrations of glycerol stabilized membrane-bound P450 against the solubilization treatments with detergents. This finding opened the way to successful solubilization and purification of many forms of P450 from microsomes and mitochondria in the middle of the 1970s: Van der Hoeven, Haugen, & Coon (1974), Imai & Sato (1974), Ryan, Lu, Kawalek, West, & Levin (1975), Takemori, Suhara, Hashimoto, Hashimoto, Sato, Gomi, Katagiri (1975), West, & Lu (1976),…….

Purification of P450 enabled the elucidation of molecular properties of P450. The optical absorption spectra of P450 were measured. Differences in the catalytic activities of various P450s were confirmed by using purified P450 preparations.

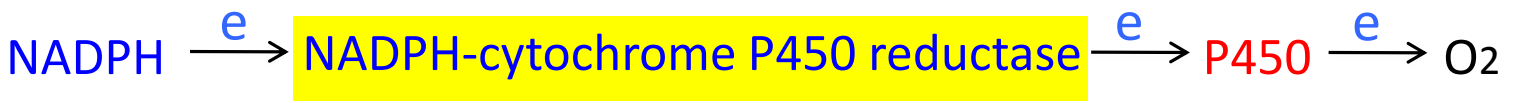


Absorption spectra of purified P450. (Imai & Sato)

Constitution of microsomal P450 enzyme system

Lu and Coon (1968) reported successful solubilization of the P450-catalyzed fatty acid ω-hydroxylation activity from rabbit liver microsomes by deoxycholate in the presence of glycerol and DTT, and separated the solubilyzed hydroxylase into three fractions: a P450-containing fraction, an NADPH-cytochrome c reductase-containing fraction, and a lipid factor. The three fractions were needed for the reconstitution of the hydoxylase activity. However, Miyake, Gaylor, & Mason reported in the same year that a purified NADPH-cytochrome c reductase preparation solubilized from microsomes by trypsin was unable to reconstitute the hydroxylase activity when added to the P450-containing fraction.

This puzzling problem was solved by the purification of detergent-solubilized NADPH-cytochrome c reductase from liver microsomes (Ichihara, Kusunose & Kusunose, 1973). The purified reductase reconstituted fatty acid ω-hydroxylase activity when added to a partially purified P450. Similar studies by several other groups confirmed the role of NADPH-cytochronme c reductase in the microsomal P450-catalyzed reactions, and the reductase was renamed NADPH-cytochrome P450 reductase.



The constitution of microsomal drug-oxidation system was elucidated!

Elucidation of the amino acid sequences of various P450s

The first complete amino acid sequences of two P450s were elucidated in 1982: a soluble bacterial P450, P450cam, by chemical sequencing by Haniu, Armes, Tanaka, Yasunobu, Shastry, Wagner, & Gunsalus, and a phenobarbital-inducible liver microsomal P450 by cDNA cloning by Fujii-Kuriyama, Mizukami, Kawajiri, Sogawa, & Muramatsu.

Elucidation of the amino acid sequences of P450s by cDNA cloning was more expedient than the chemical sequencing method. The total amino acid sequences of many P450s were elucidated in the following years, and enabled classification of the P450s based on the comparison of their amino acid sequences. Nebert et al. proposed in 1987 to classify P450s into Families and Subfamilies based on the similarities of their amino acid sequences. The symbol "*cyp*" for P450 genes was also proposed. This classification was widely accepted by P450 scientists.

Purification of various microsomal and mitochondrial P450s, and reconstitution of P450-catalyzed reaction systems elucidated the roles of various P450s in many important metabolic activities in human and some vertebrate animals. Tissue-specific expression of many P450s was confirmed. Researches on P450 expanded into various areas in medical science.

Elucidation of the structures of the genes of various P450s

The nucleotide sequences and the structures of many P450 genes were actively studied in the 1980s and the 1990s. The molecular mechanism of the regulation of the expression of various P450 genes were the major subject of research. The induction of liver microsomal drug-metabolizing activities by certain chemical compounds were explained by stimulated expression of particular P450 genes.

Examination of the nucleotide sequences of various P450 genes revealed the nucleotide polymorphism in the coding regions of many P450 genes. In the drug-metabolizing P450s, the problem of "poor metabolizers" of Debrisoquine was explained by a single-nucleotide polymorphism of the gene of microsomal P450 2D6 (Meyer, et al. : 1990). The single nucleotide polymorphism of drug-metabolizing P450s became an important subject of study in pharmacology.

Discovery of the role of microsomal P450s in chemical carcinogenesis was another Important development in P450 research in the 1970s. Chemically stable polycyclic aromatic hydrocarbons like benzopyrene were found to be activated by P450-catalyzed reactions to the DNA-binding epoxides. Activation of various "carcinogenic compounds" by particular microsomal P450s was confirmed.

Roles of Cytochrome P450 in many important metabolic activities in animals

Oxidative metabolism of various xenobiotics including drugs in liver, intestine, and some other tissues.

Activation of carcinogenic compounds in chemical carcinogenesis.

Synthesis of cholesterol from squalene in the liver.

Synthesis of endocline steroid hormones in adrenal gland and gonads.

Synthesis of local steroid hormones in various tissues including brain.

Inactivation of steroid hormones by hydroxylation reactions.

Synthesis of bile acids from cholesterol in the liver.

Synthesis of eicosanoids from arachidonic acid in various tissues.

Synthesis of a few prostaglandins (prostacyclin, thromboxane).

ω-Hydroxylation of fatty acids.

Conversion of vitamin D_3 to active 1,25-dihydroxyvitamin D_3 in liver and kidney.

Inactivation of prostaglandins and leukotrienes.

Inactivation of vitamin E, melatonin, retinoic acid, ……..

Metabolism of xenobiotics by insects, plants, fungi, and bacteria

Many P450s were found in plants and fungi, and their contribution to the synthesis of various toxic secondary metabolites, phytoallexins, to prevent the predation by herbivorous insects were elucidated. Insects developed resistance to phytoallexins in order to survive. The major mechanism of the resistance is P450-catalyzed detoxication of plant toxins. This struggle between plants and herbivorous insects is called "chemical warfare" or "arms race", and the major weapons on both sides are various P450s. Chemical warfare is also going on in the soil among many fungi and bacteria. They fight one another by producing "antibiotics" to outrival others.

Recent extensive use of chemical insecticides and herbicides in agriculture resulted in the emergence of "resistant" weeds and insects. Various mechanisms of resistance are utilized by plants and insects, and the major mechanism is detoxication of various insecticides and herbicides by P450-catalyzed reactions. Microbes in the soil degrade insecticides and fungicides. Research on P450 expanded to agricultural science.

Discovery of the roles of P450 in the synthesis of many plant secondary metabolites of complex molecular structures attracted the attention of organic chemists. Since many plant secondary metabolites are utilized as expensive medicines, possibility of the use of plant P450s for industrial production of various plant secondary metabolites is being explored. P450 is becoming a useful tool in biotechnology.

Published papers on Cytochrome P450 ("Cytochrome P450 topic" in *Web of Science*)

Areas of Research

	number of papers (%)		
Year	2000	2010	2015
Total	1,708 (100.0 %)	2,455 (100.0 %)	2,607 (100.0 %)
Pharmacology	896 (52.5 %)	1,290 (52.5 %)	1,246 (47.8 %)
Medicine	337 (19.7 %)	519 (21.1 %)	542 (20.8 %)
Biochemistry	220 (12.8 %)	252 (10.3 %)	284 (10.9 %)
Biology	144 (8.4 %)	213 (8.7 %)	270 (10.4 %)
Agriculture	27 (1.6 %)	69 (2.8 %)	125 (4.8 %)
Environment	47 (2.8 %)	43 (1.8 %)	54 (2.1 %)
Biotechnology	37 (2.2 %)	69 (2.8 %)	86 (3.3 %)

Published papers on Cytochrome P450 ("Cytochrome P450 topic" in *Web of Science*)

Substrates of P450s utilized in Research

	number of papers (%)		
Year	2000	2010	2015
Total	1,713 (100.0 %)	2,433 (100.0 %)	2,590 (100.0 %)
Xenobiotics including drugs	1,321 (77.1 %)	1,921 (79.0 %)	1,982 (76.5 %)
Endogenous substrates (fatty acids, sterols, …)	361 (21.1 %)	442 (18.2 %)	471 (18.2 %)
Synthesis of natural compounds	31 (1.8 %)	70 (2.9 %)	145 (5.6 %)