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Metabolism of Aromatic Hydrocarbons by the Cytochrome P-450 System and Epoxide Hydrolase

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It gives me great pleasure to have the honor of joining James R. Gillette (1) and Minor J. Coon (2) in being named as recipient of the third Brodie Award in Drug Metabolism. I wish to thank the American Society for Pharmacology and Experimental Therapeutics, administrators of the award, and the CIBA-GEIGY Corporation, the sponsors of the award, for their recognition of my studies in oxidative drug metabolism which began at the NIH in 1966. In the intervening years, I have had the satisfaction of enjoying the association of numerous talented colleagues and collaborators without whose skill and creativity the studies I am about to describe would not have been possible. Although their contributions are well documented in the literature, there is one individual whose contribution is not. That is Dr. J. E. Rall, the Intramural Research Director of the NIADDK, who has been instrumental in creating the environment so necessary to the pursuit of fundamental knowledge.

My entry into the field of drug metabolism followed a rather unusual path. When I came to the NIH in 1966, I had no formal training in pharmacology. In fact my graduate research with Robert Letsinger at Northwestern University was in the area of bioorganic chemistry and had as a goal the chemical synthesis of a gene. On joining Dr. Bernhard Witkop's Laboratory at the NIH, I had the good fortune of meeting John Daly. The three of us, along with Sidney Udenfriend and Gordon Guroff, began a several year study of what was to become one of the "Cinderella Stories" in oxidative drug metabolism whose eventual consequences reached as far as the biology of cancer.

These studies had begun with an attempt to develop facile assay procedures for mono-oxygenase enzymes that catalyze the hydroxylation of aromatic rings to form phenols. The principle of the assay was to tritiate specifically the ring position at which enzymatic hydroxylation was to occur. The extent to which metabolism had taken place could then be quantified based on the amount of tritiated water that was formed. Although the concept of the assay was basically sound, initial experiments with several enzyme preparations including phenylalanine hydroxylase, tryptophan hydroxylase, and hepatic liver microsomes led to the same puzzling conclusion: the amount of tritium released was less than the amount of product formed, based on chemical quantitation of the resultant phenols. Furthermore, the phenols that were formed were radioactive. Once it was established that the residual radioactivity in these phenols was not the consequence of nonspecific labeling of the substrates, we were faced with the startling discovery that a tritium substituent on an aromatic ring underwent migration and retention during the course of mono-oxygenase-catalyzed aryl hydroxylation. In rapid succession, our laboratories as well as others, demonstrated the generality of the phenomenon. Mono-oxygenase enzymes from plants, fungi, and mammals were shown to catalyze the migration and retention of aromatic ring substituents such as deuterium, tritium, halogen, and alkyl groups during the course of phenol formation. The reaction was named the NIH shift (3) and has been the subject of an extensive review (4).

In the course of the above studies, a debate had been going on between Daly and myself. What was the mechanism of this novel process? Was an arene oxide a discrete intermediate in the reaction or did the reaction proceed *via* any of a series of highly unstable species that could never be isolated (fig. 1)? The problem was sufficiently perplexing that we often changed sides in this debate, sometimes even in a single day. Interestingly, peroxyacids were found to mimic cytochrome P-450 in the hydroxylation of aromatic substrates since both deuterium and tritium were found to migrate and be retained (5). As fortune would have it, Vogel and co-workers (6) had just described the synthesis of benzene oxide, a seemingly improbable compound in which one of the formal aromatic double bonds of benzene was epoxidized. We then set out to establish, through the application of a cold-carrier trapping experiment, whether or not benzene oxide was the precursor of phenol when benzene was metabolized by hepatic microsomes (7). Although the results of these experiments were inconclusive regarding the intermediacy of benzene oxide in the formation of phenol, we were not to be without a reward for our efforts. We had discovered a new enzyme, later to be called microsomal epoxide hydrolase, which had the ability to hydrate arene oxides to trans dihydrodiols (7). The first solid evidence was in hand that, *in vivo*, dihydrodiol metabolites of the polycyclic aromatic hydrocarbons, known since the 1930's (8), were actually formed *via* arene oxides. Subsequent studies on the liver microsomal metabolism of naphthalene unequivocally demonstrated that naphthalene 1,2-oxide was the predominant metabolite of naphthalene and was the precursor of the *in vivo* metabolites naphthol, the trans 1,2-dihydrodiol, and a glutathione conjugate. In the course of these studies, Gillette and co-workers (9) had shown that one of the oxygen atoms in the dihydrodiol originated from water, providing further evidence for the arene oxide pathway. The chemistry of arene oxides (10,11) and their role in the metabolism of aromatic hydrocarbons (12) have been the subject of several reviews.

Unknowingly, Ron Estabrook then set the stage for what was to come. Through Estabrook's organization of an American Chemical Society Symposium in Washington, D. C., Daly and I were to meet for the first time Anthony Lu and Wayne Levin. Lu had recently joined Allan Conney's research group at Hoffmann-La Roche after achieving the first solubilization, purification, and reconstitution of a cytochrome P-450 with Minor Coon (13) at the University of Michigan. Subsequent meetings between myself, Levin, and Conney led to a collaboration that has produced over 200 papers in the past 8 years on the mechanism of action of the cytochrome P-450 isozymes and epoxide hydrolase and their role in the induction of cancer as elicited by the polycyclic aromatic

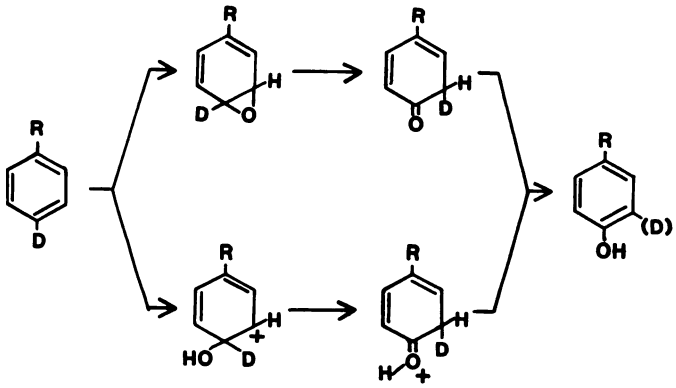

FIG. 1. *Possible mechanisms to explain the occurrence of the NIH shift.*

The upper pathway is depicted to proceed via an initially formed arene oxide whereas the lower pathway would result from initial attack of a hydroxyl cation or its equivalent.

hydrocarbons. These studies would have been impossible without the remarkable synthetic skills of Dr. Haruhiko Yagi at the NIH and the exceptional biochemical skills of Dr. Dhiren Thakker at the NIH and Dr. Alexander Wood at Hoffmann-La Roche.

Our initial goal was to define quantatively the metabolism of the widespread environmental carcinogen benzo[a]pyrene and to identify which of its metabolites were responsible for its tumorigenic activity in mammals, through systematic testing of all of its possible primary oxidative metabolites for tumorigenic activity. Perhaps because of the sheer magnitude of the problem, no other laboratory had attempted such an approach in the past, and consequently no ultimate carcinogen was known for any member of the polycyclic aromatic hydrocarbon class of carcinogens.

Over 15 man-years of effort were committed to the synthesis of various phenols, dihydrodiols, and arene oxides of benzo[a]pyrene (14–16) such that well characterized compounds were available as metabolite standards and for tumor studies. Through the use of a highly purified mono-oxygenase system reconstituted with cytochrome P-450c in the presence and absence of epoxide hydrolase, we demonstrated that several arene oxides were formed from benzo[a]pyrene and that they were precursors of various phenols and dihydrodiols (17). Tumor studies established that benzo[a] pyrene 7,8-oxide and 7,8-dihydrodiol were proximate carcinogens along the metabolic pathway leading to the ultimate carcinogen (18,19). Concurrently, studies of the ability of liver microsomes to catalyze the covalent binding of benzo[a]pyrene and certain of its metabolites to DNA suggested a benzo[a]pyrene 7,8-diol-9,10-epoxide as the reactive metabolite responsible for this binding (20,21). We succeeded in synthesizing the four metabolically possible optically active isomers of the 7,8-diol-9,10-epoxide (22), and we demonstrated that the (+)-(7R,8S)-diol-(9S,10R)-epoxide (fig. 2) was indeed the isomer responsible for nearly all of the tumorigenic activity of benzo[a]pyrene (23). This same stereoisomer predominates in the overall stereochemical course of the metabolism of benzo[a]pyrene to its 7,8-diol-9,10-epoxide (24,25).

Once it had become clear that a 7,8-diol-9,10-epoxide of benzo[a]pyrene was the most important ultimate carcinogen that was metabolically formed from benzo[a]pyrene in mammals, the stage was set for the development of a theory of polycyclic hydrocarbon-induced carcinogenesis. Reflections on the chemical reactivity of epoxides provided the crucible in which this new theory was to be formed. For any given carcinogenic polycyclic hydrocarbon in which one or more fused benzo-rings are present, several positional isomers of diol epoxides are possible. Interestingly, only one of these positional isomers is predicted to have high chemical reactivity relative to the others. This isomer is the one in which the epoxide group forms part of a bay-region (*cf.* fig. 2) of the hydrocarbon. Based on quantum mechanical considerations by myself and Dr. Roland Lehr and on a reevaluation of the limited biological data we proposed that, for those polycyclic aromatic hydrocarbons that were carcinogenic, bay-region diol epoxides would be prime candidates as metabolically formed ultimate carcinogens (26–28). As with any theory, the main value of the "bay-region theory" lies in its ability to make predictions that are then subject to experimental verification. Our laboratories at the NIH in conjunction with those of our colleagues at Hoffmann-La Roche, as well as a number of other laboratories both here and abroad, set out to test this new theory. The results of these tests have been most gratifying. To date more than a dozen hydrocarbons have been found to fit the predictions (*cf.* 29).

Once the formation of bay-region diol epoxides had been established as the primary means by which polycyclic aromatic hydrocarbons cause cancer, Drs. Sayer and Wood in our laboratories began a rational and systematic search for compounds that could block their biological activity through destruction of the reactive epoxide group. Ideally such a blocking agent should have high affinity for the diol epoxide through π-π interactions between the aromatic portions of the diol epoxide and the blocking agent. In addition, such a blocking agent should have both acidic and nucleophilic groups to aid in the destruction of the epoxide. The naturally occurring plant phenol, ellagic acid, is such a compound. It rapidly reacts (30) with the benzo[a]pyrene 7,8-diol-9,10-epoxide to form a *cis* adduct (fig. 3) and thereby markedly inhibits the mutagenic activity (31) of this compound *in vitro*. Tests to evaluate its ability to block tumorigenic activity *in vivo* are in progress.

In the course of our tumor studies on the polycyclic aromatic hydrocarbons, it has become clear that stereochemistry of bay-region diol epoxides is just as important a factor as reactivity in the elicitation of tumorigenic response. Thus, relative stereochemistry, absolute configuration and conformation (32) all play roles in influencing the tumorigenic response of bay-region diol epoxides. Our recognition of the critical role of stereochemistry in the tumorigenic response of the polycyclic hydrocarbons has led us, almost serendipitously, back to the cytochromes P-450. Having noted that single enantiomers of a given positional isomer of a bay-region diol epoxide generally possess nearly all of the tumorigenic activity, we sought to ask which of the four metabolic isomers was actually formed in mammals. As fate would have it, the highly tumorigenic enantiomer predominates in the overall metabolic sequence. Thus, in liver microsomes, the combined action of cytochrome P-450 and epoxide hydrolase results in the formation of (R,R)-dihydrodiols with bay-region double bonds. These dihydrodiols are then epoxidized, mainly by the cytochrome P-450c isozyme, to bay-region (R,S)-diol-(S,R)-epoxides (*cf.* 33). This remarkable stereochemical control has led to the development of a stereochemical model for the catalytic binding site of

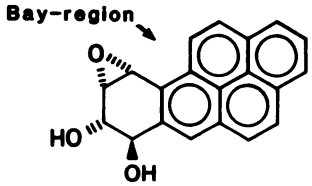

FIG. 2. *The structure of the first ultimate carcinogen identified for any member of the polycyclic hydrocarbon class: (+)-(7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene.*

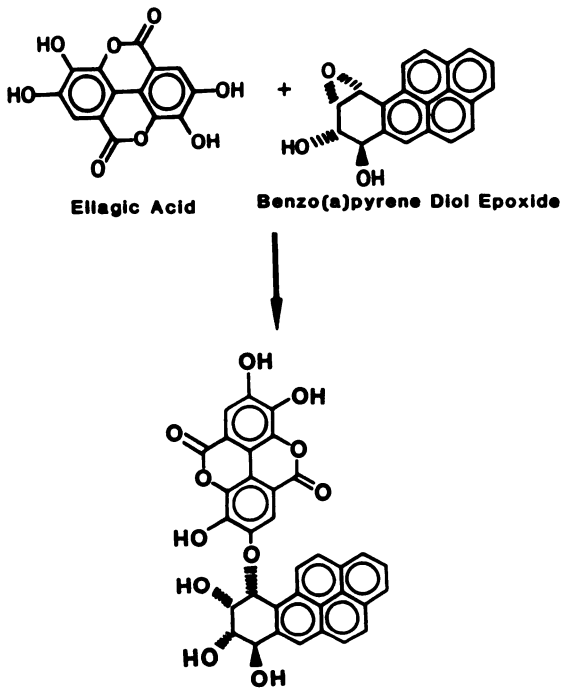

FIG. 3. *Reaction of benzo[a]pyrene 7,8-diol-9,10-epoxide with ellagic acid to form a cis-adduct.*

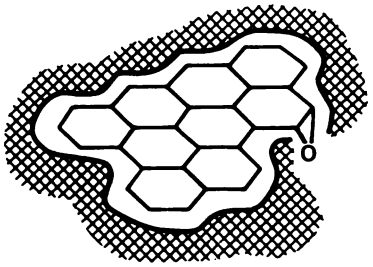

FIG. 4. *Minimum boundary of the catalytic binding site of cytochrome P-450c based on the absolute configuration of several metabolites of benzo-[a]pyrene.*

The boundary was constructed by a superimposition of these metabolites to align the epoxides that are formed. The resultant superimposition is not meant to represent a possible hydrocarbon but is meant to define the shape of the cavity.

cytochrome P-450c based on the absolute configuration of metabolites formed from benzo[a]pyrene (34). The model (fig. 4) has powerful predictive properties in terms of the absolute configuration of metabolites that are formed and in terms of the absence of otherwise reasonable metabolites that sterically could not be formed in the site.

The past 15 years have been filled with dramatic advances in the field of drug metabolism. It has passed from an area of endeavor in which few scientists found a permanant home to a broadly based field of research that has attracted the continued interest of biochemists, organic and inorganic chemists, immunologists, and geneticists, not to mention pharmacologists. An understanding of the regulation, multiplicity, structure, mechanism of action, and specificity of the cytochromes P-450 seems close at hand. To young scientists searching for an area in which to specialize, I would suggest that the cytochromes P-450 hold great potential. To those of you who have long worked in this field, you will well know what I mean when I say that the cytochromes P-450 are a class of enzymes that obey the exception rather than the rule.

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