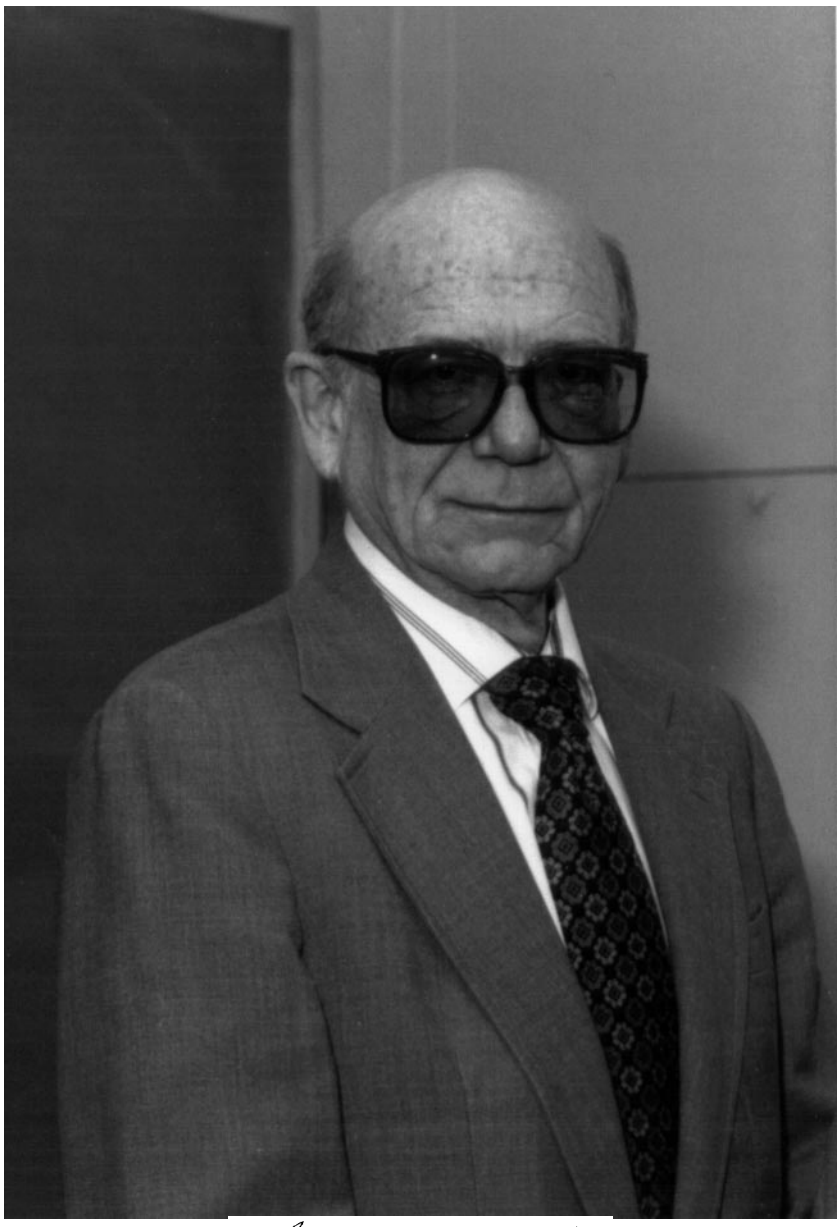

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LABORATORY OF CHEMICAL PHARMACOLOGY, NATIONAL HEART, LUNG, AND BLOOD INSTITUTE, NIH: A Short History

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■ **Abstract** The Laboratory of Chemical Pharmacology (LCP) began in 1950 as the Section of Pharmacology within the National Heart Institute, the National Institutes of Health. Its first chief was Bernard B. Brodie, considered by many to be one of the fathers of modern pharmacology. Since its inception, LCP has made many significant contributions to the fields of pharmacology and toxicology. LCP was among the first to study (*a*) the effects of drugs on the turnover of serotonin and norepineprine in brain and other tissues, (*b*) the absorption of drugs from the gastrointestinal tract and their passage across the blood-brain barrier, (*c*) the oxidation and reduction of drugs and other foreign compounds by liver microsomal enzymes (later known as the cytochrome P450 enzymes) and inhibitors and inducers of these enzymes, (*d*) the formation of toxic chemically reactive metabolites of drugs and other foreign compounds, and (*e*) mechanisms of immunological responses. Approximately 300 scientists worked in LCP during its existence, and they and their collaborators published more than 1,300 papers. This is a short history of the people who worked in it and of their contributions to biomedical sciences.

PROLOGUE

The Laboratory of Chemical Pharmacology (LCP) evolved from a small laboratory within the Goldwater Memorial Hospital in New York City during the Second World War. At that time the Japanese Army had overrun Southeast Asia, the principal source of quinine. Clearly, drugs were needed to control malaria in the Allied armies, if they were to perform effectively in malaria-infested regions. A group of scientists recruited and led by a bright young renal physiologist, James Shannon, was asked to solve the problem (1). Through the influence of EK Marshall, Shannon had become convinced that the pharmacological effects of a drug were more closely related to the concentration of the drug in the blood than to any arbitrary dose of the drug. At that time, this was a revolutionary idea. But

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showing such relationships required analytical methods capable of detecting the parent drug in absence of its metabolites in blood and other tissues.

In approaching the problem, Bernard Brodie, together with his technician, Sidney Udenfriend, developed principles that could be used to develop analytical methods for many drugs. The general approach (2) was to extract unchanged drug into ''the least polar solvent'' capable of quantitatively extracting the drug, to wash the organic extract to remove polar metabolites, to return the drug to an aqueous buffer, and to detect the unchanged drug either directly by measuring its absorbancy in a spectrophotometer or fluorescence in a fluorometer or indirectly by converting it to a detectable derivative. Using analytical procedures based on these principles, Shannon's group studied many compounds of potential use in the treatment of malaria. They discovered (3), for example, that Atabrine in dogs and volunteers had a very long half-life in the body and that the best way of dosing soldiers was to administer a loading dose followed by smaller maintenance doses. There is no doubt that the Goldwater group played a major role in assuring victory in the Pacific.

After the war, Shannon's Goldwater group dissolved. Shannon became the Director of the Squibb Institute for Medical Research. Others, including Udenfriend, dispersed to academia. But Brodie remained at Goldwater. However, the two principles that guided the anti-malarial program, (*a*) that the pharmacological effects of a drug were more closely related to the concentration of the drug in the blood than to any arbitrary dose of the drug, and (*b*) development of analytical methods based on extraction of drugs into the least polar solvent, guided Brodie's approach to pharmacology for the next several years. Indeed, from 1947 through 1953, Brodie and his associates published more than twenty papers in the *Journal of Pharmacology and Experimental Therapeutics* on the disposition of various drugs, including carbonamide, dicumarol, aminopyrine (Pyramidon), benzazoline (Priscoline), ethyl biscoumacetate (Tromexan), dibenamine, caffeine, pentobarbital, ephredine, and phenybutazone (Butazolidin).

Early Discoveries

While still in New York, Brodie & Julius Axelrod (4) discovered that acetanilide was converted, amongst other things, to both aniline and N-acetylaminophenol. Flinn & Brodie (5) further showed that N-acetylaminophenol, later known as acetaminophen or paracetamol, was a potent analgesic. Moreover, Brodie & Axelrod (6) also found that phenacetin, then a major component of many analgesic preparations, was also converted in the body to N-acetylaminophenol. These studies thus illustrated another principle that was to guide the work of LCP for several years: Pharmacological effects of drugs are frequently mediated in part by metabolites of drugs. Identification of the metabolites might therefore lead to the development of more specific and safer drugs.

Brodie et al also showed that the short action of procaine was due to its short half-life in the body. They suggested that the ester bond in procaine be replaced

by an amide bond to form procaine amide, which has a longer half-life (7) and thus was useful in the treatment of cardiac arrhythmias (8).

In another study, Brodie et al discovered that the elimination of the ''ultra-short-acting'' barbiturate, thiopental, from the body was rather slow; indeed, its short action was due to its rapid distribution into deep pharmacokinetic compartments, such as fat (9). Repeated doses of thiopental thus could result in prolonged narcosis, because the drug would accumulate in the body until the serum levels remained at pharmacologically effective levels even after the redistribution phases were complete.

In still another study, Brodie et al (10) discovered that N-acetyl 4-aminoantipyrine was not significantly bound to blood or organ components but was distributed with body water. The amount of body water thus could be calculated from measurements of the drug in blood at various times.

THE EARLY YEARS

Shannon soon became disenchanted with industry and in 1949 accepted the position of Scientific Director of the National Heart Institute. He immediately started to recruit the best people he could find for his staff. This was not as easy as it might seem. At that time, government laboratories had a poor reputation. But owing to his persistence and persuasive personality, he collected a group of scientists who would become leaders in many biomedical sciences, including pharmacology. He was aided in his recruitment by a quirk in the draft law that permitted young physicians to enlist in the Public Health Service and thereby to work at NIH rather than to serve in M.A.S.H. units in Korea. Among the scientists he recruited were Robert Berliner, James Wyngaarden, Thomas Kennedy, Christian Anfinsen, Robert Bowman, Marjorie and Evan Horning, and later, Donald Frederickson.

Shannon also was able to convince Brodie in 1950 to join him as the head of the Section of Chemical Pharmacology of the National Heart Institute, which became the Laboratory of Chemical Pharmacology (LCP) in 1952. Shannon staffed the section with people such as Drs. Sidney Udenfriend, Bert La Du, and Elwood Titus, and several ''technicians,'' including Julius Axelrod, Lewis Aronow, Herbert Weisbach, Jack Cooper, and Parkhurst Shore. The initial cadre of the National Heart Institute was housed in NIH's Building 3, which was far too small for the group envisioned by Shannon. So the move of Brodie's group from Goldwater to Bethesda was gradual until the new Clinical Center was built in Bethesda. Even after the Clinical Center was built, Brodie still maintained a group at Goldwater, headed by John Burns, whom Brodie had recruited after the war.

Drug-Metabolizing Enzymes

After Brodie became chief of LCP, he was contacted by Glenn Ullott, of Smith Kline and French, who told him of a compound that had some very strange properties. Even at fairly large doses the compound, known as SKF-525A, seemed to exert no obvious action of it own, but it prolonged the action of many barbiturates. This could readily be demonstrated by injecting the compound just prior to the barbiturate into mice and measuring the length of time that they ''slept.'' The question was whether the effect was due to the potentiation of the action of the barbiturate in brain or to a slowing of the metabolism of the barbiturate. Studies by Axelrod et al (11) revealed that the levels of the drug on the awakening were virtually unaffected by SKF-525A, but the half-life of the barbiturate was prolonged. And studies by Cooper et al (12) indicated that SKF-525A inhibited the in vitro as well as in vivo metabolism of many other drugs, suggesting that there may be something in common for the enzymes that metabolized the drugs.

But what were these enzymes? At that time the Spinco ultracentrifuge had just been invented and it became possible to isolate various fractions of liver homogenates. Using fractions separated with this instrument, Axelrod (13) discovered that amphetamine was metabolized to phenylacetone by the 9,000 x g supernatant fraction, but not by either the microsomal fraction or the soluble fraction alone. By brilliant insight he concluded that the enzyme was in the microsomes and that the soluble fraction provided NADPH (then known as TPNH). At about the same time, but unknown to Axelrod, Mueller & Miller at the University of Wisconsin (14) discovered that the N-demethylation of the carcinogen, N-dimethylaminoazobenzene, required liver microsomes, NADP, the soluble fraction, and a substrate for the generation of NADPH.

Subsequently, various members of LCP discovered that many seemingly different reactions were catalyzed by enzymes in liver microsomes in the presence of an NADPH generation system: for example, the *O*-dealkylation of phenacetin (Axelrod), the *N*-demethylation of aminopyrine and other alkylamines (Bert La Du, Leo Gaudette, and Natalie Trousof), the oxidation of hexobarbital (Jack Cooper), and the hydroxylation of aromatic compounds (Sidney Udenfriend & Chozo Mitoma). Brodie recognized the implications of these findings, and he and his colleagues published the classic paper on the oxidative microsomal enzymes (15), later to be known collectively as cytochrome P450. Shortly thereafter Axelrod left Brodie and went back to school (part time) to get his Ph.D. degree (1), which he obtained within a year.

In 1954, the Clinical Center of the NIH was completed and LCP moved to its facilities in the eastern wings of the 7th and 8th floors. Sidney Udenfriend became the first Deputy Chief of the Laboratory and had his office on the 8th floor, and Brodie had his on the 7th floor. With the increase in space, there was an increase in the number of scientists and a great increase in the breadth of the studies of biomedical problems.

Drug metabolism remained an active field. James Fouts studied inhibitors of the microsomal enzymes (16, 17) and the reduction of nitro compounds, such as chloramphenicol and ρ-nitrobenzoic acid (18), and azo-compounds, such as Prontosil (19), by liver microsomes. I was assigned by La Du to the problem of solubilizing and purifying the microsomal enzyme, a problem that defied my best efforts. Nevertheless, La Du and I were able to prove that although hydrogen peroxide was formed by liver microsomes, the enzymes were not typical peroxidases, such as horseradish peroxidase, and we obtained the first evidence that TPNH cytochrome c reductase (later known as NADPH cytochrome P450 reductase) might be involved in the reactions (20).

Other Studies During the 1950s and Early 1960s

A major objective of the laboratory at the time was to determine the factors that governed the transfer of drugs across biological membranes. In a series of papers, reviewed in (21), Lewis Schanker et al discovered that the rate of absorption of drugs from the small and large intestines depended on the pKa of the drug, the pH of the medium, and the lipid solubility of the nonionized form of the drug. Moreover, Shore et al (22) studied the absorption of drugs from the stomach and discovered that acidic conditions in the stomach could lead to the accumulation of weak bases. Thus for some drugs there may be an entero-stomachic circulation as well as an entero-hepatic circulation. In other work in the laboratory, Schanker studied mechanisms of biliary secretion (23), and Steven Mayer & Roger Maickel studied the properties of the blood-brain barrier (24, 25).

John Burns and his group in Goldwater continued to study the metabolism of phenylbutazone analogues (26), which led to the development of a new uricosuric drug. But Burns' major interest at the time was the elucidation of the synthesis of ascorbic acid (27).

La Du & Vincent Zannoni (28) became interested in the enzymes that catalyzed the metabolism of tyrosine, a problem that led to La Du's interest in pharmacogenetics. Neil Moran & Marion Cotton (29) and Cotton & Harriet Maling (30) performed studies on the effect of drugs on the cardiac contractile force. Severinghaus began studies on factors that governed the pulmonary dead space (31) and the serum pCO2 and pO2 (32).

But one of the most significant projects of the laboratory resulted from a collaboration of Sidney Udenfriend with Robert Bowman. Udenfriend had become an expert in the theory of the fluorescence of compounds, and recognized the importance of developing ways of determining both the activation and the fluorescence spectra in order to develop more specific and sensitive methods for assaying biochemicals. Bowman became intrigued with the problem and developed a spectrophotofluorometer. With this prototype, Udenfriend and his postdoctoral student, Daniel Duggan (33), began to explore the factors that governed fluorescence, and thus the usefulness of the instrument, later known as the Aminco-Bowman Spectrophotofluorometer.

With this advance in methodology, Udenfriend together with Donald Bogdanski & Herbert Weissbach were able to detect small amounts of serotonin in brain (34). Shore developed techniques for assaying small amounts of catecholamines (35), and together with Alan Burkhalter & Victor Cohn developed a method for histamine (36). These methods provided the basis of numerous papers during the next two decades by LCP and other laboratories throughout the world. For example, the methods led to the discovery by Brodie et al that reserpine caused the release of serotonin (37) and norepinephrine from tissues.

Shortly after this time, Bert La Du left the laboratory and I became head of the Section on Drug Enzyme Interactions. Sidney Udenfriend also left to form his own laboratory, within the National Heart Institute, to which incidentally he attracted the future Nobel laureate, Marshall Nirenberg, from the National Arthritis Institute. John Burns then moved to Bethesda and became the second Deputy Laboratory Chief.

At this time Allan Conney joined Burns to work on an ascorbic acid problem, but he soon convinced Burns and several others in other laboratories of NIH to study the effects of 3-methylcholanthrene pretreatment on the metabolism of different drugs, which led to a classic paper published in *Science* (38). The fact that 3-methylcholanthrene affected different reactions differently provided one of the first indications that there were multiple forms of cytochrome P450. He continued his work by showing that phenobarbital and other compounds (39; reviewed in 40) were able to induce cytochrome P450 enzymes. At about the same time, Remmer (41) in Germany and Kato & Chiesara (42) in Italy were also studying the effects of pretreatment of animals with barbiturates and were also coming to the conclusion that barbiturates could serve as inducers of liver microsomal enzymes. Later, Joan Booth (43) in LCP discovered that anabolic steroids were inducers.

Neuropharmacology

Early in the 1960s John Burns left the laboratory to join Burroughs Wellcome, and Parkhurst Shore served for a short time as the third Deputy Laboratory Chief. However, Shore soon left for the Southwestern Medical School in Dallas, Texas, and then Erminio Costa became the fourth Deputy Laboratory Chief.

During the 1960s, most of LCP focused its attention on various aspects of neuropharmacology. Brodie and Costa and colleagues continued studies on the mechanisms by which drugs affected the synthesis, transport, and storage of norepinephrine and serotonin in various organs of the body. In a series of more than 40 papers they studied the effects of reserpine, tetrabenazine, decarboxylase inhibitors (44), dopamine beta oxidase inhibitors (45), and ''false transmitters'' (46). In the late 1960s Norton Neff, Thomas Tozer, and colleagues (47) studied the rates of uptake and release of radiolabeled norepinephrine from rat heart and began to apply mathematical expressions for two-compartment systems to analyze the kinetics of these processes.

Marjorie Horning, in the Laboratory of Chemistry of the National Heart Institute, in collaboration with Maling (48), discovered that the source of fatty acids appearing in liver after alcohol administration was adipose tissue, a finding that led Brodie to his interest in lipolysis. After Sutherland made his seminal discoveries of the role of cyclic AMP, Gopal Krishna (49) developed a simple method for the assay of cyclic AMP, which greatly facilitated studies of control mechanisms of lipolysis in adipose tissues. Indeed, this became a major objective of the laboratory during this phase, resulting in more than 30 papers on the mechanism of lipolysis in adipose tissues. Frandsen & Krishna (50) subsequently developed methods for the assay of cyclic GMP and guanylate cyclase activity, which help to elucidate the role of cyclic GMP in the retina (51).

Titus' section also became interested in neuropharmacology. Lewis Ignarro (52) discovered that both alpha and beta adrenergic receptors were present in mouse spleen. Hans Dengler (53) studied the uptake of norepinephrine into isolated brain tissues. Arthur Michaelson & Palmer Taylor (54) studied uptake mechanisms in heart subcellular particles. Subsequently, Titus, with Colin Chignell (55) and later with William Hart, (56) began to purify components of the sodium potassium ATPase. Chignell soon became interested in the development and use of sophisticated analytical methods (57), including circular dichroism measurements (58) and spin-labeled probes for ESR measurements (59), for analyzing protein configurations and protein binding.

Pharmacokinetics of Parent Drugs

Although the general strategy during the 1960s was to attempt to relate either the intensity or the duration of action of a drug to its concentration in the blood, we began to wonder about the effects of reversible binding of drugs to blood components on drug action and drug metabolism. Analysis of the problem revealed that marked differences in the extent of reversible binding of a drug to blood components, especially when the drug is also extensively bound to extravascular components, may result in only trivial differences in its unbound concentration at receptor sites (60), which governs the proportion of receptor sites occupied by the drug. We also found that reversible binding of a drug to blood components may either increase or decrease the half-life of the drug, depending on the hepatic extraction ratio, and devised a nomogram for estimating where the crossover point would occur for given sets of factors (61). In attempts to relate enzyme kinetics to the clearance of drugs by the liver, Sandy Pang (62) developed an approach based on the formation and subsequent metabolism of metabolites in single-pass liver perfusion experiments that would differentiate between the ''well stirred'' and the ''parallel tube'' models. Unfortunately, the results indicated that neither model was adequate, and Pang began her life-long quest for understanding the kinetics of drug metabolism in perfused organs.

We used pharmacokinetic studies to determine the extent to which species, sex, and individual differences in drug responses could be explained by differ-

ences in metabolism of drugs. In fact, Gertrude Quinn et al (63) had published their classic paper showing that most of the species, strain, and sex differences in the pharmacologic action of barbiturates could be attributed to differences in drug metabolism. Ryuichi Kato in LCP had expanded these studies to other drugs and the effects of various treatments (64, 65). Moreover, Elliot Vesell & John Page (66) in LCP began to demonstrate, using phenylbutazone in human twins, that most of the individual differences in drug metabolism were due to genetic differences.

When attempts to relate plasma levels of drugs to pharmacologic effects failed, we looked for metabolites of the drug that might have pharmacologic activity. Accordingly, James Dingell and I found that the anti-reserpine action of imipramine, discovered by Fridolin Sulser (67), was due to its demethylated metabolite, desipramine, (68) and that species differences in the anti-reserpine action were due to differences in the relative rates of formation and elimination of desipramine (69). In addition, Folke Sjoqvist et al (70) found that desipramine exerted its ''anti-tremorine'' effect by inhibiting the conversion of tremorine to oxotremorine, its active metabolite, but desipramine actually prolonged the action of oxotremorine by inhibiting its metabolism.

But the strategy failed to explain species differences in the teratogenic effects of thalidomide. We were aware that thalidomide frequently caused teratogenic effects in humans, monkey, and rabbits, but rarely in rats. We were also aware that thalidomide in aqueous solutions is unstable at pH 7 and is converted to many hydrolysis products nonenzymatically (71). It was not surprising, therefore, that Herbert Schumacher & David Blake (72) discovered virtually no species differences in the biological half-life of the drug in rats and rabbits. But we discovered that the drug became covalently bound to proteins in liver.

We were aware of the seminal work in the laboratory of James & Elizabeth Miller (73) at the University of Wisconsin indicating that carcinogenic agents, including polycyclic hydrocarbons, azobenzenes, estradiol, and 2-acetylaminofluene, were converted to chemically reactive agents that became covalently bound to proteins and nucleic acid. Thus our finding that thalidomide could react covalently with protein prompted us to write (72), ''The radioactivity bound to the soluble fraction of liver 24 hr after thalidomide administration could not be removed by exhaustive dialysis. It therefore seems possible that after thalidomide enters the fetus it might cause teratogenesis by acylating various components which are essential for normal fetal development.'' Unfortunately, at that time we were not able to devise an experiment that would provide convincing evidence that covalent binding of the drug to macromolecules actually resulted in teratogenesis.

We were able to demonstrate that other drugs could be converted to metabolites that became covalently bound to macromolecules. But it soon became evident that before we could claim that a substance caused toxicity through the formation of a chemically reactive metabolite, we must determine whether changes in the extent of binding of the reactive metabolite, caused by changes in the metabolism

of the parent drug and the reactive metabolite, would result in parallel changes in the incidence or severity of a toxicity.

Since we couldn't think of any easily detected toxicity to explore at the time, my section of the laboratory dropped the project and studied other things.

THE LAST TWENTY-FIVE YEARS

In about 1968, Erminio Costa took a position as chief of a laboratory in the Mental Health Institute, and I became the fifth Deputy Laboratory Chief. Shortly thereafter, Brodie had his first heart attack and his health began to deteriorate.

Chemically Reactive Metabolites

Before Brodie went away to recuperate from one of his illnesses, he encouraged Watson Reid, together with Gopal Krishna, Glenn Sipes, and Arthur Cho, to explore the possibility that chemically reactive metabolites could cause toxicities. Their studies of the hepatic toxicity of bromobenzene and other halogenated aromatic compounds led to the publication of the classic paper by Brodie et al (74), showing that the hepatotoxicity of bromobenzene was mediated through a chemically reactive metabolite.

At that time we had a pretty good idea that the reactive metabolites of the halogenated aromatic compounds were epoxides. As early as 1950 Eric Boyland (75) in England had proposed the formation of epoxides of aromatic compounds, which he inferred from the finding of 1,2-dihydronaphthalene-1,2-diol in urine of animals dosed with naphthalene. In 1960 Joan Booth et al (76) in Boyland's laboratory found that 1,2-dihydronaphthalene and 1,2,3,4-tetrahydronaphthalene 1,2-oxide were converted to the same glutathione metabolite when they were incubated with glutathione and either rat liver slices or microsomes. In 1967, Jordan Holtzman et al (77) in LCP found that only one of the oxygen atoms in the 1,2-dihydronaphthalene 1,2-diol formed from naphthalene by mouse liver microsomes originated from atmospheric oxygen. And in 1968 Donald Jerina et al (78) in NIDDK, NIH, were able to trap radiolabeled 1,2-dihydronaphthalene 1,2-oxide formed from radiolabeled naphthalene by rat liver microsomes. But subsequent work by David Jollow et al (79) provided more definitive evidence that bromobenzene 3,4-oxide caused liver necrosis.

Because of deteriorating health, Brodie retired in 1971 and I became acting Chief of the laboratory until 1973, at which time I was confirmed as Chief. The size of the laboratory was reduced to the east wing of the 8th floor of the Clinical Center, and the sections headed by Elwood Titus and Watson Reid were transferred out of the laboratory.

During the 1970s, the laboratory focused most of its efforts on discovering other drugs that might cause toxicities through the formation of chemically reactive metabolites. Indeed, more than 150 papers (cited in reference 80) were published

during the following 23 years on toxicities produced by halogenated benzenes, ρ- and *o*-bromophenols, acetaminophen, ρ-chloroacetanilide, phenacetin, isoniazid, iproniazid, ipomeanol, furosemide, spironolactone, 2-acetylaminofluene, 2-hydroxyestrogens, allyl alcohol, niridazole, nitrofurantoin, nitrobenzene, nitroso compounds, carbon tetrachloride, chloramphenicol, and chloroform and other halogenated anesthetic gases.

Many of these papers were devoted to studying possible reactive metabolites of acetaminophen, which in large doses, causes liver necrosis in humans. These papers, beginning with one by Jerry Mitchell et al (81), showed that acetaminophen caused liver necrosis in animals through the formation of a chemically reactive metabolite. Although it seemed certain that the toxic metabolite was N-acetylimidoquinone, it was not certain how this metabolite was formed. At first it seemed possible that it was formed by way of N-hydroxyacetaminophen, which was known to decompose to the imidoquinone. Indeed, Jack Hinson spent considerable effort in synthesizing and studying the formation of various N-hydroxy aromatic compounds, including N-hydroxyacetyl-ρ-chloroanilide (82) and N-hydroxyphenacetin (83). Gerard Mulder & Hinson (84) demonstrated that N-hydroxyphenacetin was stable for several hours in aqueous solutions, but converting it to its NO-glucuronide or NO-sulfate markedly decreased its stability. In 1979, however, Hinson & Pohl (85) were able to show that N-hydroxyacetaminophen was a metabolite of N-hydroxyphenacetin but not of acetaminophen. Thus the N-acetylimidoquinone was formed directly from acetaminophen and not indirectly through an N-hydroxyacetaminophen. Moreover, it was also shown that phenacetin could be converted to reactive metabolites by several different mechanisms (86), which led me to call it ''fascinating phenacetin.''

Most of our studies for detecting the formation of chemically reactive metabolites depended on the detection of covalently bound radiolabeled material after the administration of radiolabeled drugs to animals. However, studies with doubly labeled chloramphenicol made us more cautious in our interpretation. These studies revealed that the half-life of the two labels were completely different. In fact, much of the covalently bound C^{14} from the dichloroethylamino group in chloramphenicol appeared in blood plasma and persisted for days. Further studies by Pohl et al (87) revealed that the dichloroethyl amino group was converted to oxamic acid, which entered the two-carbon pool, and served as a precursor of glycine and serine, which were then incorporated into albumin and other proteins. We thus became aware that other pathways of metabolism besides demethylation and deethylation pathways could lead to incorportation of C^{14} into amino acids and thence into proteins.

We became cautious in attempting to determine the mechanism by which a given chemically reactive metabolite caused toxicities. It became obvious that reactive metabolites might cause toxicities through many different mechanisms, not all of which were mediated by the covalent binding of the chemically reactive metabolite to a specific macromolecule. For example, it seemed possible that the chemically reactive metabolite might abstract a hydrogen atom from a lipid and thereby initiate lipid peroxidation, which could lead to toxicities; in this pathway

the reactive metabolite would be converted to a stable metabolite, which would not be covalently bound to macromolecules. Moreover, it also seemed likely that a chemically reactive metabolite could react with many different macromolecules, including various proteins, DNA, and RNA. The relative rates would depend on many factors, including the stability of the reactive metabolite in both lipid membranes and water, the location of formation of the metabolite within cells, the amounts of various target macromolecules, the number of target sites on the individual macromolecules, and the rates at which the altered macromolecules are repaired or replaced. To focus attention on the identification of the various altered macromolecules, as many of our friends wished us to do, seemed fruitless as means of identifying the covalently bound material that caused the toxicity. Indeed, even if we had identified several of the altered macromolecules, how were we to determine which one was the crucial altered macromolecule? We also wondered whether the toxicity might not be caused by several different injuries acting in concert. We even came to the conclusion that the magnitude of the amount of covalently bound material would not be particularly useful for predicting toxicities, because many of the altered macromolecules probably would not be essential to the life of the cells.

In an attempt to caution investigators about over-interpreting relationships between covalent bonding and toxicities, I wrote in 1974 (88): ''Studies of covalent binding to tissue macromolecules by themselves have little predictive value in determining whether a given compound will evoke a given kind of toxicity. Indeed, without correlative studies, no more emphasis should be placed on the finding that a reactive metabolite becomes covalently bound to tissue macromolecules than would be placed on the finding that a reversibly acting drug is localized in a given tissue in drug disposition studies.''

For these reasons, LCP focused on studies to identify reactive metabolites and to elucidate the mechanisms of their formation and inactivation. For example, Sidney Nelson et al (89) found that isoniazid is first acetylated to form N-acetyl isoniazid, which then undergoes hydrolysis to form acetylhydrazine. The acetylhydrazine then is converted to a chemically reactive metabolite that causes necrosis in animals. Similarly, iproniazid (89, 90) undergoes hydrolysis to isopropyl hydrazine, which in turn is converted to a toxic, chemically reactive metabolite. Pohl et al (91) were able to demonstrate that chloroform was converted to phosgene. Although carbon tetrachloride was probably converted to reactive metabolites by several mechanisms, the major pathway (92) appears to be reductive dechlorination to form trichloromethyl radical, which reacts with oxygen to form a peroxy radical, which decomposes to phosgene. Enflurane (93), halothane, and other halogenated alkanes (94) appear to undergo hydroxylation to form hydroxyhalo-intermediates, which decompose to ketones or acyl halides.

Kinetics of Reactive Metabolites

The amount of covalently bound material, measured at different times, can provide an indirect measure of the amount of reactive metabolite entering the organ,

whether generated there or carried there by the blood. For convenience, the amount may be expressed as: Amount = Dose ABC (95), where A is the fraction of the dose that is absorbed, B is the fraction of the absorbed material that is converted to the chemically reactive metabolite, and C is the fraction of the chemically reactive metabolite that becomes covalently bound. The purpose of this approach was to emphasize that ratio B could be increased either by increasing the activity of the enzyme that formed the reactive metabolite or by decreasing the activity of enzymes that catalyzed side pathways and that ratio C could be increased by decreasing mechanisms of inactivation of the reactive metabolite.

Several functional categories of reactive metabolites were envisioned based on their various stabilities (96). At one extreme, the reactive metabolite might be so stable that virtually every organ in the body would be exposed to it; indeed, some of it might be excreted into the urine and thereby constitute a potential environmental hazard. At the other extreme, the reactive metabolite might never escape the active site of the enzyme in which it was formed; in this case the precursor would be considered to be a ''suicide inhibitor'' or a ''metabolically activated inhibitor.'' Intermediate cases would include (*a*) reactive metabolites that escaped cells in which they were formed and entered other cells in either the same or different organs, where they could cause toxicities, or (*b*) the formation of stable metabolites that would leave the cells in which they were formed and be carried by the blood to other cells or organs in which the metabolites would be converted to short-lived reactive metabolites. It also seemed possible that a given reactive metabolite might be in one category under some conditions and in another category under other conditions. For example, at low doses of acetaminophen virtually all of the reactive metabolite may react with glutathione and thereby very little if any of it would escape the cells, but at high doses glutathione stores may become depleted and the life of the metabolite may be prolonged sufficiently to permit its escape.

Because the expected effects of inducers and inhibitors on the area under the curve of the reactive metabolite will differ with the category of the reactive metabolite, it seemed useful to devise methods for determining the category of given reactive metabolites. Various kinetic expressions (97) were derived for different in vitro experiments, based on the assumption of a steady-state (pseudo zero order) rate of formation of the reactive metabolite and its inactivation by a combination of a first-order reaction to form a stable metabolite and a bimolecular reaction with a nucleophile, such as a protein or glutathione. The mathematical expressions for such systems may be rearranged to provide straight-line plots of the effects of different concentrations of the nucleophile.

By using variations of this approach, Hinson & Larry Andrews (98) were able to elucidate the mechanisms of decomposition of the N-O-glucuronide and the N-O-sulfate of phenacetin; Henry Sasame et al (99) were able to demonstrate that not all phenolic metabolites of propranolol were formed from presumptive arene oxides; Terrence Monks and Serrine Lau (100, 101) were able to show that bromobenzene oxide escapes hepatocytes both in vitro and in vivo.

Renal Toxicity

Studies by Lau and Monks revealed that the bromobenzene-induced renal toxicity, discovered by Reid (102), followed a complicated series of events. Because pretreatment of animals with 3-methylcholanthrene decreased the severity of the hepatic necrosis caused by bromobenzene (103) and increased the formation of *o*-bromophenol, we had begun to view the formation of *o*-bromophenol as a protective mechanism. But Lau & Monks (104) showed that *o*-bromophenol was a more potent renal toxicant than bromobenzene. In a series of papers, they also showed that bromohydroquinone was more toxic and diglutathionyl bromohydroquinone was still more toxic to the kidney than bromobenzene. They (105) further showed that acivicin, a selective inhibitor of γ-glutathionyl transpeptidase present on the brush border cells of the kidney, prevented the toxicity. Thus it seems likely that diglutathionyl bromohydroquinone is formed predominately in the liver and is carried to the kidney where it enters brush border cells by the γ-glutathionyl transpeptidase mechanism, and is then converted to a toxic metabolite in the renal brush border cells. Since leaving LCP, Lau & Monks have greatly extended their studies to include many other hydroquinone and quinone compounds.

Suicide Enzyme Inhibitors

The major contributions of LCP to the field of ''suicide inhibitors'' of cytochrome P450 enzymes have been relatively recent. Indeed, our first experience with ''suicide inhibitors'' arose during the early 1970s when we were attempting to explain the feminizing effects of spironolactone. Although it was subsequently shown that this effect at low doses of the drug is probably due to its interaction with dihydrotestosterone receptors (106), Raymond Menard et al (107–109) found that spironolactone at high doses caused the destruction of 17 α-hydroxylase in testes and adrenals.

During the mid-1980s, Pohl became interested in mechanisms by which cytochrome P450 and other hemoproteins might be inactivated by various drugs. Clearly, reactive metabolites might react with hemoproteins in several different ways. They might react solely with the heme to form various heme derivatives; they might react solely with the protein portion of the enzyme; they might form a bridge between the heme and the protein of the enzyme; or they might react with the heme to form a heme radical that reacted with the protein. Helen Davies et al (110, 111) obtained support for the covalent binding of heme to protein by producing cytochrome P450s labeled with radioactive heme in vivo, and demonstrating that some of the radioactivity was covalently bound after treatment of tissue preparations and intact animals with various drugs. After joining Pohl, Yoichi Osawa became interested in studying the mechanisms by which heme became covalently bound to protein. As a model he studied the reaction of monobromotrichloromethane with reduced myoglobin. In these reactions, he (112)

discovered that a dichloromethyl group reacted with the heme, which in turn reacted with histidine 93 of myoglobin. Similar studies (113) revealed that the reaction of monobromotrichloromethane with hemoglobin resulted in the binding of heme to cysteine 93 of the beta chain via the ring I vinyl group.

Deuterium Isotope Effects

During the course of our studies to elucidate mechanisms of cytochrome P450 enzymes, Kiyoshi Nagata et al (114) discovered that rat liver microsomes catalyzed the conversion of testosterone to Δ^6-testosterone. After excluding the possibility that the double bond was formed by dehydration of the hydroxylated metabolites, we proposed that it was formed by a double hydrogen abstraction mechanism. At that time we were unable to devise an experiment that would prove our hypothesis. But Nagata had discovered that purified 7-α hydroxylase (CYPIIA1) could also convert testosterone to Δ^6-testosterone and its epoxide in addition to 7-α hydroxytestosterone. This finding suggested to Kenneth Korzekwa that studies of various deuterated forms of testosterone might provide the information necessary to confirm the double hydrogen extraction mechanism and even to provide clues to which hydrogen was abstracted first. After developing the appropriate mathematical equations and performing the necessary experiments with Nagata's enzyme preparation, he (115) established not only that the double extraction mechanism was valid, but also that the 6-α hydrogen was abstracted before the 7-α hydrogen.

These studies prompted my desire to understand the theory of isotope effects as applied to cytochrome P450 enzymes in the hope that many questions about the mechanisms of these enzymes might be resolved. I especially wondered about the mechanisms by which the cytochrome P450 enzymes were able to form several metabolites from the same substrate. Three general mechanisms seemed plausible. In the first mechanism (which I called the parallel pathway mechanism), the substrate combines with the ferric form of the enzyme in different orientations, and the orientations do not change during the enzymatic cycle; thus each orientation leads to a different metabolite. In the second mechanism (which I called the nondissociative mechanism), the substrate combines with ferric form of the enzyme in different orientations, but the binding site is sufficiently large that the substrate may change orientations during the enzymatic cycle. In the third mechanism (which I called the dissociative mechanism), first postulated by Gerald Miwa & Anthony Lu (116), the substrate combines with the ferric form of the enzyme, but it may also dissociate from the enzyme during the enzymatic cycle, equilibrate with the substrate in the aqueous medium, and recombine with the activated enzyme in either the same or different orientations.

Derivation (117) of the appropriate equations for these mechanisms with nondeuterated and deuterated substrates suggested experiments that would differentiate between them. Using these concepts, John Darbyshire & Katsumi Sugiyama (118) studied the mechanism by which CYP2C11 metabolizes testosterone to

2α-hydroxytestosterone, 16α-hydroxytestosterone, and androstenedione. The results clearly showed that the mechanism could not be the nondissociative mechanism, but whether it was by the parallel pathway mechanism or by the dissociative mechanism was unclear. One experiment supported the parallel pathway mechanism, but two other experiments supported the dissociative mechanism.

We also wondered about the mechanism by which testosterone was converted to 16α-hydroxyandrostenedione. Clearly, it required two enzymatic cycles to achieve its oxidation state. But does the presumptive androstenedione intermediate dissociate from the active site, equilibrate with that in the medium, and then recombine with the enzyme, or does the intermediate never leave the active site of the enzyme before it undergoes the second cycle? Although it is rather easy to evaluate the extreme cases for the formation of secondary metabolites by determining the effects of increasing concentrations of the substrate on the formation of a secondary metabolite, there was no obvious way at the time for evaluating intermediate cases. Using the relevant mathematical expressions for such cases, Sugiyama & Darbyshire studied the conversion of testosterone to 16α-hydroxyandrostenedione by P450 2C11 and found that about 15% of the 16α-hydroxyandrostenedione was formed directly from testosterone (119).

Immunology

As early as 1971 (74) we recognized that immune responses might be caused by chemically reactive metabolites. For many years, however, there seemed to be no obvious way of approaching the problem. In the mid-1980s, however, Hiroko Satoh in LCP developed an antibody that recognized the trifluoroacetyl group derived from halothane and used it to show that proteins containing the group were present on the surface of hepatocytes of animals treated with halothane (120). Pohl later learned that scientists in England (121, 122) had found that patients recovering from halothane toxicity had developed antibodies that reacted with liver proteins from animals receiving the drug. Since it seemed possible that these antibodies might play a role in the development of immune reactions, Gerald Kenna came to LCP with the patients' sera to work with Pohl. Studies by him and other members of Pohl's section revealed that the antibodies recognized several liver neoantigens, most of which contained the trifluoroacetyl group derived from halothane (123). The identification of these proteins was reported in more than thirty papers and summarized in (124).

During the mid-1960s, Michael Beaven (125) became interested in studying factors that affected levels of histamine in various organs, including the stomach (126), and retained that interest after he left LCP to join another laboratory within the National Heart Institute. His group rejoined LCP in the early 1980s, and he took a sabbatical to work in the laboratory of James C. Metcalfe in England. There he learned to measure free intracellular Ca^{++} using quin-2 (127). After returning to LCP, he and his colleagues embarked on a major program of the laboratory to study the cascade of events that occur following the reaction of

antigens with antibodies, including IgE, attached to membranes of 2H3 basophil leukemic cells (128). From the 1980s through 1994, his section published more than 70 papers that showed the involvement of the phosphatidylinositol cascade in causing increases in free intracellular Ca^{++}, phosphorylation of proteins (such as myosin) by protein kinase C, and the release of granules containing histamine and other inflammatory mediators (reviewed in 129, 130).

EPILOGUE

Because the major interests of LCP at the time of my retirement in 1994 were in various aspects of immunology, Edward Korn, the Scientific Director of NHLBI, decided to combine LCP with the group of Warren Leonard to form the Laboratory of Molecular Immunology. LCP, as such, thus ceased to exist after my retirement.

The outstanding talents of the scientists in LCP and its support staff, including our secretaries, Helen Balaguer and Bonnie (Farley) Chambers, made the achievements of LCP possible. Space does not permit a listing of all of the honors and awards received by various alumni of LCP, but the following may be noteworthy. Julius Axelrod and Lewis Ignarro are Noble Laureates. Bernard Brodie and Julius Axelrod have been Lasker awardees. The American Society for Pharmacology and Therapeutics has elected John Burns, Steven Mayer, Bert La Du, Sydney Spector, Allan Conney, Palmer Taylor, and Jerry Mitchell as presidents; The Society's award in Drug Metabolism is in the honor of Bernard B. Brodie; Bernard Brodie, Julius Axelrod, and Sidney Udenfriend have won the Sollman Award; Parkhurst Shore, Steven Mayer, James Fouts, Lewis Schanker, Ronald Kuntzman, Colin Chignell, Jerry Mitchell, and Sidney Nelson won the Abel Award; Elliott Vesell, Allan Conney, and Sydney Spector won the ASPET Award for Experimental Therapeutics; Harvey Kupferberg won the Epilepsy Research Award; John Burns won the Weiker Memorial Award; Elliott Vesell won the Gold Award; I won the first Brodie Award. Glenn Sipes has served as president of the Society of Toxicology; Allan Conney, Michael Boyd, and Alan Buckpitt have received the SOT Achievement Award; Allan Conney received the Arnold J. Lehman Award; Erik Dybing and Sidney Nelson received the Frank R. Blood Award. Robert Smith and Ryuichi Kato have served as presidents of the International Society for the Study of Xenobiotics, which has selected Ryuichi Kato and me as honorary members.

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