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Title Page

The development of drug metabolism research as expressed in the publications of ASPET

Part 2-1959-1983

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Running title page

Drug Metabolism Research in ASPET 1959-1983

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Abstract

In 25 years drug metabolism research went from using sub cellular particles of undefined content to an understanding of metabolism at the molecular level. The discovery of cytochrome P450, enzyme induction, reactive intermediates, and genetic polymorphisms provided milestones in the field. New publications from ASPET chronicled the discoveries and provided communications to advance the science of drug metabolism.

This 25-year period in the development of drug metabolism research involved a greater expansion of knowledge than in all of the previous history of the field. Moving from the subcellular level to the molecular level involved a number of significant discoveries and the development of exciting new technologies. Recognition of the changing science was reflected in two new publications from the American Society for Pharmacology and Experimental Therapeutics (ASPET). Molecular Pharmacology was launched in 1965 focusing on research at the enzyme and receptor level. Avram Goldstein was the founder of this journal and editor for its first three years. In 1973 ASPET launched Drug Metabolism and Disposition (DMD) under the editorship of Ken Leibman. Ken served the society well, emphasizing scientific scholarship and diversity of ideas. He even provided short summaries of articles published in other journals when he thought they might be of interest. During his 10 years as editor, DMD became the premier publication in the field. In 1978, ASPET introduced the Bernard B. Brodie Award to "recognize outstanding original research contributions in drug metabolism and disposition". The recipients of this biennial award represent some of the most outstanding contributors to the progress of drug metabolism science.

As 1959 began, it was clear that the microsomal fraction of the homogenized liver cell consisted mainly of fragments of the endoplasmic reticulum (Palade, 1956)[1]. This fraction contained both drug metabolizing activity and a unique red pigment that had some of the properties of a cytochrome (Murphy, 2008). Two major discoveries in the

early 60s shaped the nature of research for the next 50 years. The discovery of P450 eventually led to an understanding of the enzymes involved in oxidative drug metabolism and the nature of the catalytic reaction. The discovery of the inducibility of those enzymes initiated the concept of the existence of multiple hepatic P450s and led to the research efforts aimed at deciphering the controlling mechanisms in DNA expression linked to drug metabolizing enzymes.

Discovery of P450

The discovery of P450 and its function evolved from observations by Ryan and Engel that C-21 hydroxylations of progesterone and hydroxylated progesterones were catalyzed by a CO inhibitable enzyme in the adrenal cortex (Ryan, 1957). They characterized the reaction as belonging to the class of enzymes categorized by Mason as “mixed-function oxidases” (Mason, 1957) and by Hayaishi as “monoxygenases” (Hayaishi, 1962). These enzymes required molecular oxygen and a reductant such as NADPH. While Ryan and Engel thought a cytochrome in the adrenal microsomes might be involved in the reaction, their methods did not permit measurement of the pigment in its active state. Meanwhile the pigment in liver microsomes which had been shown to bind CO was isolated and characterized by Omura and Sato as a cytochrome, referred to as Cytochrome P450 (Omura, 1962). The story of the experiments characterizing the microsomal cytochrome as the terminal oxidase for mixed function oxidases is best told by the participants. David Cooper has detailed the meandering path that led him from his role as a surgical resident to his interest in the response of animals to stress, and to the

studies on the biosynthesis of steroids in the adrenal (Cooper, 1973). Collaborating with Otto Rosenthal, Cooper was frustrated in his attempts to define the stoichiometry of the C-21 hydroxylation and shared his efforts with Ron Estabrook. Estabrook invited Cooper to the Johnson Research Foundation for what proved to be an exciting and productive collaboration. They solved the problem of the exact stoichiometry of the reaction (Cooper et al., 1963) and went on to study the nature of the CO inhibitable reaction. In his detailed and enjoyable reminiscences presented on the occasion of his Brodie award, Ron Estabrook[2] has paid particular homage to the advances in instrumentation that allowed them to perform their groundbreaking experiments (Estabrook, 2003). The photochemical action spectrum of the CO inhibited adrenal microsomes proved that P450 was the oxygen carrier for this class of reactions that included both steroid hydroxylation and the oxidation of a multitude of drugs and other xenobiotics. Their landmark papers provided the foundation for studies of P450 related reactions involved in an ever-expanding list of transformations (Estabrook, 1963; Cooper, 1965).

Induction of metabolizing enzymes

The second major breakthrough regarding microsomal metabolism came with the observations by Alan Conney in the US and H. Remmer and co-workers in Germany that the microsomal activity could be stimulated by pre-administration of various xenobiotics. Conney, working with the James and Elizabeth Miller, found that polycyclic hydrocarbons could markedly increase the N-demethylation of the aminoazo dye 3-methyl-4-monomethylaminoazobenzene (Conney, 1956). Subsequent studies showed

increases in drug metabolizing enzymes following induction by phenobarbital or 3,4 benzpyrene (Conney, 1960). Remmer and Merker also showed changes in enzyme activity and the endoplasmic reticulum in rabbits after treatment with phenobarbital and other drugs (Remmer, 1963). These discoveries stimulated the field and by the time Conney wrote his classic review on induction, over 200 compounds had been shown to cause induction of the microsomal enzymes (Conney, 1967). Studies on the mechanisms of induction were initiated in this period. Poland and co-workers found an hepatic cytosol component that bound 2,3,7,8-tetrachlorodibenzo-p-dioxin and other polycyclic hydrocarbons and suggested that it was the receptor for induction of aryl hydrocarbon hydroxylase (Poland, 1976). A summary of the advances was presented by Bresnick et al. in their 1984 review (Bresnick, 1984). Induction by both 3-methylcholanthrene (3-MC) and phenobarbital had been shown to involve increases in protein synthesis, RNA polymerase isozymes and nuclear RNA precursors. The field was poised for breakthroughs in not only the understanding of P450 but also the factors controlling cellular activity at the most basic levels.

Progress with P450

As the list of structurally diverse substrates for cytochrome P450 catalyzed oxidations grew, investigators became perplexed by the broad specificity of the enzyme system. The inability to free the enzyme system from the ER membrane without loss of activity severely hampered progress. While the finding that a soluble, active P450 system was present in *Pseudomonas putida* (Masayuki, 1968) greatly advanced our understanding of the cytochrome and its structure, it did not shed light on the specificity

of the mammalian system. Scientific meetings in this time frame were characterized by the arguments as to whether there was one P450 with membrane-controlled specificity, or multiple P450s (maybe 2!) lending various activities. The early evidence for at least 2 enzymes was mainly spectrophotometric. Imai and Sato found that when microsomes were treated with ethyl isocyanide two peaks were observed at 430 and 455 nm (Imai, 1966). The size of the peaks was dependent on pH. Sladek and Mannering[3] looked at microsomes from inducer-treated animals and found that after 3-MC induction the ratio of the two peaks differed from that of control or phenobarbital induced rats (Sladek, 1966). They suggested that there were at least two P450s. They referred to the 3-MC induced form as P_1-450. Alvares and co-workers found that after 3-MC induction the peak absorbance of microsomes after reduction and addition of carbon monoxide was found at 448 nm rather than the 450 nm of the controls (Alvares et al., 1968). They termed this pigment P448. Schenkman, Remmer and co-workers found that when substrates or inhibitors were added to microsomes there were spectral shifts (Remmer et al., 1966; Schenkman et al., 1967). These shifts were of two general types termed type I and type II. These spectral shifts were later shown to be related to the spin state of P450 iron (Kumaki et al., 1978).

The field struggled with the concept of one or two or more P450s for a number of years. In 1972, the second symposium on microsomes and drug oxidation was held at Stanford University. It was decided that this symposium would be an excellent first publication for the new ASPET journal, Drug Metabolism and Disposition. Browsing through this first

volume gives good insight to the state of knowledge of P450 in 1972. There was evidence for two distinct forms of mammalian P450 and solubilization efforts were underway (Lu, 1973). The chemistry of P450 was being explored especially with the soluble P450 from *P. putida* (Lipscomb and Gunsalus, 1973). Studies on the mechanisms of P450 inhibitors (Gillette et al., 1973), and inducers (Conney et al., 1973) were presented. The meeting encompassed virtually all aspects of P450 research. The discussions were included in the publications helping to illustrate the ongoing thought processes.

The initial breakthrough in solubilization of membrane bound P450 came in Jud Coon's laboratory in the study of ω-hydroxylation of fatty acids[4]. Anthony Lu, was able to obtain solubilized fractions of rabbit liver microsomes that consisted of lipid, P450, and reductase. When combined these fractions were able to catalyze the ω-hydroxylation of lauric acid (Lu, 1968). Using variations of this methodology, it became possible to apply standard protein purification methods to P450s[5]. After his post-doctoral studies, Lu moved to Alan Conney's laboratory at Hoffman La-Roche and began a series of collaborative studies on the drug metabolizing system in liver microsomes. The solubilization and partial separation of cytochromes "P450" and "P448" showed that the substrate specificity of these preparations resided in the cytochrome fraction (Lu, 1972). These techniques, along with the application of immunological assays and SDS gel separation, led to the separation of six forms of P-450 (Thomas, 1976)[6]. Lu and West summarized the state of the art in the field in 1979 making the case for multiple P450s

with species and organ specific forms (Lu and West, 1979). A unique inducible P450 was isolated from rats treated with pregnenolone-16α-carbonitrile (Elshourbagy NA, 1980). Guengerich and co-workers were able to isolate 8 P450 forms from rats pretreated with phenobarbital or beta-naphthoflavone (Guengerich FP, 1982) and Wang et al. purified 6 P450s from human liver microsomes (Wang PP, 1983). The first amino acid sequence of a cytochrome P450 was determined by Fujii-Kuriyama and co-workers(Fujii-Kuriyama, 1982). As a prelude to our increased understanding of individual diversity, the relative levels of P450 forms in human liver were estimated by using warfarin metabolism as a diagnostic probe (Kaminsky et al., 1984). Soon the question moved from "more than one P450?" to "how many P450s?" The methodology available in 1983 for answering that question was beginning to show promise. The definitive answer, however, was to be found only after the molecular biological revolution occurring over the next 25 years.

Biotransformation

The advances in technology during this period dramatically changed how and to what extent we were able to determine the structure of metabolites formed during the course of metabolism. Mass spectrometry moved from the hands of specialists to the analytical lab with the launch of the LKB 9000 GC/MS. Spectrometry in biological media became routine with the Cary 14 spectrophotometer and later with the Shimadzu instrument containing larger photomultiplier tubes ideal for turbid solutions. Thin layer chromatography came and went during this period being displaced by GC and HPLC. The Varian A-60, introduced in 1961, was the first widely accepted proton NMR

instrument operating at 60 MHz. By 1983, a typical metabolism project utilized HPLC for separation and NMR and Mass Spectrometry for structure identification.

There have been numerous studies on diphenylhydantoin (DPH) throughout this time period, and they provide a good picture of the changing technology. In 1960, Maynert examined the fate of DPH in dog, rat and human (Maynert, 1960). He was expanding the findings of Butler that the main metabolite of DPH in human and dog was the p-hydroxylated analogue (Butler, 1957). He prepared ^{15}N labeled DPH and assessed the excretion by isotopic dilution. Metabolites in ether extracts of urine were resolved by paper chromatography and, after derivatization by diazotized sulfanilic acid, eluted from the paper, and monitored in a Beckman B spectrophotometer. His results showed that the p-HO-DPH and its glucuronide were the main products of metabolism. In 1970 Atkinson and co-workers found that meta hydroxy DPH was actually the major primary metabolite of DPH in the dog (Atkinson AJ Jr, 1970). Atkinson used thin layer chromatography followed by elution of selected zones from the plates and analysis of the methylated samples by GLC to identify the meta-substituted metabolite. This is still one of the most striking examples of species specificity in the literature. The dihydrodiol metabolite of DPH was initially reported by Chang et al (Chang, 1970). Chang isolated the radiolabeled metabolite by silica column chromatography, countercurrent extraction, and thin layer chromatography. The isolated metabolite was analyzed by IR, NMR, UV and polarimetry. In a study of the absolute configuration of the dihydrodiol metabolites of DPH, they were isolated by extraction and preparative thin layer chromatography and analyzed using ORD, CD and GC/MS of silylated derivatives (Maguire JH, 1980). In 1983, evidence for an arene-3, 4-oxide was obtained using deuterated DPH and isolating

metabolites by HPLC and analyzing them by GC/MS (Moustafa MA, 1983). As with many other projects our level of understanding advanced with the advances in technology. Yet, despite all these advances, metabolism scientists in 1983 were still dreaming of the direct combination of the separation powers of HPLC and the analytical powers of Mass Spectrometry.

Advances in analytical technology along with a greater understanding of the enzymes involved in transformation allowed exploration of the actual intermediates involved in metabolic pathways. A breakthrough in the understanding of aromatic hydroxylation came with the discovery of the "NIH shift". This reaction was characterized by the movement of a hydrogen to an adjacent ring position during aromatic hydroxylation (Guroff G, 1967). Jerina[7], Daly and others were able to demonstrate that an epoxide intermediate would explain the products of the microsomal oxidation of benzene and naphthalene (Jerina, 1974). This led to studies on polycyclic hydrocarbons and the unraveling of the diol-epoxide pathways. It is thought that the diol epoxide of benzo(a)pyrene is the ultimate carcinogenic metabolic of this potent carcinogen (Buening and Akagi, 1978).

The formation of reactive intermediates and suicide substrates became a major area of research. The formation of "green pigments" during administration of 2-allyl-2-isopropylacetamide was shown by de Matteis to be due to destruction of P450 (De Matteis, 1971). Ethylene and other olefins were found to have similar destructive properties (Ortiz, 1980). 1-aminobenzotriazole was oxidized in microsomal incubations with concurrent loss of P450 (Ortiz, 1981). The progress on suicide inhibition of P450

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was summarized Ortiz de Montellano and Correia in 1983 (Ortiz, 1983)[8].

The classic story of reactive intermediates and drug therapy arose from a series of studies on acetaminophen in Bernard Brodie's lab. In a series of four papers, Brodie and co-workers were able to show that 1. acetaminophen liver necrosis was dependent on the extent of metabolism and activity of P450 (Mitchell et al., 1973a), 2. acetaminophen liver necrosis was correlated with covalent binding of radiolabeled acetaminophen (Jollow et al., 1973), 3. radiolabeled acetaminophen was covalently bound to microsomes when metabolized by a P450 dependent reaction (Potter et al., 1973), and 4. the covalent binding of acetaminophen and hepatic necrosis was diminished by high levels of glutathione and exacerbated by depletion of glutathione levels (Mitchell et al., 1973b). These studies are the basis for current studies on liver toxicity due to acetaminophen overdose, the effect of induction and inhibition on that toxicity and the role of P450 forms in metabolism. In many respects this system has become a model for all potential P450 interactions, and it is a staple at all drug metabolism scientific gatherings.

The role of glutathione as a precursor of mercapturic acids was confirmed in 1959 (Bray, 1959a; Bray, 1959b) 80 years after the discovery of these important elimination products by Baumann and Preuss (Baumann, 1879). In the following 25 years, there were over 150 papers in the ASPET journals referring to aspects of glutathione in metabolism. The role of glutathione as a scavenger of reactive intermediates was of primary interest as exemplified by the finding of the glutathione conjugate of acetaminophen by Hinson et al. (Hinson et al., 1982) or the formation of mercapturic acids from cyclohexene epoxide in the rat (van Bladeren et al., 1981).

It was becoming clear that in the P450 system there were multiple enzymes with the same mechanism but differing specificities. Once past that mental hurdle, it was necessary to deal with a second issue, the redundancy of enzyme types in the metabolism of a given class of compounds. Two significant examples illustrate this stretch in thinking. Ziegler and co-workers studied the N-oxidation of dimethylaniline and found a flavin containing enzyme free of heme that catalyzed the reaction (Machinist, 1968). Initial work was performed in pig liver but soon moved to other species including human (Gold, 1971). The enzyme was found to be distinct from other microsomal flavoproteins (Masters BS, 1971). This was a surprising finding, not only for Dan Ziegler[9], but for many in the metabolism field who had ascribed that activity exclusively to the P450 system (Ziegler, 1991). The second example of expanding the thought process came from the lab of Charles Lieber. Rubin and Lieber found that ethanol was an inducer of the microsomal oxidizing system (Rubin E, 1968). Lieber went on to describe a microsomal system capable of oxidizing ethanol (Lieber, 1970) and to show that the metabolism of ethanol by this system was a significant factor in ethanol disposition (Lieber and DeCarli, 1972; Lieber and Decarli, 1973). In 1977, a reconstituted P450 system was shown to oxidize ethanol (Ohnishi and Lieber, 1977). This finding silenced skeptics who had doubted the relevance of the earlier findings. In both of these examples, the lesson of keeping an open mind to new discoveries was brought home with sound data and determination.

The establishment of the role of P450 in metabolism indicated that variability in metabolism between and within species could be caused by different numbers of P450s

or P450s differing in structure, specificity, activity or any combination of these. A new aspect of variability was uncovered in the labs of R.Smith and M. Eichelbaum. Smith's group found that a significant portion of humans (including R. Smith) were deficient in the ability to hydroxylate debrisoquine (Mahgoub, 1977). It had previously been shown that 4-hydroxylation was the major pathway of debrisoquine metabolism in humans (Allen et al., 1975). Eichelbaum's group had similar findings using sparteine (Eichelbaum, 1978). This genetic deficiency, which was soon be traced to P450 opened a new era in our understanding of the role of P450 (Davies, 1981). Examination of additional drugs both old (Boobis et al., 1983; Mellstrom et al., 1983) and new (Wang et al., 1984) helped to expand the numbers of agents affected by this genetic polymorphism. The P450 catalyzing the reaction was isolated using specific antibodies and electrophoretic separation (Distlerath LM, 1984).

The discovery of p-glycoprotein in 1976 and its subsequent purification in 1979 was the opening of a new area of research in the study of drug disposition (Juliano, 1976; Riordan and Ling, 1979). The magnitude of the contribution of transporters to drug effectiveness and/or toxicity would only be realized with the soon to be explosive growth of this field.

In 25 years the science of drug metabolism had moved from struggling with membrane preparations to an understanding of the molecules involved in transforming drugs and foreign substances. Methodology for separation and identification of metabolites and intermediates had been greatly improved and had great potential for even further advances. Scientists were asking new questions regarding human variability in metabolism. The P450 field had moved rapidly and was getting cluttered with enzymes of

varying terminology. If only someone would invent a nomenclature system to systematize further research! With so many questions answered it would seem that the field was at a peak. However, as with all science, the greater the knowledge, the more numerous and sophisticated were the next generation of questions.

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Footnotes

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[1]An historical overview of the isolation and identification of the intracellular components of the liver cell is found in the three nobel lectures of the pioneers in this field, Claude, de Duve and Palade which can be found online at http://nobelprize.org/nobel_prizes/medicine/laureates/1974/

[2]A recent symposium honoring Ron Estabrook and his scientific contributions was published in drug metabolism reviews 39:251-283(2007)

[3]Gil Mannering has detailed his entrance into the field of drug metabolism and his work with microsomes and metabolism inhibitors in his 1984 Brodie award lecture(Mannering, 1986).

[4]Jud Coon discussed how his interest in fatty acid oxidation led him into the world of P450 and studies on the mechanism of P450 catalyzed oxidations in the 1980 Brodie award lecture. (Coon, 1981)

[5] Anthony Lu has provided an overview of the work leading to solubilization of P450 and

an understanding of its role in fatty acid and drug metabolism (Lu, 1998)

[6] The collaborative efforts leading to the resolution of rat liver P450s has been described in Wayne Levin's Brodie Award Lecture (Levin, 1990)

[7] Don Jerina was recipient of the 3rd Brodie award. His Brodie lecture details the path from a puzzling retention of radioactivity during p-hydroxylation of p-labeled aromatic rings to the identification of dihydrodiol epoxides as ultimate carcinogenic moieties formed from benzo(a)pyrene and other aromatic hydrocarbons (Jerina, 1983).

[8]Paul R. Ortiz de Montellano has detailed the development of this field in his 1994 Brodie lecture. (Ortiz, 1995)

[9]Dan Ziegler looks back on the discovery of the Flavin Mono-oxygenase system in his Brodie Award lecture.(Ziegler, 1991)