ENZYME-CATALYZED DETOXICATION REACTIONS: MECHANISMS AND STEREOCHEMISTRY

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I. AN OVERVIEW OF DETOXICATION

A. Defense Against Chemical Insult

Modern industrial society has spawned spectacular technological progress that many contend is often at the expense of increasingly serious threats to the environment through inadequate control of chemical emissions, improper disposal of waste, and inappropriate or uninformed use of agricultural chemicals. Social, political, and economic issues aside, concern about the impact of environmental pollution on human health and the desire for rapid and rational development of useful chemical agents such as drugs and pesticides has appropriately prompted an enormous research effort by the biomedical community in the last two decades toward an understanding of how biological systems defend themselves against foreign chemicals. The relatively recent emergence of large-scale pollution from man-made sources should not be taken to suggest that selective pressure for organisms to develop mechanisms for the detoxication of foreign or xenobiotic compounds is also recent. Indeed, most organisms educated through that ultimate school-of-hard-knocks, evolution, "know" in exquisite molecular detail how to deal with many chemical and biological hazards.

Organisms have developed a number of defensive measures in response to a wide variety of molecular and supramolecular threats from hostile environments. In addition to the obvious physical barriers, which serve the integrity of biological systems, higher organisms have developed sophisticated and very flexible immune systems to render large molecules and particles harmless. In contrast, defense against the hazards of small molecules more often than not relies on metabolism to prepare substances for excretion via the normal routes of waste disposal. There is little doubt that the large share of the metabolic detoxication of small molecules is carried out by a family of enzymes specifically designed for that purpose.[1] It has therefore been suggested by Jakoby[1] that these enzymes be called detoxication enzymes.

B. The Metabolic Strategy of Detoxication

A central challenge for an organism in the detoxication of xenobiotics is the ability to metabolize for excretion a seemingly limitless number of molecules of all possible physicochemical descriptions; inert, reactive, electrophilic, nucleophilic, lipophilic, hydrophilic, and so on. These properties dictate how a molecule is absorbed and distributed in addition to the type of biotransformation necessary for detoxication. Molecules which pose the most difficult problems for an organism are lipophilic compounds which can penetrate physical barriers such as skin, mucous, and cell wall membranes to become distributed throughout the organism. Both reactive and inert lipophilic molecules must ultimately be transformed into hydrophilic species of low chemical potential for effective detoxication and elimination. Given the general nature of this metabolic problem it is not surprising that organisms rely on a family of enzymes capable of catalyzing a diverse group of chemical transformations.

Flexibility and insensitivity to substrate structure are keys to a successful strategy for the metabolism of foreign compounds. Such properties derive from three levels of molecular design. First, insensitivity to substrate structure is obtained, in part, through multiple forms or isozymes of the same enzyme which have different and sometimes overlapping substrate preference. For instance, at least eight isozymes of glutathione *S*-transferase composed of binary combinations of six subunit types have been isolated from rat liver cytosol.[2] Furthermore, microheterogeneity of the subunits appears to contribute even further to the diverse substrate specificity of this group of enzymes.[3,4] Second, the specific expression of detoxication enzymes is in many instances induced by exposure of an organism to a xenobiotic.[5] This genetic control allows organisms to respond to chemical insult in a more specific and flexible manner which is loosely analogous to the immune response of organisms toward large molecules. Finally, as is discussed in more detail below, individual detoxication enzymes generally show very loose constraints on substrate recognition. This relieves an organism, to a large extent, from the infeasible task of having to manufacture custom-designed enzymes for each foreign compound encountered.

The chemical transformations catalyzed by detoxication enzymes can be conveniently divided into two major classes, often referred to as phase I (functionalization) and phase II (conjugation) reactions.[6] Phase I reactions (oxidations, reductions, hydrolyses) often serve to add or unmask electrophilic or nucleophilic chemical handles on the xenobiotic molecule. These functional groups can then be used to append other, usually hydrophilic moieties such as glutathionyl-, glucuronyl-, or sulfuryl-groups in phase II transformations. In this fashion lipophilic xenobiotics can in principle be transformed into water-soluble products capable of being transported and excreted in the urine or bile. Once a hydrophilic metabolite is formed it is pertinent to ask, how is it transported out of the cell for excretion? Recent investigations[7] have clearly demonstrated conscription of specific or existing active transport systems for this purpose. It should be clear that the outcome of this metabolic scenario depends to a large extent on the chemical structure of the parent compound and the catalytic properties of the participating enzymes. It should be equally evident that given the limited resources of even the most sophisticated organisms a failsafe scheme for the detoxication of every xenobiotic is not possible. Paradoxically, enzyme-catalyzed detoxication reactions occasionally lead to metabolic activation of foreign compounds. That is, the chemical transformation leads to a metabolic intermediate of high chemical and/or biological activity. Metabolic activation can result from a single simple enzyme-catalyzed reaction as in the epoxidation of vinyl chloride, **1**, by cytochrome P-450 to the mutagenic chloroethylene oxide, **2**[8] (Figure 1) or from a serial combination of enzyme-catalyzed and spontaneous reactions. Examples of the latter are also shown in Figure 1. For instance, reaction of the soil fumigant 1,2-dibromoethane, **3**, with glutathione (GSH) catalyzed by GSH *S*-transferase gives 1-bromo-2-(*S*-glutathionyl)ethane, **4**, which rapidly rearranges by loss of bromide to the highly reactive episulfonium ion, **5**.[9] One of the most notorious cases of metabolic activation is the transformation of the relatively inert polycyclic aromatic hydrocarbon benzo[*a*]pyrene, **6**, to the highly tumorogenic 7,8-diol-9,10-epoxide, **9**, by the serial action of cytochrome P-450 and epoxide hydrolase.[10]

It sometimes appears that efforts to understand the mechanistic basis for detoxication dwell excessively on metabolic failures deleterious to the health of an organism. Regardless of this apparent emphasis it is clear that any real understanding of the metabolic basis of detoxication requires a thorough knowledge of the molecular details of the mechanisms of action of the enzymes involved. From this, perhaps the coherence of enzyme-catalyzed detoxication reactions can be thoroughly understood.

C. Catalytic Character of Detoxication Enzymes

Although exceptions certainly exist, several generalizations can be made concerning the catalytic character of detoxication enzymes. Unlike most enzymes which catalyze a specific

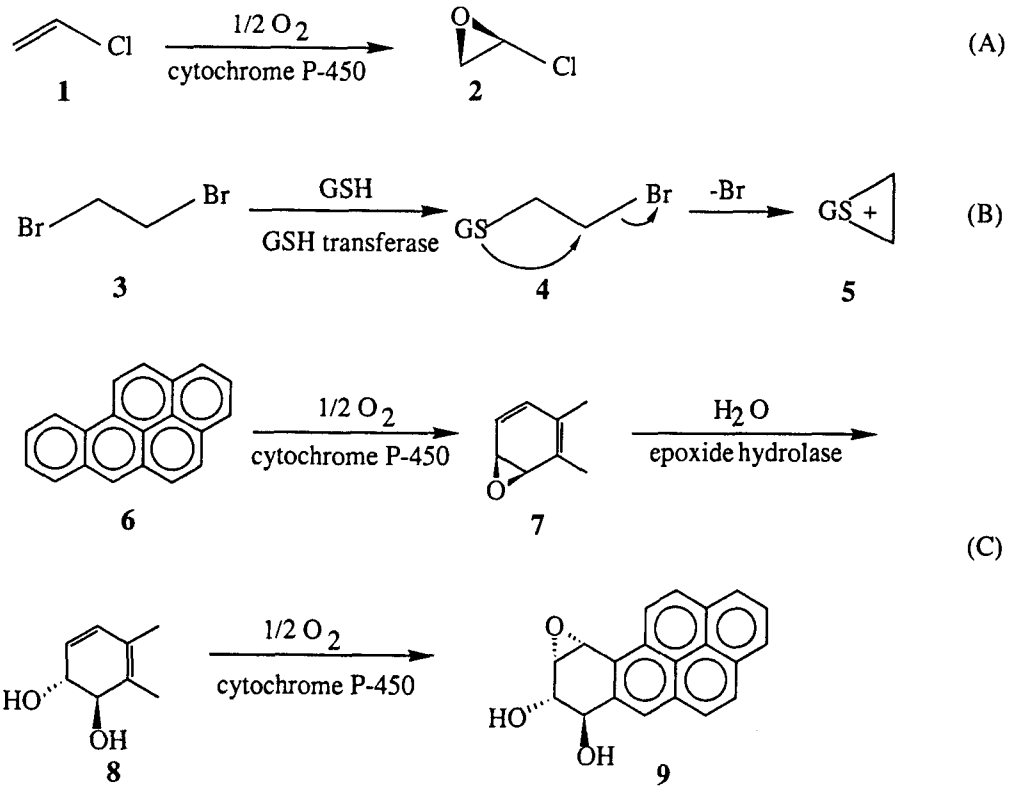


FIGURE 1. Examples of metabolic activation of xenobiotics by detoxication enzymes. (A) Oxidation of chloroethylene by cytochrome P-450. (B) Activation of 1,2-dibromoethane by glutathione *S*-transferase. (C) Oxidation of benzo[*a*]pyrene by cytochrome P-450 and epoxide hydrolase.

metabolic reaction with a high degree of substrate selectivity, detoxication enzymes exhibit rather broad selectivity at least toward the xenobiotic substrate. A major driving force for substrate binding and orientation with these enzymes is mutual desolvation of the lipophilic substrate and an often structurally ill-defined lipophilic surface on the protein. Such a simple yet general mechanism for substrate recognition is an obvious advantage to enzymes which must recognize structurally diverse molecules.

It is to be expected, however, that detoxication enzymes exhibit pronounced kinetic, stereochemical, or regiochemical discrimination toward a variety of substrates. One well-studied example is the metabolism of benzo[*a*]pyrene by rat liver microsomes to the 7,8-diol-9,10-epoxide illustrated in Figure 1. Cytochrome P-450 catalyzed oxidation of **6** occurs enantioselectively to give predominately the (7R,8S)-benzo[*a*]pyrene-7,8-oxide, **7**.[11] Regiospecific hydration of **7** by epoxide hydrolase gives the (7R,8R)-dihydrodiol, **8**.[11,12] Finally, oxidation of **8** at the 9,10-position occurs stereoselectively to give the (7R,8R,9S,10R)-benzo[*a*]-pyrene 7,8-diol-9,10-epoxide, **9**.[11,13] Interestingly, this series of stereochemical choices, dictated in large part by the topologies of the active sites of the enzymes involved, are not in the best interest of the organism inasmuch as 9 is at least an order of magnitude more potent as a tumorigen that the other three possible stereoisomers.[14] Methods which probe the topology of the active sites of detoxication enzymes are then quite useful in unraveling molecular mechanisms of metabolic activation and detoxication.

The ability of detoxication enzymes to accommodate a variety of substrates appears at first glance to occur at the expense of catalytic efficiency. Typical turnover numbers (k_c) are on the order of a few per second at best and quite often much lower. Arguments that

such low turnover numbers suggest that detoxication enzymes have rather crude, undeveloped catalytic machinery are not very convincing when one considers the difference in the nature of their task as compared to other enzymes. It is difficult to imagine how an enzyme might evolve to utilize substrate structure and binding energy to enhance k_c when the substrate structure is variable. In addition, it is plausible that low turnover numbers are in some measure a manifestation of a high degree of nonproductive substrate binding. After all, binding and binding in the correct orientation for catalysis are not one in the same.

Significantly, the catalytic efficiency of detoxication enzymes is more often than not manifest in k_c/K_m, the second-order rate constant for substrate recognition and catalysis. These enzymes often exhibit rather low values of K_m for the xenobiotic substrate, an optimum situation with respect to catalytic efficiency under conditions where the substrate concentration, $[S] < K_m$. Under normal conditions it is reasonable to expect that foreign compounds are found only at relatively low concentrations in organisms, hence low values of K_m offer an obvious catalytic advantage in their metabolism. Rather than squander binding energy to pay for higher turnover numbers which would be largely unutilized in the face of $[S] < K_m$ detoxication enzymes simply use it for seeking and holding the rare substrate molecule. Indeed a cogent argument has been made by Palcic and Klinman[15] that detoxication enzymes may have evolved toward tight substrate binding in response to the physiological conditions of $[S] \ll K_m$. Such tight binding should also appear in the transition state for the reaction.

Not to be overlooked is the fact that detoxication enzymes are very specific for the physiologic cosubstrates in their reactions. For example, cytochrome P-450, epoxide hydrolase, glutathione *S*-transferase, sulfotransferase, and UDP-glucuronosyltransferase show quite narrow specificity toward their respective cosubstrates oxygen, water, glutathione, 3′-phosphoadenosine 5′-phosphosulfate, and UDP-glucuronate. Such behavior is the norm for enzymes. Of course using carefully designed molecules these enzymes can be tricked into using alternative substrates, a valuable technique for elucidating mechanism. Experimental evidence[16] has suggested UDP-glucuronosyltransferase utilizes the intrinsic binding energy[17] of UDP-glucuronate to stabilize the transition state for the transferase-catalyzed reaction. So it would seem that detoxication enzymes like most others are really quite highly evolved catalytic machines. From the perspective of fundamental enzymology they represent an opportunity to investigate catalytic efficiency and specificity that arise from decreases in K_m (tight substrate and transition state binding) with xenobiotic substrates as well as the utilization of intrinsic binding energy (loose substrate binding) with the physiologic cosubstrates.

D. The Study of Detoxication Enzymes

Virtually all fields of biochemistry and the biological sciences including genetics, immunology, pharmacology and toxicology, medicinal chemistry, and enzymology are contributing crucial pieces to the current understanding of the puzzle of detoxication. Extremely important advances[18-21] in the genetics and regulation of expression of detoxication enzymes are elucidating how cells respond to chemical insult in a specific but flexible manner through the phenomenon of induction.[5] Immunological techniques are providing insite into the intracellular localization[22] and molecular structural relationships between the myriad isoenzyme forms of detoxication enzymes.[23,24] The more holistic approaches of pharmacology and toxicology are giving a unique view of the biochemical and temporal aspects of how enzymes and transport systems cooperate in the metabolism of xenobiotics. Finally, organic and medicinal chemical and enzymological approaches are illuminating the more microscopic and molecular aspects of the metabolic basis of detoxication.[25] Although all of these fields of endeavor are critical to a complete understanding of how organisms deal with foreign chemicals, a complete discussion of their respective contributions is well beyond the purview of a single review. This review instead will concentrate on state-of-the-art endeavors of the disciplines of bioorganic chemistry and enzymology to comprehend at the molecular level the details of enzyme-catalyzed detoxication reactions.

Mechanistic and stereochemical studies of detoxication enzymes have been and continue to be very useful in attempts to understand the fundamental nature of enzyme-catalyzed reactions and the role of catalysis in the metabolism of xenobiotics. Most studies of enzyme mechanism seek insight into the factors that determine the rate and course of enzyme-catalyzed reactions. Answers as to how the enzyme leads the substrates to the transition state for reaction are generally obtained from several types of observations including (1) the kinetics of individual reaction steps, (2) the influence of substrate reactivity and geometry on the reaction course, and (3) the molecular architecture of the enzyme-substrate complex. In the absence of X-ray crystal structures knowledge of chemical mechanism and stereochemical outcome of reactions can be used to piece together the molecular layout of active sites. These microscopic observations are quite useful in the somewhat larger context of understanding metabolic pathways. Kinetic studies suggest how effectively an enzyme may compete for substrate in a cellular milieu of other detoxication enzymes. Stereochemical and regiochemical choices by individual enzymes often dictate "channeling" of xenobiotics to particular metabolites.

A considerable number of mechanistic investigations of detoxication enzymes have been carried out on microsomes, partially purified enzymes or mixtures of isozymes. Although such studies can offer insight into the competition of isoenzymes for substrates, the organization of metabolic pathways and their overall outcome they are of limited use in elucidating chemical and kinetic mechanisms of catalysis. In fact, they are often misleading. Conversely, knowledge of the mechanism of an enzyme-catalyzed reaction in exquisite detail is no guarantee that one fully understands its role in metabolism. It is, however, a big step in the right direction. Through the application of modern, high-resolution protein purification techniques and the dogged efforts of many researchers it has been possible in recent years to obtain satisfactory quantities of highly purified isozymes of many detoxication enzymes for structural and mechanistic investigations. This review will focus primarily on mechanistic inquiry with homogenous enzymes.

Over two dozen enzymes can reasonably be said to be involved in the detoxication of foreign compounds to the extent that it is their primary function. Five of these, chosen mostly for their metabolic importance, representative nature and, of course, their familiarity to the author, are discussed here in some detail. For a more complete survey of enzyme-catalyzed detoxication reactions the reader is refered to two exellent series of monographs edited by Jakoby et al.[25,26]

It is perhaps desirable at this juncture to point out some of the more general aspects of the five enzymes discussed. Of the five, cytochrome P-450, is involved in phase I metabolism. Two of these, epoxide hydrolase and glutathione *S*-transferase, catalyze conjugation reactions toward molecules with electrophilic functional groups while the others, sulfotransferase and UDP-glucuronosyltransferase, catalyze group-transfers to nucleophiles. Three of the enzymes, cytochrome P-450, epoxide hydrolase, and UDP-glucuronosyltransferase, are membrane-bound enzymes found largely in the endoplasmic reticulum. Sulfotransferase and glutathione *S*-transferase are, for the most part, cytosolic proteins. Although epoxide hydrolase is highly functional group specific the others show little or no preference for specific functional groups. It is hoped that these enzymes fairly represent the diversity of catalytic chemistry encountered in metabolic detoxication reactions.

II. CYTOCHROME P-450

A. Monooxygenation

Monooxygenation is one of the most important and often seminal reactions in the metabolism of xenobiotic compounds. The reaction catalyzed by monooxygenases, the stoichiometry of which is shown in Equation 1, results in the insertion or addition of one atom of

oxygen from dioxygen to a substrate molecule and the other to water. Monooxygenases catalyze a rather diverse

$$NADPH + O_2 + S \rightarrow S-O + H_2O + NADP^+ \quad (1)$$

group of reactions in metabolism including hydroxylation, epoxidation, heteroatom oxidation, oxygen and nitrogen dealkylations, deaminations, heteroatom release, and reductions. Most of these reactions, with the obvious exception of the last, result either directly from oxygen atom insertion or addition or by subsequent rearrangement or reaction of a reactive intermediate. Perhaps the best testament to the importance and catalytic potential of monooxygenases and more specifically the cytochromes P-450 is that they alone are able to catalyze the initial step required in the metabolism of many relatively inert molecules such as hydrocarbons.

Quite a number of enzymes are capable of catalyzing monooxygenations either as their primary function or through mechanistic fortuity. Apart from the cytochromes P-450 most other hemeproteins that bind oxygen are capable of catalyzing some degree of monooxygenation chemistry given the required reducing equivalents. Thus, hemoglobin, myoglobin, horseradish peroxidase, and the dioxygenases all catalyze monooxygenation of organic molecules to some extent.[27-33] In addition, FAD-containing monooxygenases contribute significantly to the metabolsism of xenobiotics.[34] Considering the rather high concentration of some of these proteins, particularly the ones of oxygen transport, it is not unreasonable to expect that, even with relatively low catalytic rates, they may contribute significantly to detoxication reactions.

It is, however, widely believed that the cytochromes P-450 are the group of heme-containing proteins primarily responsible for the monooxygenation of foreign compounds. The proteins are widely distributed in nature, occurring in plants, animals, and microorganisms. Their central importance to metabolism, their ability to catalyze an amazing variety of often difficult reactions, and the rich chemistry associated with their catalytic mechanism has prompted a staggering amount of research over the last 10 years. It is obviously not possible to site all of the elegant and informative studies performed even in the last few years. Fortunately, several recent, excellent review articles[35-37] and a series of monographs[38] are available for the reader to consult.

B. An Overview of the Mechanism of Cytochrome P-450-Catalyzed Oxidations

The generally accepted mechanism for the red-ox cycle of cytochrome P-450 illustrated in Figure 2 consists of two substrate binding steps (steps 1 and 3) and two electron transfer or reduction steps (steps 2 and 4) which lead to the first central complex, A. Two product release steps (steps 5 and 6) through a second central complex B, where oxidation of the organic substrate occurs, completes the catalytic cycle. Addition of substrates is rapid. In biological systems reducing equivalents derived ultimately from NADPH are supplied in steps 2 and 4 by NADPH cytochrome P-450 reductase, or in the bacterial system by putidaredoxin. Although the electron transfer steps are rate limiting in some cases,[35] turnover of the catalytic cycle is often limited by chemistry occurring in the second central complex.

The three questions most relevant to any investigation of the mechanism of cytochrome P-450 catalysis concern: (1) the mechanism of formation of the second central complex (oxygen activation), (2) the mechanism(s) of decomposition of the central complex (oxygen transfer), and (3) the influence of physical constraints of the enzyme active site on the course of the oxygen transfer. The latter two questions are perhaps of the greatest interest to metabolic detoxication. Of these two questions it is usually reasonably assumed that the basic features of oxygen transfer chemistry are similar regardless of which particular isoenzyme is under study. In contrast, the regiochemical or stereochemical course of oxygen

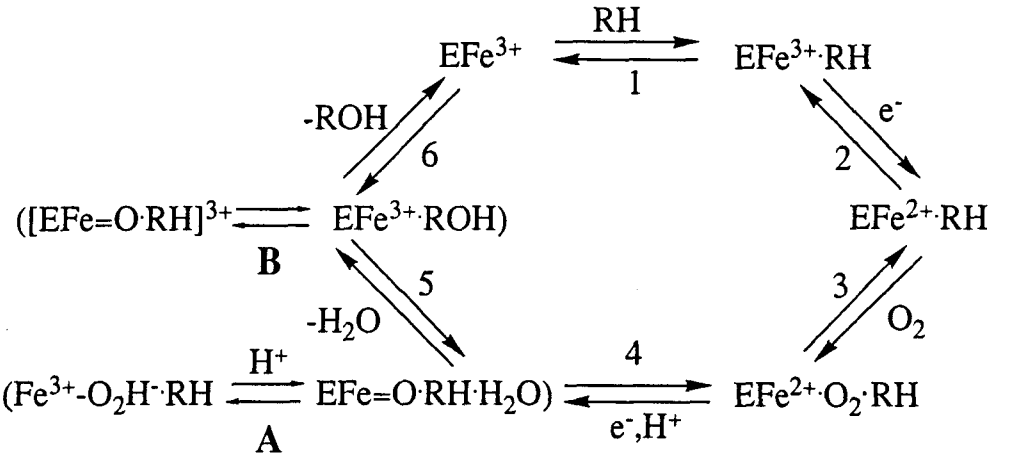


FIGURE 2. Proposed catalytic cycle for cytochrome P-450.

transfer is, in part, a structural question over which an individual isoenzyme may indeed exert a great deal of physical control.

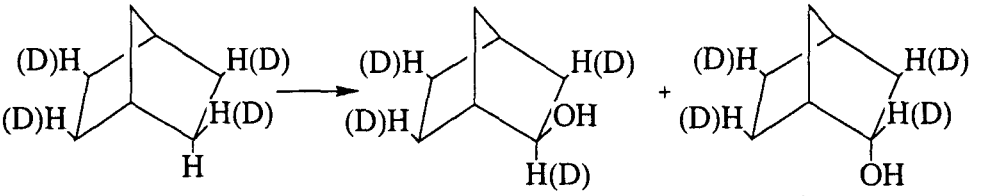
It is now generally accepted that the oxidizing species in the catalytic cycle is an iron-oxo intermediate, or oxene complex.[39,40] It is often convenient if not accurate to think of cytochrome P-450-catalyzed reactions as oxene reactions analogous to those of the isoelectronic carbene species. The events leading to formation of the iron-oxo complex are by no means as clear as illustrated in Figure 2. A variety of side reactions or uncoupling reactions may occur under the right circumstances. For instance, in the absence of oxygen the one electron-reduced $EFe^{2+}\cdot RH$ complex can catalyze reduction of a bound substrate, e.g., benzo[*a*]pyrene 4,5-oxide to benzo[*a*]pyrene.[41] Furthermore, the one electron-reduced $EFe^{2+}\cdot O_2\cdot RH$ species can decompose to give peroxide radical and the $EFe^{3+}\cdot RH$ complex in the absence of a second electron transfer (step 4). Although there is a rich variety of chemistry in the steps leading to oxygen activation the focus of the discussion below will be on the oxygen transfer step and the influence of the protein on the course of oxygen transfer.

C. Oxygen Transfer: Hydroxylation

Since the original studies by Groves and co-workers[42] it has become widely accepted that hydroxylation of aliphatic hydrocarbons proceeds as shown in Equation 2. The first step, homolytic cleavage of the C-H bond is slow and

$$([EFe=O,\ HCR_3] \rightleftarrows [EFe\text{–}OH,\ \cdot CR_3] \rightleftarrows [EFe^{3+},\ HOCR_3]) \qquad (2)$$

forms a short-lived carbon radical-ferric hydroxide radical pair which collapses to the enzyme-product complex. The original evidence for this mechanism derived from observations on the cytochrome $P\text{-}450_{LM2}$ hydroxylation of norbornane, **10**, and all-*exo*-2,3,5,6-tetradeuteronorbornane, D_4-**10**. As illustrated in Figure 3, hydroxylation of **10** is stereoselective giving predominately the *exo*-product, **11**. Quite in contrast, the enzyme exhibits a switch in stereoselectivity toward D_4-**10** giving a slight excess of the *endo* D_4-**12** product. More interestingly, the *exo*-product from D_4-**10** was a 75/25 mixture of D_3-**11** and D_4-**11**. Formation of D_4-**11** is most reasonably attributed to initial abstraction of the *endo*-hydrogen followed by in-cage epimerization to *exo*-radical before radical recombination. From these data an intrinsic isotope effect of 11.5 ± 1 can be calculated for C-H bond cleavage. Interestingly, the isotope effect is not manifest in the overall rate of product formation, so C-H bond breaking is, in this case, not rate limiting.



	11	12
10	3.4	1
$D_4$10	0.76	1

FIGURE 3. Stereoselective hydroxylation of norbornane, **10**, and all-*exo*-2,3,4,5,6-tetradeuteronorbornane, D_4-**10** by cytochrome P-450$_{LM2}$. Data from Reference 42.

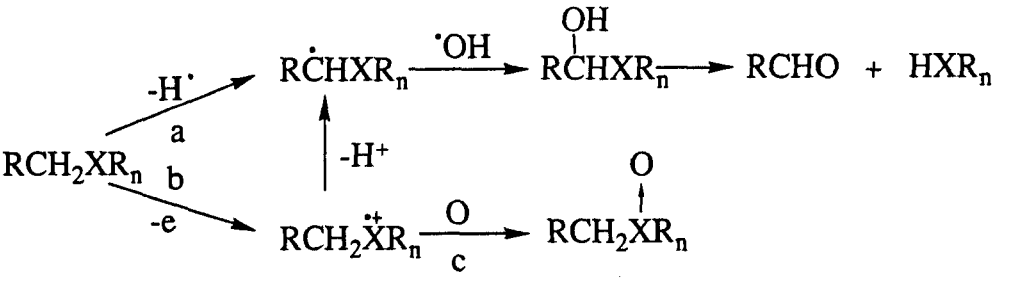


FIGURE 4. Pathways for oxidative dealkylation (a and b) and heteroatom oxidation (c) of heteroatom containing molecules. n = 2, 1, and 0 for group V, VI and VII elements, respectively.

D. Oxygen Transfer to Molecules Containing Heteroatoms

The cytochromes P-450 catalyze oxidation of organic molecules containing group V, VI, and VII elements, the consequences of which vary dramatically with the heteroatom and the structure of the alkyl group. The two usual reactions are heteroatom dealkylation and heteroatom oxygenation as shown in Figure 4. Evidence exists for two pathways for heteroatom dealkylation, which differ in the initial path of radical formation. Partitioning of substrate between α-hydrogen atom abstraction (path a) or single electron transfer (SET) to form a heteroatom cation radical (path b) followed by loss of an α-proton is thought to depend to a large extent on the first electron ionization potential of the heteroatom.[36] Thus, molecules with heteroatoms of low ionization potential (P,N,S,I) prefer cation radical formation. Heteroatomic molecules which have atoms of low ionization potentials and lack α-hydrogens or form cation radicals which are relatively stable (e.g., -$S^{+\cdot}$-) with respect to the α-carbon radical may trap atomic oxygen in the central complex thus accounting for heteroatom oxygenation (path c).

There is good evidence that oxidative cleavage of heteroatomic species with atoms of high ionization potential (e.g., oxygen as in ethers) proceeds via hydrogen atom abstraction (path a, Figure 4). Miwa and co-workers have recently reported an elegant demonstration of the power of primary kinetic isotope effects in distinguishing between mechanisms as in Figure 4 but also, just as importantly in identifying just when, in the catalytic cycle, the enzyme is committed to catalysis.[43,44] Observed kinetic deuterium isotope effects ($^{D}V/K$) in the O-dealkylation of 7-ethoxycoumarin (**13**, Figure 5) catalyzed by rat liver cytochromes P-450 and P-448 were 3.8 and 1.9, respectively, and very much lower than the intrinsic isotope effects (k_H/k_D = 12.8 and 14.0) found for C-H bond cleavage. The large intrinsic isotope effects are similar in magnitude to that found for aliphatic hydroxylation via hydrogen abstraction followed by radical recombination[42] and strongly suggest a similar rate-limiting

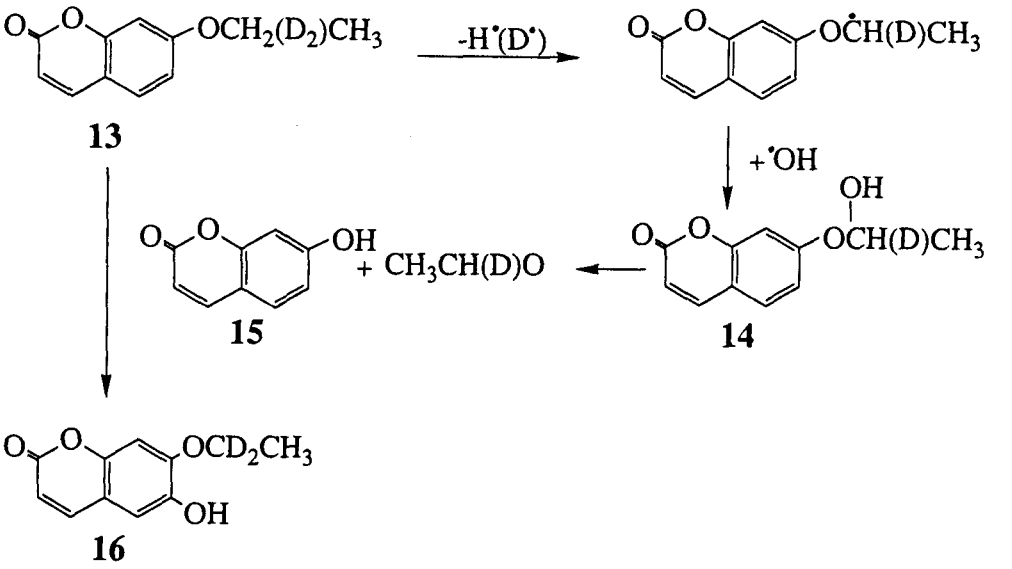


FIGURE 5. Proposed mechanism for the oxidative dealkylation of 7-ethoxycoumarin, **13**.[43,44]

step in formation of the hemiacetal intermediate, **14**. Of course these results do not unequivocally rule out the alternative of rapid, reversible single electron transfer from oxygen followed by slow loss of the α-proton.

Diminution of the intrinsic isotope effect indicates that the second central complex (B of Figure 2) tends to partition toward product (C-H bond cleavage) rather than toward dissociation of the substrate. The enzyme-substrate complex is, at this point, committed to catalysis. An interesting alternative for supression of the intrinsic isotope effect has been demonstrated in the cytochrome P-448-catalyzed reaction.[44] When D_2-**13** was used as substrate a fivefold increase in a minor reaction product, 6-hydroxy-7-ethoxycoumarin, **16**, was observed, with no change in either the turnover rate of the cycle or in the concentrations of oxo-enzyme intermediates. This regiochemical switching from a heavy to light isotopic site may be ascribed to dissociation of the enzyme-substrate complex away from the isotopically sensitive step and is reminiscent of the stereochemical switching observed in norbornane hydroxylation.[42] Finally these observations clearly demonstrate that an irreversible step exists between the oxy-enzyme intermediates formed in steps 3 and 4 of Figure 2 and substrate oxidation. That step is likely to be dioxygen bond cleavage in the decomposition of the first central complex.

That dealkylations of thioethers and amines occur by cation-radical formation (path b, Figure 4) is supported by two lines of evidence. First, intrinsic kinetic isotope effects are much smaller than those observed for ethers,[45,46] consistent with a cation-radical deprotonation mechanism. Second, the suicide inhibition of cytochrome P-450 by substituted cyclopropylamines gives a clear indication of one electron oxidation chemistry.[47,48] Both **17** and **18** (Figure 6) are mechanism-based inhibitors of cytochrome P-450. Although α-hydroxylation and dehydration of **17** to the Schiff's base **19** may account for some or all inactivation by **17** this is not possible with **18**. In as much as **18** is just as effective as an inhibitor, single electron transfer to the cation-radical followed by rapid rearrangement to a carbon radical (**20** or **21**) which inactivates the enzyme is implicated.[47,48]

Heteroatom oxygenation is likely a closely related process to dealkylation. Indeed, Sligar and Bruice[49] have found evidence for the possible involvement of *N*-oxides and the *N*-dealkylation of amines. The crucial finding is that *N*-oxides of dimethylaniline can serve as the oxidant for the demethylation of the *N*-oxide. Unlike iodosobenzene, which can act as

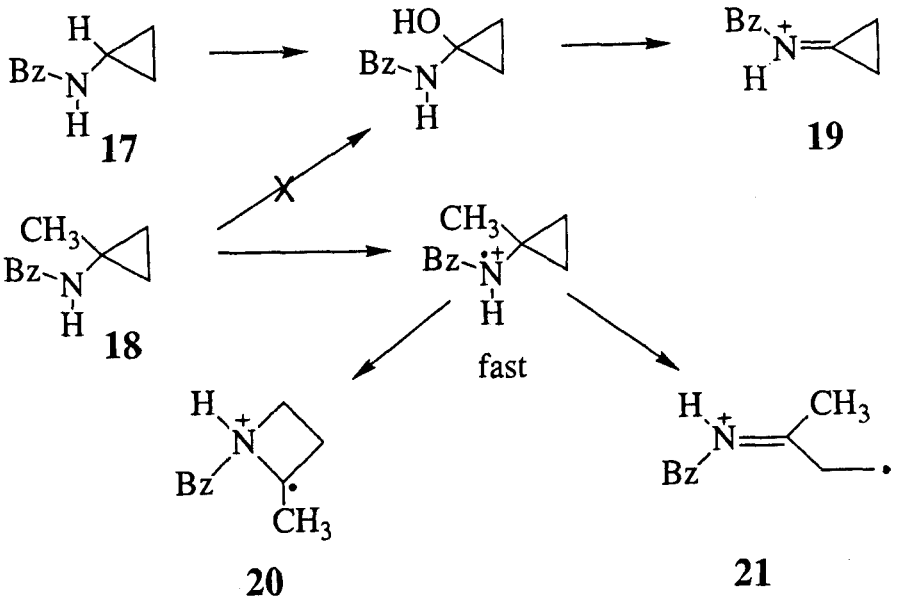


FIGURE 6. Alternative mechanisms for generation of reactive species in the suicide inactivation of cytochrome P-450 by substituted cyclopropylamines.[47,48]

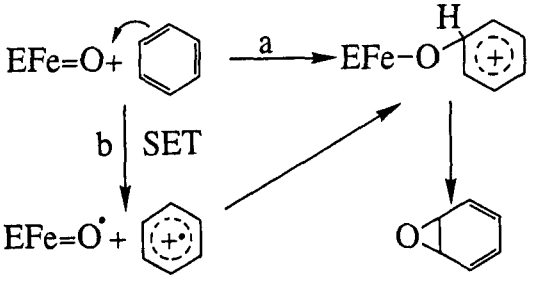


FIGURE 7. Electrophilic (a) and radical (b) mechanism for the cytochrome P-450-catalyzed epoxidation of arenes.

an alternative oxygen source for substrate oxidation (both of itself and other substrates)[50] the *N*-oxides do not support normal substrate oxidation suggesting that hydroxylation at the α-position occurs very rapidly relative to dissociation of the iron-oxo dimethylanaline complex.

It has been pointed out by Guengerich and Macdonald[36] that both pathways of single electron transfer or α-hydrogen atom abstraction can, and probably do occur to some extent in most heteroatomic molecules. The major pathway for a given molecule including heteroatom oxidation is, in general predictable from structure and ionization potential.

E. Oxygen Transfer to Alkenes and Arenes

Oxidation of unsaturated compounds by cytochrome P-450 often yield epoxides and arene oxides which are important reactive intermediates in many detoxication pathways. In addition, arene oxides are, more often than not, intermediates in the hydroxylation of aromatics. Both concerted and stepwise mechanisms have been considered for epoxidation reactions catalyzed by the enzyme. Arguments for a concerted mechanism with simultaneous formation of both carbon-oxygen bonds have been based primarily on the observation that olefin stereochemistry is retained in the product. Of course retention of stereochemistry may simply be enforced by steric constraints in the active site. Some evidence but no general consensus has emerged for stepwise mechanisms outlined in Figure 7 involving either electrophilic

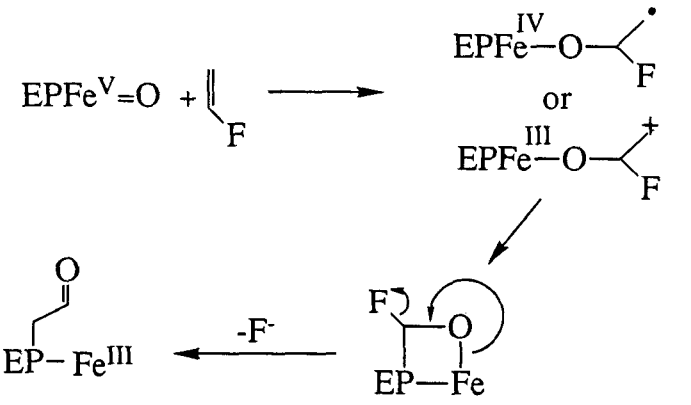


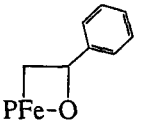
FIGURE 8. Possible intermediates in the suicide inactivation of cytochrome P-450 by vinyl fluoride.[51]

addition of activated oxygen with an ionic intermediate (path a) or by radical formation.[36] Formation of a radical intermediate can occur by a sequential single electron transfer radical recombination pathway (path b) or by a direct mechanism.

Compelling evidence as to mechanism has again been obtained by studies of the suicide inactivation of cytochrome P-450 during the oxidation of substituted olefins. For instance, vinyl fluoride inactivates cytochrome P-450 during turnover to yield *N*(2-oxoethyl)protoprophyrin IX[51] as shown in Figure 8. A cogent argument has been made for a radical intermediate based on the expected stabilities and reactivity of possible cation and radical intermediates and the regiochemistry of the inactivation. Thus, electrophilic formation of the carbon oxygen bond is expected to occur at the unsubstituted carbon, regiochemistry opposite to that shown in Figure 8. Less discrimination would be expected for radical formation. Alternatively, heme alkylation could occur by reaction of an epoxide intermediate with one of the pyrrole nitrogens. The involvement of epoxides in cytochrome P-450 inactivation seems unlikely inasmuch as epoxides have not been shown to inactivate the enzyme by alkylation of the porphyrin.[36]

It is not possible, at this time, to say without equivocation that both epoxide formation and heme alkylation with olefins occur via a common radical intermediate. Indeed, recent studies of the oxidation of phenylacetylenes suggest that the mechanisms for formation of phenylacetate and heme alkylation diverge very early in catalysis.[52] Direct stepwise oxygen addition remains a distinct possibility in epoxidations.

The recent, exciting observation[53] of an intermediate in the epoxidation of styrene by a model heme system, the iron-oxo complex of 5,10,15,20-tetramesitylporphyrin at cryogenic temperatures is the strongest indiction yet that epoxidation is a stepwise process. The intermediate has been proposed to be either a metallooxophenylcyclobutane, **22**, or an iron-oxo ρ-olefin complex. Furthermore, the rate constant for formation of the intermediate correlated well with σ^+ for a series of substituted styrenes with a ρ value of -1.9, consistent with initial single electron transfer between the iron-oxo complex and the substrate.



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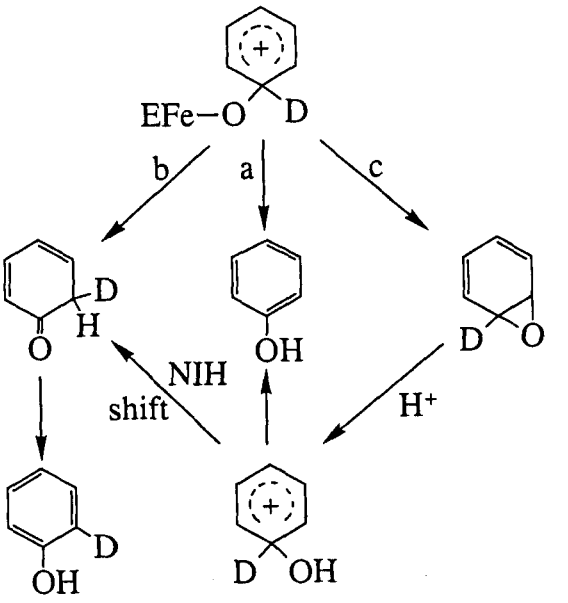


FIGURE 9. Mechanisms for hydroxylation of deuterobenzene by (a) direct formation of phenol, or (b) its ketotautomer, or (c) an arene oxide intermediate.

Historically, one of the most significant findings in cytochrome P-450-catalyzed oxidations of unsaturated compounds was the migration of substituents from the para to the meta position during the para hydroxylation of aromatics. This phenomenon, called the "NIH shift", along with the first isolation of an arene oxide in metabolism was taken to suggest the intermediacy of arene oxides in the hydroxylation of aromatic hydrocarbons.[54-56] Arene oxide chemistry and biochemistry has been recently reviewed.[57]

In principle, the hydroxylation of aromatics can occur directly as in the case of aliphatic hydroxylation (Figure 3), by direct formation of phenol or its keto tautomer from an [EFe-O-Ar] complex, or via an arene oxide intermediate as shown in Figure 9. Hanzlik and co-workers[58] have recently reported evidence for hydroxylation of substituted benzenes involving both NIH shift and loss of deuterium label. Results of their studies suggest observation of the NIH shift does not necessarily require involvement of an arene oxide intermediate, but does implicate a cyclohexadienone intermediate in all NIH shift-like migrations. Such results are consistent with the view that the exact mechanism of aromatic hydroxylation is a sensitive function of substrate reactivity and orientation of the substrate in the enzyme-substrate complex.

F. Structure of Cytochrome P-450

The structural characteristics, particularly those of the active site, are obviously important for any rigorous analysis of the mechanism and stereochemistry of cytochrome P-450-catalyzed reactions. The enzymes from animal sources are membrane-bound enzymes usually located in the smooth endoplasmic reticulum. This fact as well as the existence of multiple isoenzymes creates difficulties in the solubilization and separation of individual enzymes. In spite of this, a great deal is known about the primary sequence of the enzymes from various sources.[18,59-61] These studies have been particularly significant in that they support the implication of a highly conserved cysteinyl peptide which acts as the fifth (axial) ligand to the heme iron in the active site. In oxygen-binding proteins such as hemoglobin this ligand is a histidyl side chain. The importance of the presence of a fifth ligand in catalytic turnover of a model heme system has been demonstrated.[52]

Although much is known of the primary sequence of the cytochromes P-450 relatively little was known, until recently, about the three-dimensional structure. Fortunately, the soluble bacterial cytochrome P-450 from *Pseudomonas putida* (cytochrome P-450_{cam}) has yielded to three-dimensional structural analysis by X-ray crystallography. Cytochrome P-450_{cam} functions in nature as a rather specific 5-*exo*-hydroxylase, the initial step in the metabolism of camphor. There is every reason to believe that the basic mechanistic features of catalysis are the same as those for the eukaryotic enzymes. Given the right substrate the enzyme will, in fact, catalyze dealkylation and epoxidation reactions.[49,62,63] The bacterial enzyme has been deduced to have a shape in solution that approximates a cylinder 3.0 nm in diameter and 8.0 nm in length.[64] More to the point, however, is the recent solution of the crystal structure of cytochrome P-450_{cam} at 2.6 Å and later at 1.7 Å resolution.[65] The structural data from X-ray crystallography is of invaluable help in understanding the mechanism of oxygen activation, transfer, and substrate accomodation by cytochrome P-450. Structural features of the protein are described very briefly below.

The ferric cytochrome P-450_{cam}-camphor complex is roughly the shape of a triangular prism about 3.0 nm thick and 6.0 nm on a side, not unlike the dimensions determined in solution.[64] The heme is wedged between two parallel helical segments and surrounded by largely hydrophobic amino acids except for two arginine residues (112, 299) and His 355 which interact with the propionates of the heme group. Significantly, the edges of the heme are not exposed suggesting electron transfer must involve contacts with the protein surface. The axial heme ligand is the thiolate side chain of Cys 357 which is coordinated to the α-face (as defined by Rose et al.[66]) of the heme group. The camphor molecule appears to be oriented about 0.4 nm from above the A ring of the porphyrin and adjacent to the oxygen binding site. The molecule is oriented for 5-*exo*-hydroxylation by contact with a group of hydrophobic residues and perhaps most importantly by an apparent hydrogen bond between the hydroxyl group of Tyr 96 and the carbonyl group of the substrate. This later feature is shown in an approximate representation (Figure 10) of the orientation of camphor, the heme, and the axial thiolate ligand in the complex.

The structures of the eukaryotic cytochromes P-450 beg comparison with the bacterial enzyme. Sequence comparisons suggest that considerable homology exists between the cystienyl peptides which supply the axial ligand to the heme as well as similarities in the distal helix-heme contact region.[65] Perhaps more important are the differences in the polypeptides that compose the substrate-binding sites of the various enzymes. It has been pointed out that from sequence alignments that neither the Tyr 96 or Val 295, two regions that are critical for camphor orientation, are conserved in eukaryotic enzymes.[65] It is perhaps in these regions that control of substrate specificity is at least in part, dictated in the enzymes from higher organisms.

The solution and continued refinement of the three-dimensional structure of cytochrome P-450_{cam} is a major advance in our understanding of the chemistry of this class of enzymes. In the absence of similar structural data for the eukaryotic enzymes it may be possible to approximate some features of their structure by computations based on the bacterial data set. At present, more indirect techniques discussed below must be used to obtain a much lower resolution picture of the active site of these cytochromes P-450.

G. Stereoselectivity and Regioselectivity of Cytochromes P-450

Investigations of the stereo- and regioselective behavior of monooxygenases are valuable in discerning the contributions of various isoenzymes to particular metabolic reactions and in mapping the topologies of substrate-binding sites in favorable instances. To date such studies have employed two types of chemistry. On the one hand Ortiz de Montellano and co-workers have utilized mechanism-based chemical modification to explore the immediate environment of the heme. Most other investigations have focused on the stereo- and regio-

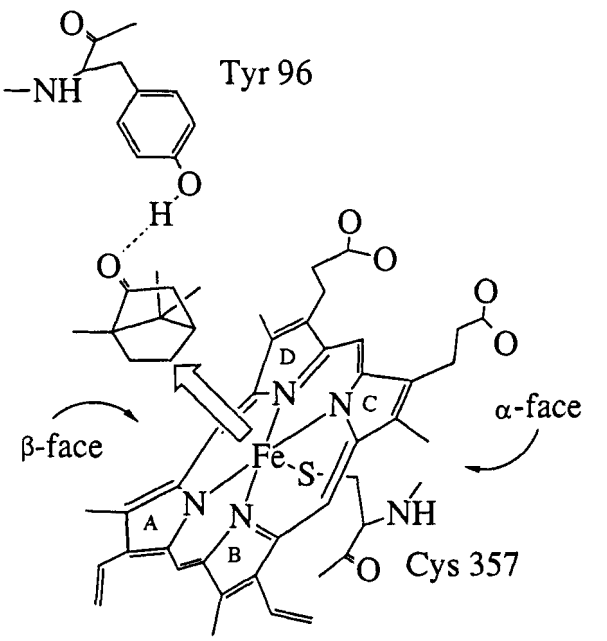
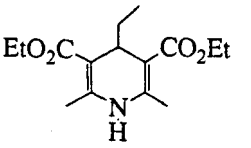


FIGURE 10. Approximate orientation of heme, cysteine 357, camphor, and tyrosine 96 in the cytochrome P-450$_{cam}$-camphor complex.[65] Note that the designation of the prochiral faces of the heme is after Rose et al.[66]

selective turnover of substrates. The latter technique has been used successfully by Jerina et al. to construct a model of the substrate-binding site for cytochrome P-450$_c$.



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1. Mechanism-Based Chemical Modification

The observation[67] that the cytochromes P-450 from rat liver catalyzed transfer of the 4-alkyl group from 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine, **23**, to the pyrrole nitrogens of heme was subsequently exploited to determine the facial orientation of the heme group in the active site of the eukaryotic enzymes.[68] The substitution pattern of the heme group renders the two faces of the molecule prochiral as illustrated in Figure 10. Alkylation by **23** during catalysis should occur on the same face of the heme where oxygen activation and transfer take place. Chiroptical comparison of the C-ring alkylated *N*-ethyl-protoprophyrin IX with the same compound obtained from the ethylation of hemoglobin (in which the absolute orientation of the heme is known) with ethyl hydrazine clearly established that heme alkylation occurred on the β-face[66] of the prosthetic group.[68] This is fully consistent with the absolute configuration of heme coordination in cytochrome P-450$_{cam}$ by X-ray crystallography.[65]

In principle, all four regioisomeric *N*-alkylated protoprophyrins can be obtained during

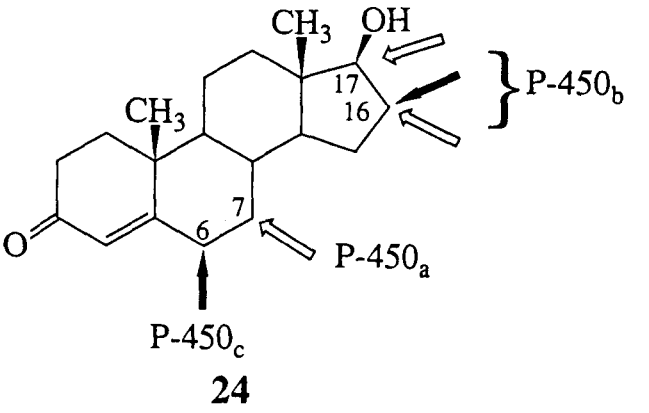


FIGURE 11. Summary of the regio- and stereoselective hydroxylation of testosterone by rat liver cytochromes P-450$_o$, P-450$_b$, and P-450$_c$.[71,72]

catalytic alkylation. This is in fact observed with **23**. However, mechanism-based alkylation of heme by olefins and acetylenes is markedly regioselective and dependent on the substrate.[69] Thus, alkylation of microsomal cytochrome P-450 by alkenes occurs on the D-ring and by alkynes on the A-ring. Interestingly, partitioning of 1-octene between epoxidation and heme alkylation is stereospecific. Heme alkylation occurs only if initial oxygen addition occurs on the *re* face of the double bond.[70] In contrast, epoxidation occurs on both faces with retention of substrate stereochemistry. The results cannot be unambiguously interpreted since heterogeneous microsomal enzyme was used. These results and the lack of B-ring alkylation have been taken to suggest that the B-ring is buried in the protein matrix, a conclusion consistent with the heme environment in cytochrome P-450$_{cam}$.[65]

2. Stereo- and Regioselective Hydroxylations

The stereo- and regioselectivity of monooxygenation has been examined in a number of reactions. Steroids are hydroxylated to a large extent in liver to give hormone metabolites with dramatically altered biological activity and are thought in some instances to be physiological substrates for cytochrome P-450. Not surprisingly, then, the regio- and stereoselectivity of several isozymes of cytochrome P-450 from rat liver has been investigated.[71,72] These reactions tend to be more or less regioselective depending on the isoenzyme and substrate. For example, testosterone, **24** (Figure 11) is hydroxylated in a highly regio- and stereoselective fashion ($\geq$ 95%) at the 7 α position and (~100%) at the 6 β position by cytochromes P-450$_a$,[71] (PB-3),[72] and P-450$_c$, respectively. In contrast, the P-450$_b$ isozyme is less discriminate, catalyzing hydroxylation at the 16α, 16β, and 17 positions with respectable turnover numbers. Hydroxylation of **24** at the 17-carbinol position gives after dehydration androstenedione which is rapidly hydroxylated at 16β by the same isoenzyme (P-450$_b$). One wonders if the first product is necessarily released from the enzyme surface before the second cycle of oxidation.

Investigations of a similar nature have revealed that the cytochromes P-450 from rat and rabbit liver can distinguish between the two antipodes of the anticoagulant warfarin.[73,74] For example, cytochrome P-450 (MC-A) from rat liver catalyzes the efficient hydroxylation of both R and S warfarin but clearly differentiates between the two enantiomers in hydroxylation at the 8-position where the R antipode reacts approximately eightfold more efficiently than the S. More extensive exploration of the regio- and stereochemical preferences should facilitate the construction of models for the substrate binding sites of these enzymes.

An interesting illustration of the contrast between the highly specific cytochrome P-450$_{cam}$ and rabbit liver P-450$_{LM2}$ is found in the hydroxylation of *d*-camphor, adamantane, and adamantanone.[75] Cytochrome P-450$_{cam}$ hydroxylations are regiospecific and predictable based on the natural substrate structure as well as the three-dimensional structure of the enzyme.[65] The rabbit enzyme exhibited little or no regioselectivity toward the same molecules.

3. Chiral Sulfoxidation

Oxygenation of heteroatoms has also been shown to be enantioselective. Walsh and co-workers demonstrated that thioethers (e.g., *p*-tolyl ethyl sulfide) are preferentially (80 to 85%) oxygenated at the pro-S lone pair on sulfur by phenobarbital-induced isozymes from rat liver.[76] Quite in contrast, the FAD-containing monooxygenase from hog liver gave almost exclusive (~ 95%) oxygenation at the pro-R enantiotopic position.[77]

4. Chiral Epoxidations

The most extensively studied aspect of the stereospecificity of the cytochromes P-450 is the oxidation of aromatic hydrocarbons to arene oxides. Oxidation of prochiral double bonds in polycyclic aromatic hydrocarbons can lead to two possible enantiomeric arene oxide products differing as to which side of the molecule the oxene is added. The recognition[78] that the enantioselectivity exhibited by rat liver cytochrome P-450$_c$ in the oxidation of polycyclic aromatic hydrocarbons could be exploited in defining the substrate binding site of the enzyme lead Jerina and co-workers to examine the chiral epoxidations of a number of such molecules.[78-83]

Before summarizing the results of these investigations it is beneficial to discuss the techniques for determining the stereochemistry of chiral epoxidations. Obviously any method for elucidating the enantioselectivity of cytochrome P-450 greatly benefits from if not requires synthetic products of known absolute configuration. Given these resources the real problem is one of how to efficiently analyze reactions for small amounts of chiral and often labile arene oxides. The methods most often used are nucleophile trapping with a chiral nucleophile,[78] regioselective opening of the oxirane with a chiral catalyst (e.g., epoxide hydrolase)[11,12,84] or regioselective trapping with an achiral nucleophile and stereochemical analysis of the more favored product.[83]

Trapping of arene oxides with chiral nucleophiles such as glutathione,[78,80] *N*-acetyl-L-cysteine,[82,84] or polyguanylic acid[85] and analysis of the diastereomeric addition products has proven most successful. The analysis is illustrated in Figure 12 for the oxidation of benz[*a*]anthracene, **25**, at the 5,6 double bond by cytochrome P-450$_c$ and trapping of the 5,6-oxide, **26** with glutathione. Reaction of racemic **26** with glutathione gives two diastereomeric pairs of positional isomers, **27** and **28**. In contrast, trapping of **26** generated by rat iver cytochrome P-450$_c$ gives predominately (> 97%) (5R,6R)-**27** and (5S,6S)-**28**,[79] clearly demonstrating a large stereochemical preference for the β-enantiotopic face[66] of the 5,6 double bond.

Stereochemical analysis of a number of such oxidations have been collated to derive a rough model of the substrate binding site of cytochrome P-450$_c$ as illustrated in Figure 13. Note that α-face oxidation of **25** to **26** requires accommodation of the substrate outside the boundries of the binding site. It is also interesting to note that oxidation of smaller substrates might be expected to occur with lower enantioselectivity. This is indeed observed with naphthalene.[82] It should be obvious that this type of analysis does not distinguish the forces that enforce the stereoselectivity. Unfavorable van der Waal's contacts with the protein and/or mutual desolvation of the hydrophobic substrate and enzyme surface may be responsible for orientation of the substrate or transition state. Nonetheless the Jerina model has proven to be a powerful predictive tool in the stereochemistry of metabolism of polycyclic aromatic hydrocarbons.

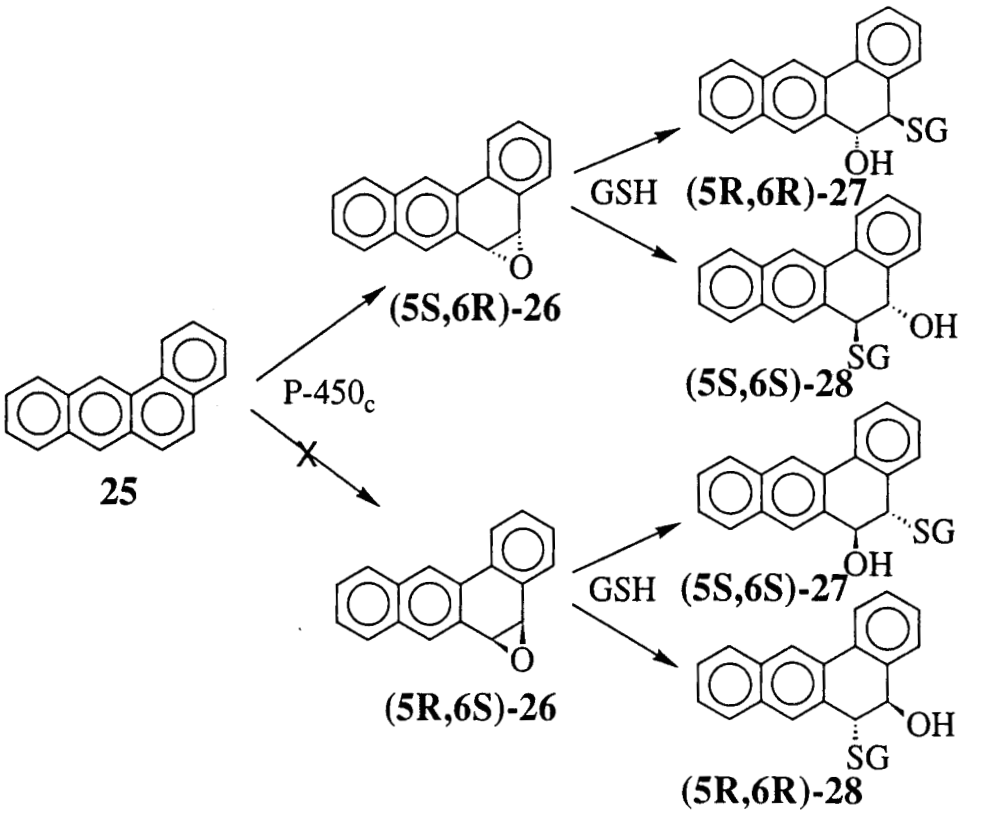


FIGURE 12. Nucleophile trapping of the benz[*a*]anthracene 5,6-oxide, (5S,6R)-**26** generated by cytochrome P-450$_c$ with glutathione (GSH).

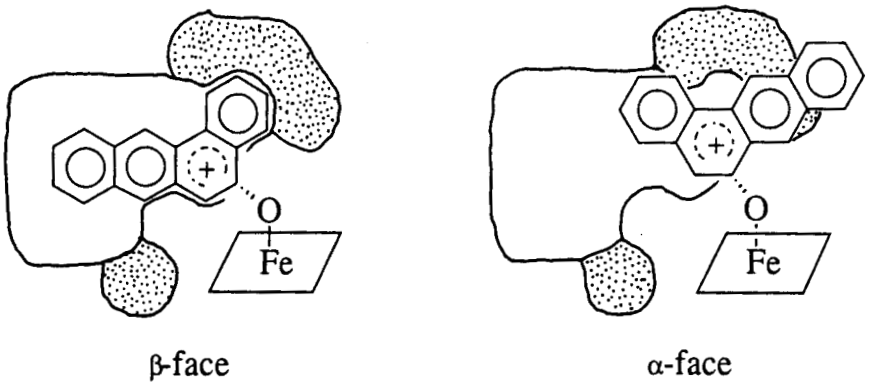


FIGURE 13. Proposed model for the active site of cytochrome P-450$_c$ by Jerina et al.[79] Productive binding of benz[*a*]anthracene for oxidation of the β-prochiral face of the 5,6-bond is shown on the left. Shaded region is unavailable for binding.

III. EPOXIDE HYDROLASE

A. The Enzymes

Epoxide hydrolase (EC 3.3.2.3) is ubiquitous in mammals. For some time the enzyme was thought to be exclusively a microsomal protein. However, several epoxide hydrolases have been purified to homogeneity from eukaryotic sources,[86-89] and it is now clear that

several distinct microsomal and soluble or cytosolic isoenzymes exist.[90-93] Although it has been pointed out that chromatographic evidence for different microsomal isoenzymes is not always unambiguous,[94] it is quite clear that at least two and perhaps three microsomal enzymes exist,[88,95] one of which is more or less specific for cholesterol-epoxide.[96,97] Inasmuch as the microsomal enzyme, which is thought to be largely responsible for xenobiotic metabolism, is the best characterized both in terms of mechanism and physical properties, it will be the focus of most of the discussion here. Recent reviews are available.[98-100]

B. Structure of Microsomal Epoxide Hydrolase

Nothing is known of the three-dimensional structure of microsomal epoxide hydrolase. However there is a considerable amount known concerning the primary sequence of the microsomal protein from rat and rabbit liver. The entire sequence of the rabbit enzyme has been determined and the amino terminal sequence of the rat enzyme is known.[101-103] One striking feature of both enzymes are the hydrophobic amino terminal sequences, reminiscent of signal sequences. In solution, the enzyme, which has a monomer molecular weight of ~50 kdaltons, appears to exist as a decameric to dodecameric aggregate of approximately 600 kdaltons. In the presence of nonionic detergents the enzyme behaves as though it were in an enzyme-micelle complex. For instance, in the presence of Brij 35 micelles the rat liver enzyme has an apparent Stokes radius of 6.02 nm while the Brij 35 micellar radius is 5.05 nm.[104] Perhaps the hydrophobic *N*-terminal tail of the protein is responsible for anchoring the enzyme in the microsomal membrane as well as in enzyme-micelle complexes and enzyme aggregates. The microsomal enzyme contains no prosthetic groups or metal ions.[86]

C. Mechanism of Epoxide Hydrolase

1. Mechanistic Alternatives

Epoxide hydrolase catalyzes the *trans*-addition of water across the oxirane ring with inversion of configuration at the oxirane carbon being attacked. There is no documented case of *cis*-addition of water in the enzyme catalyzed reaction. The basic mechanistic questions concerning this reaction is how the enzyme fixes and activates water at the active site and how it destabilizes the oxirane ring toward hydrolytic attack. Three general mechanisms have been considered for the reaction. The first, involving a metal ion either for fixing and activation of water or as a Lewis acid for activation of the oxirane[105] can be ruled out since the enzyme is not a metalloprotein.[86] Another possible mechanism considered is the formation of a covalent enzyme-substrate intermediate. The most reasonable senario for this mechanism consistent with *trans*-addition of water is Sn2 attack of an active site carboxylate to give an ester intermediate followed by hydrolysis of the ester. This mechanism is unlikely since there is no kinetic evidence for an intermediate and experiments to trap an ester intermediate have failed.[106] It is apparent that the oxygen incorporated into the diol product would derive from the carboxylate group in the first catalytic turnover and ultimately from solvent in subsequent turnovers due to washing of ^{18}O into the carboxyl group in ester hydrolysis. That ^{18}O from solvent is incorporated into the product in multiple turnover experiments has been incorrectly used as evidence to rule out an ester intermediate.[107] The critical experiment, a single turnover reaction in $H_2{}^{18}O$, that would clearly rule out such an intermediate, has not, to the author's knowledge, been done. The third mechanism for which there is considerable circumstantial evidence involves general base-general acid catalysis of the nucleophilic addition of water. This mechanism is discussed in some detail later.

2. General Base-General Acid Catalysis

Two extreme mechanisms for epoxide ring opening involving acid-base catalysis can be envisioned for the enzyme. For one, the protonation of the epoxide by the enzyme to form

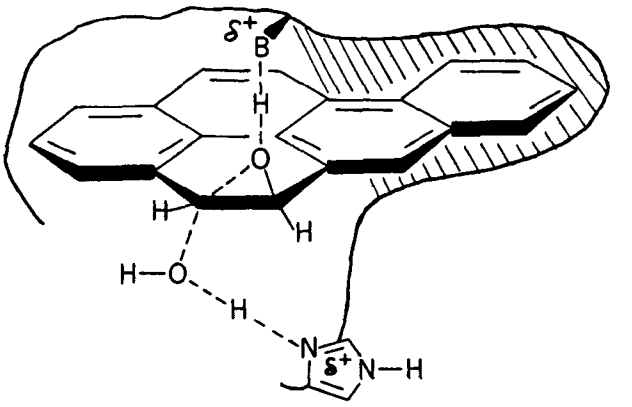


FIGURE 14. Possible transition state for the reaction of an epoxide hydrolase·arene oxide·H_2O complex illustrating general-base catalysis and general-acid assistance in the protonation of the incipient oxyanion.

a carbocation which is stereospecifically captured by water, there is little or no experimental support. In fact, the regiochemistry of the enzyme-catalyzed reaction with a number of substrates as well as the inability of nucleophiles other than water (e.g., CH_3OH, F- ect) are inconsistent with carbocation formation.[105,108] Furthermore, recent attempts to design mechanism-based inhibitors of epoxide hydrolase through the rearrangement of cyclopropylmethyl carbocations potentially formed in the hydration of cyclopropyl oxiranes indicate that carbocation formation does not occur.[109] This does not rule out general acid catalytic assistance in dispersal of electron density on the oxirane oxygen in the transition state for a nucleophilic addition mechanism.

In contrast, there is a large body of experimental evidence supporting a general-base-catalyzed nucleophilic addition of water to the oxirane by the enzyme as illustrated in Figure 14. A histidine residue has been implicated as essential for catalysis from chemical modification studies.[110] In addition, the pH rate profile for decomposition of the ternary enzyme·phenanthrene 9,10-oxide·H_2O complex shows a dependence on the dissociation of an acid group with a pKa = 6.7.[106] This is consistent with an unionized histidine side chain acting as a general base for the deprotonation of the attacking water molecule (Figure 14). Furthermore, a solvent deuterium kinetic isotope effect of 1.5 suggests proton transfer occurs in the transition state for the rate-limiting step. A substantial negative $\Delta S\ddagger$ for the reaction (-9 to -13 eu) also indicates the transition state is more polar than the reactants.[106] A linear-free energy relationship using substituted stilbene oxides shows a small negative rho value ($\rho = -1.2$) for electron withrawing substituents for attack at the α-carbon, an indication of developing carbocation character at the α-carbon.[111] Taken together the evidence cited above make a very strong circumstantial case for general-base-catalyzed nucleophilic addition of water to epoxides by the enzyme.

It is relevant to consider at this point if general-base catalysis alone can account for the rate accelerations observed with epoxide hydrolase. There are two ways in which enzymic catalysis can be compared to the analogous uncatalyzed spontaneous reactions. The first-order rate constant for turnover (k_c) or decomposition of an enzyme-substrate complex can be compared to the second-order rate constant k_s for the spontaneous reaction through the concept of "effective molarity" derived from the quotient of the two rate constants [($k_c(sec^{-1})$/ k_s (M^{-1}·sec^{-1}) = EM].[112] Although this concept has been generally used to express the degree of "approximation" in catalysis it clearly includes all contributions to the decomposition of enzyme-substrate complexes. One may also directly compare k_c/K_m to k_s to access catalysis at low substrate concentration.

Table 1
COMPARISON OF RATE CONSTANTS FOR THE HDYRATION OF PHENATHRENE 9,10-OXIDE

Reaction	k_c (sec^{-1})	k_c/K_m or k_s (M^{-1} sec^{-1})	Effective molarity, k_c/k_s (M)	Relative rate
Epoxide[a] hydrolase	0.6	3.4×10^5	—	7.6×10^{11}
H_3O^+	—	1.2×10^2	—	2.7×10^8
OH^-	—	4.7×10^{-4}	1.3×10^3	1.0×10^3
H_2O	—	4.5×10^{-7}	1.3×10^6	1

[a] Data from Reference 106, 25°C, pH 8.5.
[b] Data from References 113 and 114, 30°C.

Rate accelerations by epoxide hydrolase range from modest to substantial depending on the substrate. One of the more quantitatively understood examples is that of phenanthrene 9,10-oxide, **29.** Comparison of the rate constants for the enzyme, and acid-catalyzed reactions and the nucleophilic addition of water and hydroxide is given in Table 1.[113,114] The effective molarity for the enzyme-catalyzed reaction ranges from 10^3 to 10^6 M depending on whether it is compared to the H_2O or OH^- reactions. General-base catalysis might reasonably be expected to contribute 10 or 10^2 to the catalyzed reaction[17] with H_2O leaving a factor of 10^4 to 10^5 of rate acceleration in k_c to be accounted for. General-base catalysis is certainly not the sole contributor to the efficient decomposition of the enzyme-substrate complex. Comparison of k_c/K_m to k_s for the H_2O catalyzed reaction dramatically illustrates the ability of the enzyme to sequester substrate at low concentration and convert it to product. Clearly, a great deal of the catalytic potential of epoxide hydrolase is manifest in k_c/K_m.

It has been argued and it is certainly reasonable that general-acid catalysis may be involved in dissapating negative charge at the oxirane oxygen in the transition state (Figure 14). Although Prestwich and Hammock[115] have argued that the effective reversible inhibition of the cytosolic enzyme by α,β-epoxyketones can be interpreted to suggest an active-site protonated base proximal to the oxirane oxygen, no compelling evidence for the participation of a general acid has been reported for the microsomal enzyme. Quite to the contrary Berti and co-workers have argued that epoxide hydrolase-catalyzed formation of 2,3-epoxycyclohexanol, **31**, from (±)-*trans*-3-bromo-1,2-epoxycylohexane, **30**, precludes participation of a general acid in the hydration reaction.[107] The idea, illustrated in Figure 15 was to place an electrophilic center with a good leaving group adjacent to the incipient oxyanion to capture excess electron density developing in the transition state. The result should be a walk of the oxirane to the adjacent position. This presumes that in the presence of general-acid catalysis proton transfer would effectively compete with the second displacement reaction and the oxirane walk would not occur. That microsomes catalyze formation of **31** to a greater extent than **32**, the normal hydrolytic product, argues against effective protonation of the oxirane. It should be noted that these experiments have not been repeated with purified enzyme.

3. Strain

When it is considered that epoxide hydrolase is a highly functional-group-specific catalyst it makes sense to presume that evolutionary development may have provided the enzyme with molecular gismos for imposing strain on the oxirane in the ground state which may be relieved in the transition state. Preliminary work of Hanzlik and co-workers[116] using ^{18}O, ^{13}C, and ^2H kinetic isotope effects to examine the transition states for hydration of R- and S-*p*-

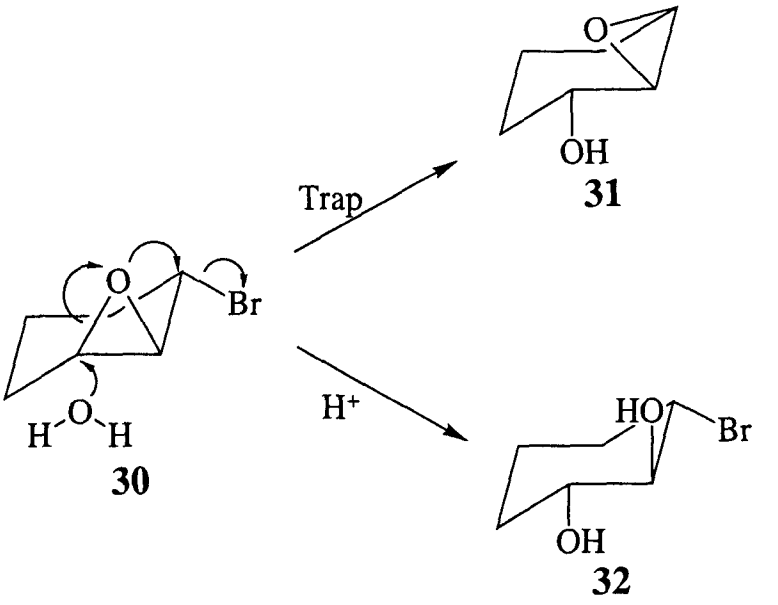


FIGURE 15. Trapping of incipient oxyanion in the epoxide hydrolase-catalyzed hydration of *trans*-3-bromo-1,2-epoxycyclohexane, **30**.[107]

nitrostyrene oxide provides some support for this point of view. The two antipodes of *p*-nitrostyrene oxide, **33**, are substrates for epoxide hydrolase and are kinetically distinguished by the enzyme. The S- and R-antipodes have V_{max} and K_m values of 116 nmol/min/mg and 7.6 μM and 34 nmol/min/mg and 1.6 μM, respectively.[117] Kinetic istopic effects on V_{max} for the enzyme catalyzed reactions suggest strain may be important in catalysis. In particular, the secondary ^2H isotope effects which are almost always inverse (<1) in epoxide ring openings are, with one exception, normal (>1) in the enzyme catalyzed reactions of 33.[116] It can be inferred that the hydrogens are gaining more freedom of motion in the transition state relative to the ground state. This suggests strain about the oxirane moiety in the ternary ground state complex is relieved in the transition state.

The importance of strain in the catalytic mechanism of epoxide hydrolase needs to be further assessed. The comparative manifestation of strain in the antipodes of **33** are modest and very probably not a true indication of the full contribution of strain to catalysis. The relationship between strain, substrate topology, and enzyme stereoselectivity is an area of obvious and relevant interest.

D. Stereoselectivity

It has been recognized for some time that the stereoselectivity of epoxide hydrolase has a profound influence on the metabolic activation or detoxication of aromatic hydrocarbons. For this reason, a good deal is known about the stereoselective behavior of the microsomal enzyme toward arene oxide substrates. Considerably less is known about the cytosolic enzyme.

The stereoselectivity of microsomal epoxide hydrolase can vary considerably with substrate structure. In principle, the enzyme should be able to recognize the chemically identical but stereochemically distinct oxirane carbons of nondissymmetric or *meso*-oxiranes. This is found to be the case (Figure 16) with *cis*-stilbene oxide, **34**, where asymmetric induction by the enzyme gives almost exclusively the (R,R)-1,2-dihydroxy-1,2-diphenylethane.[118] Conversely, very little asymmetric induction is seen with phenanthrene 9,10-oxide, **29**,[12] where the aromatic rings are coplanar.

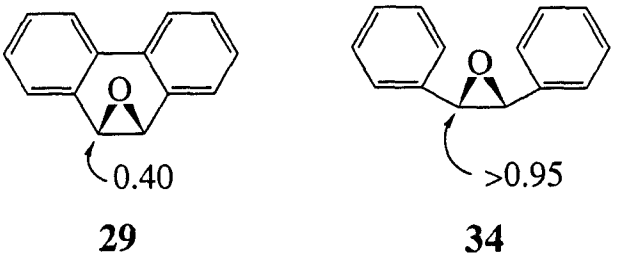


FIGURE 16. Stereoselectivity of microsomal epoxide hydrolase toward phenathrene 9,10-oxide **29** and *cis*-stilbene oxide, **34**. Numbers indicate the mole fraction attack at the oxirane carbon of S absolute configuration.

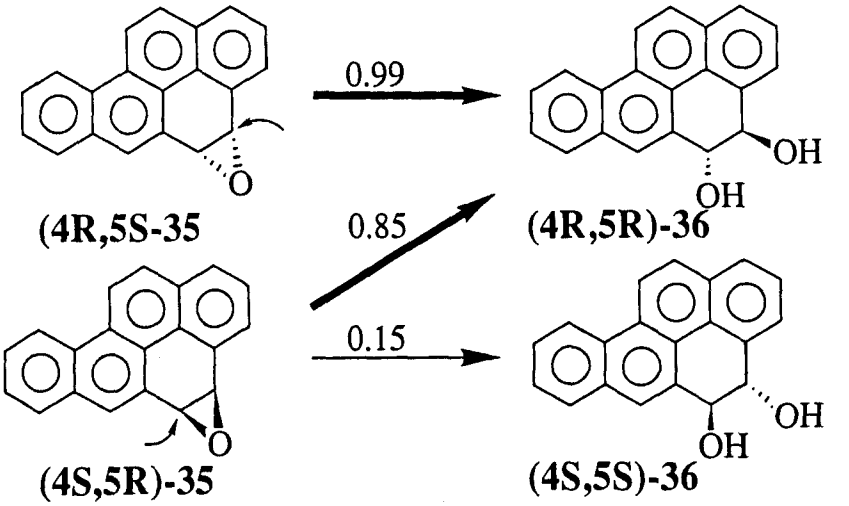
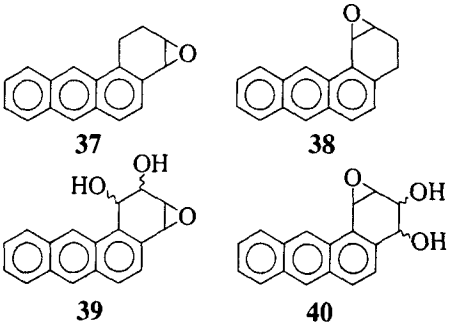


FIGURE 17. Regiochemistry of hydration of benzo[*a*]pyrene 4,5-oxides by epoxide hydrolase. Numbers over the arrows mole fraction for partitioning of attack at the two oxirane carbons.

The stereo- and regioselectivity of the enzyme toward chiral substrates is dependent on both substrate topology and the electronic character of oxirane carbons. Some generalizations can be made. With K-region arene oxides, that is, where both oxirane carbons are benzylic the enzyme usually shows a preference for catalyzing attack at the oxirane carbon of S-absolute configuration. Thus, the enzyme switches its regiochemical preference in response to the chirality of substrates such as benzo[*a*]pyrene 4,5-oxide, **35**, as shown in Figure 17, and generates predominately the 4R,5R-dihydrodiol from both antipodes. Quite to the contrary the regiochemistry of hydration of non-K-region arene oxides appears to be controlled by the electronic nature of the oxirane. In general, these substrates are hydrated at the allylic oxirane carbon.[57,84]

This situation is even more complex with diol-epoxides and tetrahydro epoxides. Regioselectivity with tetrahydroepoxides is markedly dependent on the location of the oxirane. As an example tetrahydrobenz[*a*]anthracene 3,4-epoxides, **37**, undergo preferential attack at the benzylic position whereas with the corresponding 1,2-epoxides, **38**, nonbenzylic attack predominates.[119] The corresponding diol-epoxides **39** and **40**, which are considerably poorer substrates, are hydrated exclusively at the benzylic positions.



Kinetic measurements combined with regiochemical data have been used to derive a picture of the active site of epoxide hydrolase.[12,119] It has been reasoned that if relative k_c/K_m values reflect the ability of the active site to stabilize the transition state then they may be extrapolated to suggest a structure reflecting the goodness of fit between enzyme and substrate. The original hypothesis[12] that the enzyme has a hydrophobic pocket, shown as a shaded region in Figure 14, to accommodate large hydrophobic groups of the substrate appears to have some predictive merit. Others have come to the same conclusions with completely different substrates.[120-122]

The model indicates nothing about the details of catalysis such as the ability of the enzyme to utilize substrate structure to induce strain in the oxirane or compress the reactants toward the transition state. Although these matters are of tremendous interest from a mechanistic perspective it is not clear at what point it is no longer beneficial (in a metabolic sense) for the enzyme to use substrate-binding energy for increasing k_c. Under normal metabolic (subsaturating) conditions k_c/K_m is the important kinetic constant. The development of a unified understanding of epoxide hydrolase catalysis from the mechanistic and metabolic perspectives will enhance the understanding of the evolution and role of detoxication enzymes in general.

IV. GLUTATHIONE *S*-TRANSFERASE

A. The Proteins

The glutathione *S*-transferase (EC 2.5.1.18) are a ubiquitous family of proteins whose primary functions are involved in the chemical and physical disposition of toxic substances. The chemical function of the enzyme is to catalyze the nucleophilic addition of the thiol of glutathione (γ-L-Glu-L-CysGly) to electrophilic acceptors, the first step in mercapturic acid biosynthesis. In addition, it is proposed that the proteins also serve as depots for the storage of toxic substances,[2,123-126] as high capacity steroid-binding proteins,[127] as heme-binding and transport proteins,[128,129] and perhaps as intracellular transporters of lipophilic substances.[126-128,130,131] Both the abundance of the enzymes, comprising 3 to 10% of the soluble protein in liver and the high concentrations (5 to 10 m*M*) of glutathione attest to the importance of glutathione *S*-transferases in the maintenance of health.

Glutathione *S*-transferases have been isolated and characterized from a number of sources including rat[2-4] and human tissues,[129,132,133] as well as plants.[134] The enzymes are largely found in the cytosol though a microsomal enzyme has been isolated and thoroughly characterized.[135] The most thoroughly studied enzymes are from rat liver cytosol from which eight isozymes have been isolated and characterized[2] and evidence for other microheterogeneous forms has been obtained.[3,4] The rat liver enzymes are dimeric proteins with subunit molecular weights of 25 to 30 kdaltons. Recently, a new nomenclature was proposed for the rat liver enzymes based on their binary subunit composition.[136] This nomenclature will be used throughout this review. Six of the dimeric proteins derive from binary combinations

of four subunits giving two families of isozymes designated 1-1, 1-2, 2-2 and 3-3, 3-4, and 4-4, each having two homodimeric and one heterodimeric members.[2] Two other homodimers have also been reported, isozymes 5-5 and 6-6.

Although next to nothing is known of the three-dimensional structure of the glutathione *S*-transferases the primary sequences of the major subunits have been elucidated primarily through in vitro translation of the glutathione *S*-transferase mRNAs and cloning of their corresponding cDNAs.[19,135,137-143] It is clear from these studies that subunits 1,2,3, and 4 and the microsomal enzyme are distinct gene products. Most of the limited information available on the structure of the active sites of the enzymes is from indirect evidence.

B. Reactions

1. Electrophilic Substrates

As a group of enzymes the glutathione *S*-transferases catalyze the nucleophilic addition of electrophiles including aryl and alkyl halides,[144] sulfate esters,[145] phosphate and phosphorothioate triesters,[146,147] nitrate esters,[148] esters,[149] oxiranes,[150-152] olefins,[125,149,153] lactones,[125] organic peroxides,[154] disulfides and thiocyanates,[148,149] and quinones.[155] The substrate selectivities exhibited by various isozymes overlap considerably but are nonetheless distinct. The subject of substrate selectivity of individual isozymes has been discussed in a recent review.[2] Although considerable attention has been devoted to determining the substrate selectivities of the enzymes, it has not provided much useful information concerning the topologies of the different active sites.

Most of the above reactions can be classed as simple nucleophilic displacements or Michael additions to unsaturated systems. Given the correct substrate other interesting and metabolically important chemistry can occur after addition of glutathione. This includes addition followed by rearrangement of the initial glutathione conjugate and addition-elimination reactions where glutathione acts in a catalytic manner.

Probably the most interesting example of nucleophilic addition followed by rearrangement is the metabolic activation of vicinal dihaloalkanes to give a reactive episulfonium ion species previously illustrated in Figure 1. Alkylation of DNA by **5** has been shown to give *S*-[2-(N^7-guanyl)ethyl]glutathione as the major adduct[156] perhaps accounting for the mutagenic activity of vicinal dihaloalkanes. Enzyme-catalyzed reactions of α,ω-dihaloalkanes with glutathione leads to sulfonium ions of different ring size and electrophilic reactivity. The relationship between structure, biotransformation, and mutagenic activity of α,ω-dihaloalkanes has been studied in some detail.[157-159] Subsequent rearrangement of the Michael addition product of glutathione with 4-hydroxyalk-2-enals, has been shown to produce the *S*-glutathionyl-substituted cyclic hemiacetal.[160]

Catalytic participation of the glutathione *S*-transferases in the isomerization of Δ^5-3-ketosteroids, **41**, is an example of a reaction in which glutathione is not consumed.[161] The mechanism is likely to involve nucleophilic addition of glutathione to the olefin, loss of a proton, and elimination of glutathione in a retro-Michael fashion to give the Δ^4-product, **42**, as shown in Figure 18. The source and stereochemistry of delivery of the proton to C-6 are unknown.

2. Metabolic Reactions

Glutathione conjugates formed by action of the transferase can suffer a number of metabolic fates at the hands of several enzymes. Serial cleavage of the peptide bonds by γ-glutamyltranspeptidase and hydrolysis of the cysteinyl-glycine bond gives cysteinyl conjugates. In this way, for example, the leukotrienes C, D, and E are formed.[162,163] N-Acetylation of cysteine S-conjugates[164] yields another common metabolite, a mercapturic acid. Cleavage of cysteine S-conjugates to the thiol, pyruvate, and ammonia by cysteine β-lyase[165] and subsequent methylation and oxidation or glucuronidation of the thiol can lead to a variety of other products. These metabolic events have been reviewed elsewhere.[2,26]

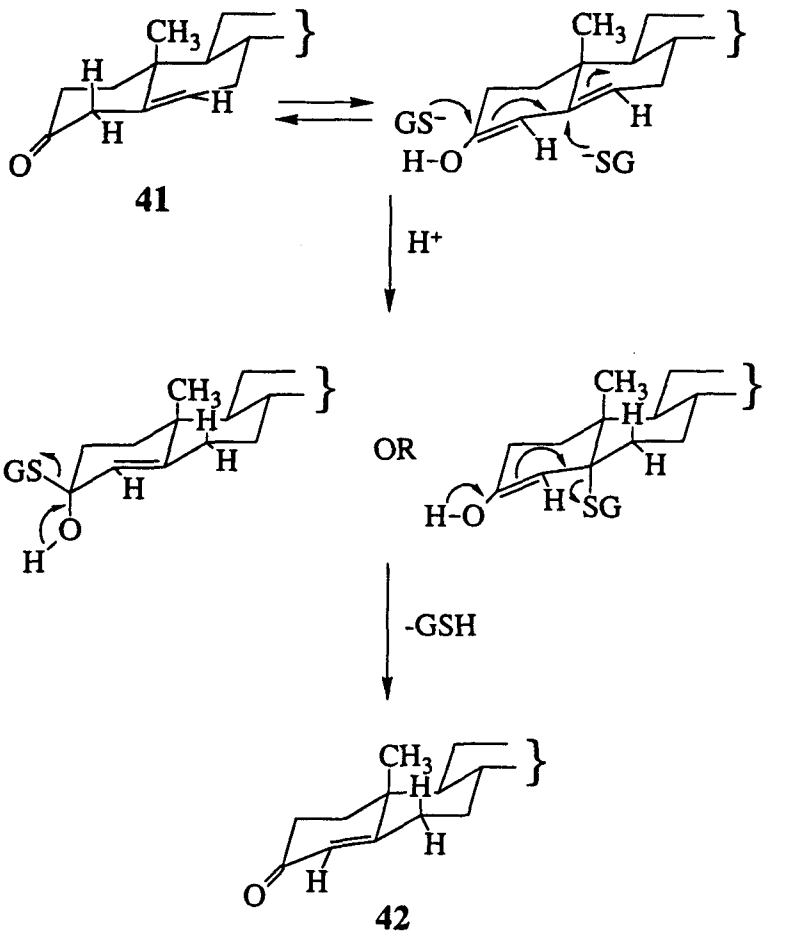


FIGURE 18. Possible mechanism for the glutathione *S*-transferase-catalyzed isomerization of Δ^5-3-ketosteroids.

C. Mechanism of Action of Glutathione *S*-Transferase

1. Kinetic Mechanism

The steady-state kinetic mechanism of two isozymes of glutathione *S*-transferase have been investigated extensively. Early studies of isozyme 3-3 concluded that initial velocity patterns at high glutathione concentrations were consistent with an ordered sequential mechanism in which glutathione binds first while at low concentrations of glutathione a ping-pong pathway pertains in which the electrophile (1,2-dichloro-4-nitrobenzene) is added and the leaving group released before addition of glutathione.[166] No evidence for a kinetically competent covalent enzyme-electrophile intermediate has been forthcoming. A later more convincing series of studies suggest a steady-state random mechanism for isozyme 3-3, with kinetically significant product complexes.[167-169] Furthermore, recent results with isozymes 3-3 and 4-4 using the oxygen (γ-L-glutamyl-L-serylglycine) and desthio (γ-L-glutamyl-L-alanyl-glycine) analogues of glutathione are consistent with either a random sequential mechanism or an ordered sequential mechanism with glutathione binding first and clearly rule out ordered or ping-pong kinetic mechanism where the electrophile adds first.[170] A rapid-equilibrium random mechanism has been proposed for isozyme 1-1 based on product inhibition studies.[171]

Further evidence supporting a sequential mechanism is obtained from consideration of the

stereochemical consequences of displacement reactions at saturated carbon centers catalyzed by the enzyme. The observation of inversion of configuration at the benzylic carbon in the enzyme-catalyzed reaction of glutathione with 1-halo-1-phenylethanes[172,173] as well as the formation of *trans*-addition products with arene oxides[152] strongly suggest a single displacement mechanism. The intervention of a covalent intermediate in a double displacement mechanism would give retention of configuration.

In certain circumstances the glutathione *S*-transferases appear to undergo slow conformational transitions which alter the kinetic characteristics of the enzyme. Thus, it has been shown that various combinations of ligands such as bilirubin and glutathione can induce, depending on their order of addition, different conformational states of the enzyme that have significantly different catalytic activities.[174-176] At least two conformational states of isozyme 1-1 have been postulated to explain altered substrate dissociation constants depending on the order of addition substrate and a product analogue.[171] The significance of these observations to the often complex kinetic behavior of the enzyme is not at all clear.

What is quite clear from all available data is that glutathione *S*-transferase-catalyzed reactions are sequential. That is, bond breaking and formation occur in a ternary central complex. The order of addition of substrates is likely to be random but may vary with substrate type. In any case, the order of substrate addition is not particularly relevant to the chemistry that occurs in the central complex.

2. *Chemical Mechanism*

Most proposals for the catalytic mechanism of glutathione *S*-transferase suggest that the enzyme provides catalytic machinery which first facilitates deprotonation of the thiol of bound glutathione and second provides an adjacent hydrophobic substrate-binding site for positioning the electrophile. This rather simple view of catalysis suggests that the catalytic mechanism can be dissected experimentally. Furthermore, it suggests that the transition states for the enzyme-catalyzed and spontaneous reactions are similar.

Original results of the most serious attempt to probe the rate-limiting transition state of the enzyme-catalyzed reaction by examining Hammett linear-free energy relationships with a series of 4-substituted-1-chloro-2-nitrobenzenes were consistent with a simple nucleophilic mechanism much like that of the spontaneous reaction.[148] Both k_c for the enzyme-catalyzed and k_s for the spontaneous reactions correlated with the σ^- substituent constants with ρ values of 1.6-1.8 and 3.1, respectively. Unfortunately, these studies were done using the heterodimeric isozymes 1-2 and 3-4. This fact probably explains why poor correlations were obtained when k_c data for all substrates were used. This type of experiment has been recently repeated with the homodimeric isozyme 4-4 giving an excellent corelation of k_c with σ^- ($\rho = 1.2$, $r = 0.99$), results which are clearly distinguishable from the spontaneous reaction.[177] Diminution of the ρ value in the enzyme catalyzed reaction could suggest that chemistry in the central complex is not entirely rate limiting. However, the good correlation and lower sensitivity of the enzymatic reaction to σ^--argues that the transition state is different, perhaps earlier, than the spontaneous reaction.

Rate accelerations in the enzyme-catalyzed reactions at neutral pH are substantial. Inasmuch as the thiolate anion is the most reasonable candidate for the nucleophilic species in the central complex its effective pKa is of considerable interest. Few pH vs. rate studies of the glutathione *S*-transferases have been attempted[144,150,166] and none have been interpreted rigorously. However, very recently isozyme 4-4 catalyzed reaction of glutathione with 3-nitro-4-chlorobenzaldehyde, **43**, revealed a dependence of both k_c and k_c/K_m^{GSH} on an acidic group with a pKa = 6.8 as shown in Figure 19.[177] The simplest functional interpretation of this result is that this is the effective acid dissociation constant for the nucleophile in the ternary complex. Thus, a good deal of the catalytic advantage of the enzyme at neutral pH can be viewed as due to a shift in the pKa of the nucleophile from approximately 9 in free solution to 6 to 7 on the enzyme surface.

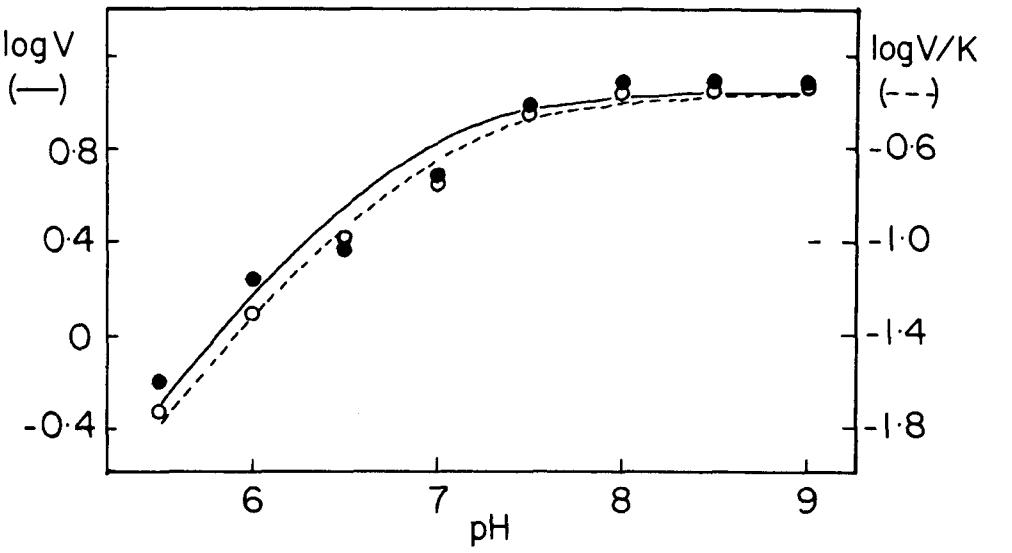


FIGURE 19. pH vs. rate profile for isozyme 4-4 catalyzed reaction of glutathione with 3-nitro-4-chlorobenzaldehyde. Experimental points are for V_{max} (●) and V_{max}/K_mGSH (○). Lines are computer fits of the data with apparent pKas of 6.8.

Comparison of the rate constants for reaction of the thiolate (GS^-) in free solution (k_s) to that on the enzyme surface (k_c) is instructive in terms of the source of rate acceleration as manifest in k_c or V_{max}. The effective molarity for the decomposition of the enzyme·GS^-·**43** complex is close to unity ($k_c^{GS-}/k_s^{GS-} = 1.5\ M$).[177] This can be interpreted to indicate much of the catalysis in V_{max} is simply due to enzyme-facilitated ionization of the thiol in the ternary complex. It appears that the enzymes do little to specifically orient the electrophile in the enzyme substrate complex. It is also instructive to compare the second-order rate constant for catalysis at low electrophile concentration to that of the spontaneous reaction. Thus $(k_c^{GS-}/K_m^s)+/k_s^{GS-} = 5.7 \times 10^5$, where K_m is the Michaelis constant for the electrophile. Clearly much of the catalytic efficiency of the enzyme is manifest in k_c/K_m.

The experimental dissection of catalysis above is consistent with the view that active site of glutathione *S*-transferase provides for the effective deprotonation of glutathione at physiological pH and serves as a hydrophobic net to capture xenobiotic substrates. The atomic and molecular details of the structure of the active site are currently unknown.

D. Structure of the Active Site

1. Specificity for Glutathione and the Structure of the Glutathione Binding Site

Surprisingly, little is known about the fundamental question of how the enzyme recognizes the tripeptide, glutathione. The ability of the transferase to extract the hydrophilic peptide from aqueous solution ($K_D \sim 3.5\ \mu M$ for isozyme 3-3)[2] certainly suggests a highly specific recognition of glutathione by the enzyme. What role the various structural features (charged groups, amide bonds, and van der Waal's surface) of the peptide play in binding is not known. What is known is that simple mercaptans such as mercaptoethanol, L-cysteine, *N*-acetyl-L-cysteine are not substrates.[144]

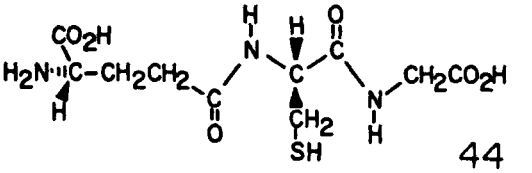
Until recently the only substrate analogue known for glutathione was homoglutathione (γ-L-glutamyl-L-cysteinyl-β-alanine) in which the C-terminus of the peptide is extended one methylene unit.[144] That the glycine residue is not essential for peptide recognition by the enzyme is indicated by the recent observation that the dipeptide γ-L-glutamyl-L-cysteine is an alternative substrate for several of the rat liver isozymes.[178] It should be pointed out that, a previous report suggesting the 6-*n*-propyl-2-thiouracil is an alternative substrate,[179] is incorrect.[180]

Table 2
KINETIC PARAMETERS FOR *RETRO*-GLUTATHIONE ANALOGUES AS SUBSTRATES FOR ISOZYMES 3-3 AND 4-4 OF RAT LIVER GLUTATHIONE *S*-TRANSFERASE

Enzyme	Peptide	k_c (sec^{-1})	K_m (μM)	k_c/K_m M^{-1} sec^{-1}
Isozyme 3-3	Glutathione, **44**	2.6	60	43,000
	Retro-glutathione, **45**	0.036	770	47
	46	0.013	3300	4.0
Isozyme 4-4	Glutathione, **44**	0.63	57	11,000
	Retro-glutathione, **45**	0.027	7700	3.5
	46	0.019	2100	9.0

From Chen, W.-J., Lee, D. Y., and Armstrong, R. N., *J. Org. Chem.*, 51, 2848, 1986. With permission.

A prominent structural feature of glutathione, **44**, which may be intimately involved in substrate recognition is the peptide backbone. The recent synthesis of two retro-peptide analogues of glutathione (**45** and **46**) and the demonstration that they are substrates for at least two isozymes of glutathione *S*-transferase sheds some light on this issue.[181] *Retro*-glutathione, **45**, in which the direction of the peptide bonds have been reversed resulting in inversion of configuration at the α-carbon of cysteine is topologically similar to glutathione except for the location of the amide NH and carbonyl groups.



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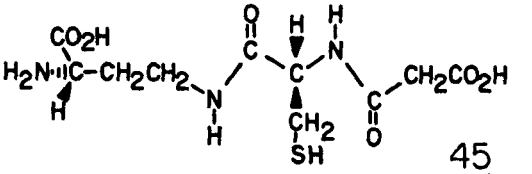

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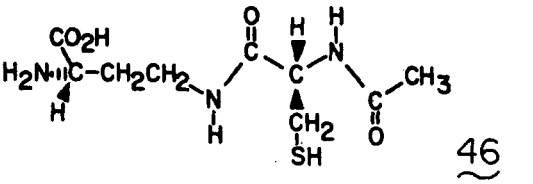

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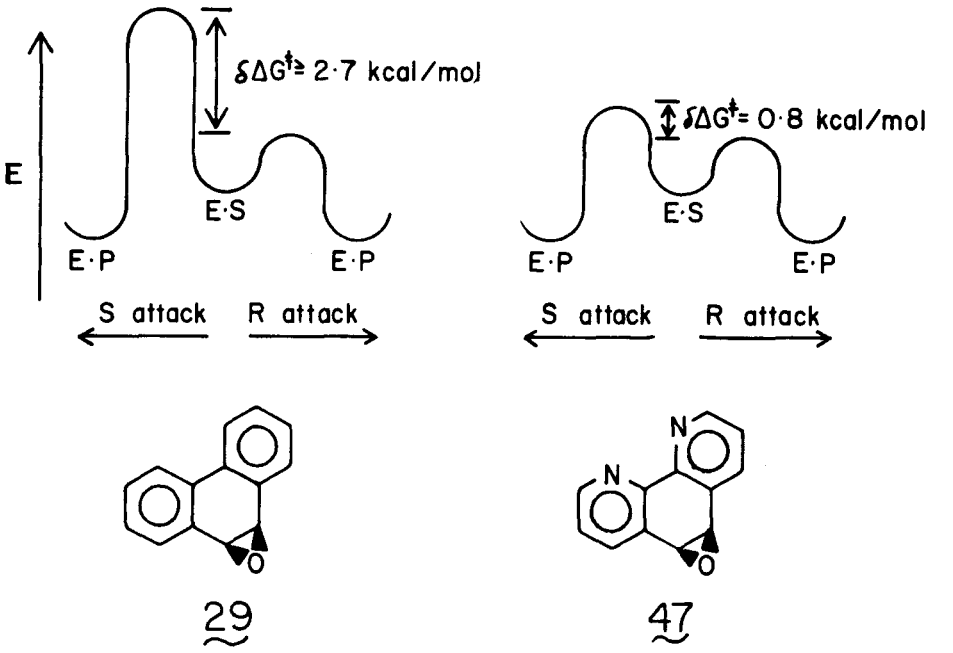
The data compiled in Table 2 clearly shows that the correct orientation of the peptide bonds in glutathione are important though not essential for substrate recognition and catalysis by the enzyme. Moreover, it is apparent that, as is the case with glutathione, the carboxylate of the glycine (malonate) residue is not essential. It should also be noted that *retro*-glutathione conjugates of electrophiles should be resistant to processing of the peptide by γ-glutamyl-transpeptidase and the dipeptidases involved in mercapturic acid formation.



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47

FIGURE 20. Partitioning of enzyme·glutathione·electrophile (ES) complexes between nucleophilic attack on the oxirane carbons of R and S absolute configuration with phenanthrene 9,10-oxide, **29**, and 4,5-diazaphenanthrene 9,10-oxide, **47**.

It has recently been suggested that glutathione binds to the enzyme in an extended conformation.[2] This notion has precedence in the X-ray crystallographic data for the peptide[182] as well as glutathione complexes with glutathione peroxidase[183] and glutathione reductase,[184] but has not been rigorously demonstrated for glutathione transferase.

2. Catalytic Locus

Nothing really is known about functional groups in the active site which may be involved in catalysis. A number of molecules have been shown to irreversibly or covalently modify and partially or wholly inactivate the enzyme.[2,166,185] As yet chemically modified residues have not been identified nor implicated as essential to catalysis.

3. Stereoselectivity and the Structure of the Xenobiotic Substrate Binding Site

The stereo- and regioselectivity of the glutathione *S*-transferases toward a considerable number of substrates have been investigated.[146,152,173,186-190] It has been demonstrated that in favorable instances the stereoselectivity of the enzyme toward arene oxides can be used to map the hydrophobic substrate-binding site.[152,187]

Isozyme 4-4 of rat liver glutathione *S*-transferase can distinguish between the stereochemically distinct oxirane carbons in phenanthrene 9,10-oxide, **29**, to the extent that it is stereospecific in generating the 9S,10S-diastereomeric product.[152,187] Expressed another way, the ternary enzyme substrate complex partitions between two possible diastereomeric transition states as shown in Figure 20. That the enzyme loses its stereospecificity toward 4,5-diazaphenanthrene 9,10-oxide, **47**, giving only 78% attack at the oxirane carbon of R-absolute configuration has been interpreted as an indication that mutual desolvation of the substrate and a hydrophobic binding site on the enzyme is largely responsible for the observed stereoselectivity.[152,187] With chiral K-region arene oxides the enzyme maintains its stereospecificity at the expense of the regiochemistry of the reaction. This behavior suggests that

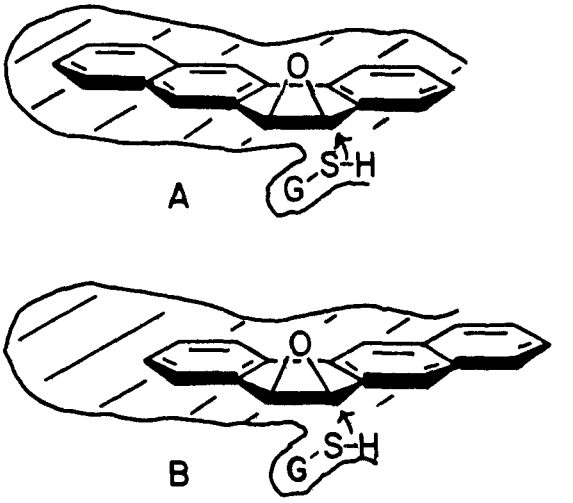


FIGURE 21. Proposed hydrophobic surface asymmetry in the active site of isozyme 4-4 of glutathione *S*-transferase. Diagram A shows productive binding of (5R, 6S)-benz[*a*]anthracene 5,6-oxide for glutathione attack at the 5-position. In (B) maximum hydrophobic overlap between enzyme and substrate dictates attack at the 6-position of the 5S,6R-enantiomer. (From Cobb et al., *Biochemistry*, 22, 805, 1983. With permission.)

the hydrophobic binding site on the enzyme is asymmetrically disposed with respect to the catalytic locus as shown by benz[*a*]-anthracene 5,6-oxide in Figure 21.

Changing the orientation of the peptide thiol appears to alter the stereoselectivity of the enzyme. Thus, the enzyme-catalyzed reaction of the glutathine analogues **45** and **46** with **29** gives two to one mixtures of the S,S and R,R-diastereomeric products.[177]

4. Relative Orientation of the Active Sites in the Dimeric Enzyme

Inasmuch as the glutathone *S*-transferases are dimeric enzymes it is entirely possible that active sites are shared among subunits. It has been pointed out that if this were true then the kinetic[191] and stereochemical[187] behavior of heterodimeric isozymes 1-2 and 3-4 might differ from that predicted from results with the homodimers. The stereoselectivity of isozyme 3-4 toward both **29** and **47** can be predicted from the kinetics and stereoselectivity of the two homodimeric proteins.[187] Furthermore, the k_c/K_m values of homodimeric isozymes 1-1, 2-2, 3-3, and 4-4 toward a number of substrates have been used to successfully predict k_c/K_m for isozymes 1-2 and 3-4.[191] The subunits in the dimeric enzymes appear to be kinetically independent. It is therefore doubtful that the enzymes have shared active sites at the subunit interfaces.

V. SULFOTRANSFERASE

A. The Enzymes

The sulfotransferases catalyze the transfer of the sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate to nucleophiles such as alcohols, phenols, and amines. Two recent reviews on the isolation, properties, and reactions of sulfotransferase are available.[192,193] Several sulfotransferases which are active with xenobiotic substrates have been isolated as pure proteins and characterized. The isoenzymes catalogued to date have been grouped into two classes based on substrate specificity toward phenols and alcohols.

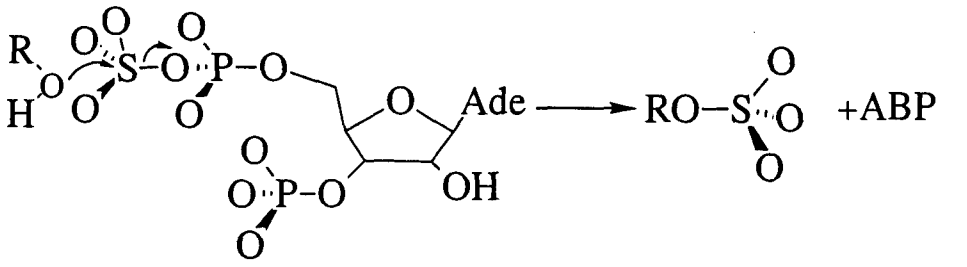


FIGURE 22. Reaction catalyzed by sulfotransferases.

Three alcohol sulfotransferases have been isolated from rat liver.[194-196] Two appear to be large multimeric proteins with molecular weights of 180 and 290 kdaltons composed of identical subunits of 28 and 32 kdaltons, respectively. Another isozyme is a dimer of apparently identical 60-kdalton subunits. Whether the aggregation state of the two high molecular-weight proteins is indicative of a particular multimeric structure in vivo is unclear since these proteins tend to aggregate on isolation.

At least four aryl sulfotransferases have been isolated from rat liver. Three purified by Jakoby and co-workers are dimeric proteins with subunit molecular weights of 30 to 32 kdaltons.[197,198] Another enzyme has been isolated as a monomer with a molecular weight of 69 kdaltons.[199]

B. Substrates and Xenobiotic Metabolism

The alcohol sulfotransferases catalyze the sulfation of a wide variety of primary and secondary alcohols and nonaromatic hydroxysteroids. Good substrates generally have a large hydrophobic group with little steric hinderance about the hydroxyl group. Interestingly phenols are not substrates for these isoenzymes. On the other hand the aryl sulfotransferases catalyze sulfuryl-transfer to phenols and not to simple primary or secondary alcohols.

The role of sulfotransferase in xenobiotic metabolism is probably much more complex than just the attachment of a hydrophilic group to a nucleophilic handle on lipophilic molecules. Inasmuch as sulfate is a good leaving group it is not surprising that some sulfate esters behave as electrophilic metabolic intermediates. For instance, the metabolism of 4-nitrotoleuene to *S*-(4-nitrobenzyl)glutathione occurs by oxidation of the methyl group to the benzyl alcohol followed by sulfation and glutathione conjugation.[145] Metabolic activation of 7,12-dimethylbenzanthacene has been demonstrated to occur by oxidation to the 7-hydroxymethyl-12-methylbenz[*a*]anthracene followed by sulfation and alkylation of DNA via the sulfate ester.[200-202]

C. Catalytic Mechanism

1. Reactions and Kinetics

The sulfotransferases catalyze the transfer of a sulfuryl group from the phosphosulfate mixed anhydride of adenosine 5′-phosphate 3′-phosphosulfate to hydroxyl groups yielding a sulfate ester and adenosine 3′,5′-biphosphate (ABP) as shown in Figure 22. The reaction appears to require no other cofactors.

Most mechanistic information concerning the sulfotransferases has been obtained with aryl sulfotransferases, primarily because of the ease with which they can be assayed. The kinetic mechanism of aryl sulfotransferase IV from rat liver has been studied in detail.[203] The reaction is readily reversible (Equation 3) with 2-chloro-4-nitrophenol as the substrate. The enzyme-catalyzed reaction has been investigated in both directions. The equilibrium favors the reverse reaction as shown in Equation 3.

$$R\text{–}OH + ABP\text{–}SO_3^- \rightleftarrows R\text{–}OSO_3^- + ABP \quad \Delta G = +1.8 \text{ kcal/mol} \qquad (3)$$

In addition, the enzyme catalyzes the sulfuryl exchange reaction between two phenols in the presence of adenosine 3′,5′-bisphosphate as shown in Equations 4 and 5. The enzyme will also catalyze the irreversible hydrolysis of the sulfate ester (Equation 6) at rate about 1% of the forward sulfation reaction.

$$R\text{–}O\text{–}SO_3^- + ABP \rightleftarrows R\text{–}OH + ABP\text{–}SO_3^- \quad (4)$$

$$ABP\text{–}SO_3^- + R'\text{–}OH \rightleftarrows R'\text{–}O\text{–}SO_3^- + ABP \quad (5)$$

$$RO\text{–}SO_3^- + H_2O \rightarrow ROH + HSO_4^- \quad (6)$$

Initial rate kinetics for isozyme IV with 2-chloro-4-nitrophenol are consistent with a rapid equilibrium random sequential mechanism with two dead-end complexes, (E·ABP-SO_3^-·RO-SO_3^-) and (E·ABP·ROH). The rate-limiting step is proposed to be the sulfuryl transfer chemistry of the central complex (E·ABP-SO_3^-·ROH ↔ E·ABP·RSO_3^-).

2. Chemical Mechanism

Very little is known about the chemical mechanism of the sulfotransferases. It is most reasonable to view the chemistry occurring in the central complex as a single displacement reaction involving nucleophilic attack of the phenol on the sulfuryl group of adenosine 3′-phosphate-5′-phosphosulfate as illustrated in Figure 22. Such a displacement would be expected to occur with inversion of configuration at sulfur were it labeled with oxygen isotopes. As of yet, no information is available on the stereochemistry of this sulfuryl group transfer reaction.

The transition state for the reaction is thought to be rather product like. Attempts to correlate V_{max} with the Hammett σ substituent constant for a series of para-substituted phenols has given variable results. In one instance no correlation was found for aryl sulfotransferases I and IV.[192,193,197] However, more recently, a relatively poor correlation of V_{max} for isozyme IV with σ_p^- was reported ($\rho = -0.25$, $r = 0.89$). The small negative ρ value has been taken to suggest a product-like transition state for the sulfuryl transfer reaction. Given what is known about substituent effects on sulfate ester hydrolysis and the endothermic nature of the sulfuryl transfer reaction this is a logical interpretation of the linear free energy relationship.[193] On the other hand, the rather poor correlation suggests that perhaps some alternate step other than the chemistry in the central complex is at least partially rate limiting with various substrates, or that geometric factors are important in determining V_{max} in the enzyme-catalyzed reaction.

3. Chemistry of the Active Site

How the active sites of the sulfotransferases participate in catalysis is not known. Recent chemical modification studies of the monomeric aryl sulfotransferase suggest that perhaps two sulfhydryl groups on the enzyme are required for activity.[199,204] Furthermore, the enzyme is inactivated by modification with phenylglyoxal implicating arginine residues in substrate binding.[204] Finally, the enzyme has been irreversibly inactivated by nucleoside 2′,3′-dialdehydes though the identity of the residues involved in this particular chemistry have not been identified. Further study is obviously necessary to delineate the role of various active site residues in substrate recognition and catalysis.

4. Catalysis

The observation that the product ABP binds considerably more tightly to the enzyme than does the substrate ABP-SO_3^- might suggest that the enzyme utilizes the intrinsic binding

energy of the substrate to stabilize the transition state for sulfuryl transfer. This possibility needs investigation. Like most xenobiotic metabolizing enzymes the sulfotransferases exhibit only modest rate accelerations particularly in k_c or V_{max}. In fact, k_c for aryl sulfotransferases is remarkably insensitive to substrate structure having values of generally between 0.1 and 2 sec^{-1} for a wide range of phenols. However, k_c/K_m values vary over almost three orders of magnitude. Very similar behavior is seen with the alcohol sulfotransferases, where k_c values for isozyme 1 varies over the limited range of 0.1 to 2 sec^{-1} while k_c/K_m varies by a factor of 10^6 going from methanol to dehydroepiandrosterone. It is quite clear that the catalytic potential of the sulfotransferases is manifest to a great degree in k_c/K_m with respect to the xenobiotic substrate.

VI. UDP-GLUCURONOSYLTRANSFERASE

A. The Enzymes

The UDP-glucuronosyltransferases (EC 2.4.1.17) are a group of membrane-bound proteins responsible for the transfer of the glucuronyl group from uridine 5′-diphosphoglucuronate to a large number of different nucleophilic acceptors. The enzymes are located primarily in the endoplasmic reticulum of eukaryotic cells. There is no doubt that several isozymes of UDP-glucuronosyltransferase exist. Indirect evidence as to the heterogeneity of this enzyme has been obtained by a number of techniques including direct kinetic analysis of reactions catalyzed by microsomes[205-208] and radiation inactivation analysis of a number of microsomal UDP-glucuronosyltransferase activities.[209] More to the point, however, is the fact that several isozymes have been purified to apparent homogeneity. Recent cell-free translation of mouse liver mRNA has also provided firm evidence for multiple forms of the enzyme.[210] Like most detoxication enzymes the UDP-glucuronosyltransferases are inducible by xenobiotic compounds. Some general aspects of UDP-glucuronosyltransferase have been recently reviewed.[211,212]

Purification and characterization of several UDP-glucuronsyltransferase have been reported in spite of considerable difficulties in solubilization of the active enzyme and its notorious instability once isolated from its native environment. Most successful purifications have relied on affinity chromatography on a UDP-hexanolamine-sepharose 4B matrix. In this way, several homeogenous isozymes from mouse and rat liver have been isolated.[213-219]

Very little is known about the structure of the enzymes. Most of the enzymes isolated above have subunit molecular weights of between 50 and 57 kdaltons. Radiation inactivation analysis indicates that the microsomal holoenzymes have molecular weights between 41.5 and 175 kdaltons suggesting that they may exist in the microsomal membrane as monomeric to tetrameric aggregates depending on the particular isozyme.[209] Physical evidence seems to suggest that the membrane environment alters the conformation of the polypeptide.[220,221] The primary sequence of a phenobarbital-inducible form of a rat liver enzyme has been deduced from a cDNA clone.[222,223] The clone indicates the polypeptide has an amino-terminal sequence characteristic of a signal peptide and a carboxy-terminal segment which could be a transmembrane-anchoring structure.

B. Substrates and Metabolic Reactions

The enzymes catalyze the glucuronidation of a tremendous number of lipophilic molecules having nucleophilic functional groups of oxygen, nitrogen, sulfur, and carbon. The UDP-glucuronosyltransferases are known to participate in metabolism of drugs, xenobiotics, and endogenous compounds.

The important role of glucuronidation in the metabolism of foreign compounds has been recognized for some time. Very early on it was clear that glucuronidation participates in the

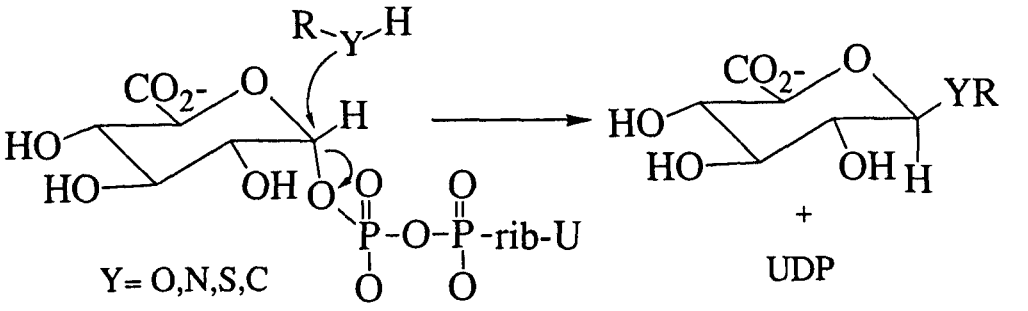


FIGURE 23. Reaction catalyzed by UDP-glucuronosyltransferase.

metabolism of polycyclic aromatic hydrocarbons.[224] In the last few years it has been unambiguously established that phenol, dihydrodiol, and quinol metabolites of polycyclic aromatic hydrocarbons are substrates for the mirosomal and purified enzymes.[225-231] The observation[232] that benzo[*a*]pyrene oxides are conjugated with glucoronic acid is almost certainly incorrect and probably due to spontaneous isomerization of the oxides to phenols which are substrates for the enzyme. A number of drugs containing oxygen, nitrogen, sulfur, and carbon nucleophiles provide interesting examples of glucuronidation in drug metabolism. For instance, tripelennamine, and antihistamic tertiary amine is quaternized by glucuronidation at nitrogen.[233] A rare example of glucuronidation at acidic carbon is found in the metabolism of phenylbutazone.[234] Formation of the carbon-carbon bond is unique in that the β-glucuronide at carbon is not hydrolyzed by β-glucuronidase.

There is some evidence that glucuronidation is involved in the metabolic activation of xenobiotics.[211] However, the role of glucuronidation is indirect and probably involves enhanced transport of metabolites and subsequent spontaneous or β-glucuronidase catalyzed decomposition to a toxin or proximate toxin.

UDP-glucuronosyltransferase is also involved in the metabolism of endogenous compounds such as steroids and bilirubin. In mammals, bilirubin is excreted primarily as a diglucuronide ester. Early work suggested formation of the diglucuronide by monoglucuronidation followed by an enzyme-catalyzed dismutation of the monoglucuronide to the diglucuronide and bilirubin.[212] More recent evidence suggests that formation of the diglucuronide involves the sequential action of two distinct UDP-glucuronosyltransferases.[209,235-238] Bile acid elimination and steroid metabolism also appear to depend in part on glucuronidation.

C. Mechanism

1. Reaction and Kinetics

UDP-glucuronosyltransferases catalyze the reversible transfer of glucuronyl group from UDP-glucuronate to a nucleophile with inversion of configuration at the anomeric carbon to give a β-glucuronide and UDP (Figure 23). The enzyme-catalyzed reaction does not appear to require additional cofactors or metal ions though it has been observed that the enzyme is greatly stimulated by the presence of phospholipid and that divalent metal ions (Mg^{2+}, Mn^{2+}) stimulate the reaction in the forward direction.[239,240] The purified pig liver isozyme (GT_{lp})[241] exhibits only about a 30% increase in V_{max} in the presence of Mn^{2+} so the mechanistic role of divalent cations in the reaction, while intriguing, may not be an important issue. Further studies are clearly warranted. The profound effect of phospholipid on the reactions have been treated in some detail by Zakim and co-workers and will be discussed below.

Kinetic studies of the microsomal[242] and purified enzymes[241] are consistent with a rapid

equilibrium random sequential mechanism. It should be pointed out that in no case has the kinetic mechanism of a homogeneous purified enzyme been rigorously defined.

The enzyme also catalyzes the slow hydrolysis of UDP-glucuronate to UDP and glucuronic acid, a reaction which has been observed with both purified pig liver[243] and rat liver[244] enzymes. This UDP-glucuronidase activity is also stimulated by phospholipids.[243]

2. Catalysis and Phospholipids

The detailed studies of Zakim and co-workers have advanced the understanding of how UDP-glucuronosyltransferase catalyzes its reaction, and further, how phospholipids effect the reaction rate.[16,243] The observation that UDP binds considerably more tightly to the pig liver enzyme than does the substrate, UDP-glucuronate, provides the first clue to a fundamental feature of catalysis by the enzyme, the utilization of intrinsic binding energy. From the kinetic constants for inhibition of the enzyme, maximum potential binding energies of UDP and glucuronic acid can be calculated.[16] These values can be used to estimate a minimum intrinsic binding energy[17] for UDP-glucuronate (minimum because it does not take into account the entropy loss associated with the covalent linking of the two ligands in UDP-glucuronate). Comparison of the minimum intrinsic binding energy with the observed binding energy for UDP-glucuronate allows the estimation of the minimum amount of intrinsic binding energy not utilized for substrate binding and therefore available for catalysis or transition state stabilization. Although the absolute value of these estimates cannot, for the reasons of entropy cited above, be taken too seriously it has been pointed out that differences in the calculated intrinsic binding energies available for catalysis between a delipidated enzyme and an enzyme phospholipid complex can be used to determine the influence of phospholipid on the ability of the enzyme to utilize intrinsic binding energies of substrates in catalysis. It is reasonable to assume the entropy changes that accompany the binding of UDP and glucuronate are similar in delipidated and phospholipid reconstituted enzyme. Therefore fairly accurate estimates of the relative binding energy utilized to enhance catalysis can be obtained. The calculated differences in intrinsic binding energy available for catalysis is gratifyingly close to the actual observed differences in free energy of activation. For example addition of oleoyl lysophosphatidylcholine lowers the free energy of activation for GT_{2p} catalyzed glucuronidation of 4-nitrophenol by -2.8 kcal/mol. The calculated phospholipid enhanced availability of intrinsic binding energy for catalysis is -3.3 kcal/mol. Similar results are observed for other lysophospholipids.[16]

It appears then that phospholipids are capable of altering the transition state for the glucuronidation reaction by increasing the efficiency with which the enzyme utilizes the intrinsic binding energy of the substrates. The exact structural nature of this phenomenon requires more experimental attention.

3.Chemical Mechanism

The majority of mechanistic information on the UDP-glucuronosyltransferases comes from work on two isozymes designated GT_{1p} and GT_{2p} isolated from pig liver microsomes.[241] Although very little is known about the active sites of these enzymes some interesting and provocative, indirect observations have been made concerning the mechanism of catalysis and the structure of the transition state, particularly with respect to the influence of phospholipids. The simplest mechanism consistent with the kinetic mechanism and stereochemistry of the reaction is a single displacement, Sn2 nucleophilic attack of the aglycone on UDP-glucuronate in a ternary central complex.

Linear free-energy relationships have been used in examining the transition states for the reactions catalyzed by isoenzymes GT_{1p} and GT_{2p}.[241,245] The results of these investigations are very interesting but, as discussed below, extremely difficult to interpret with any degree of confidence. The original observation that the V_{max} of isozyme GT_{2p} in the presence of

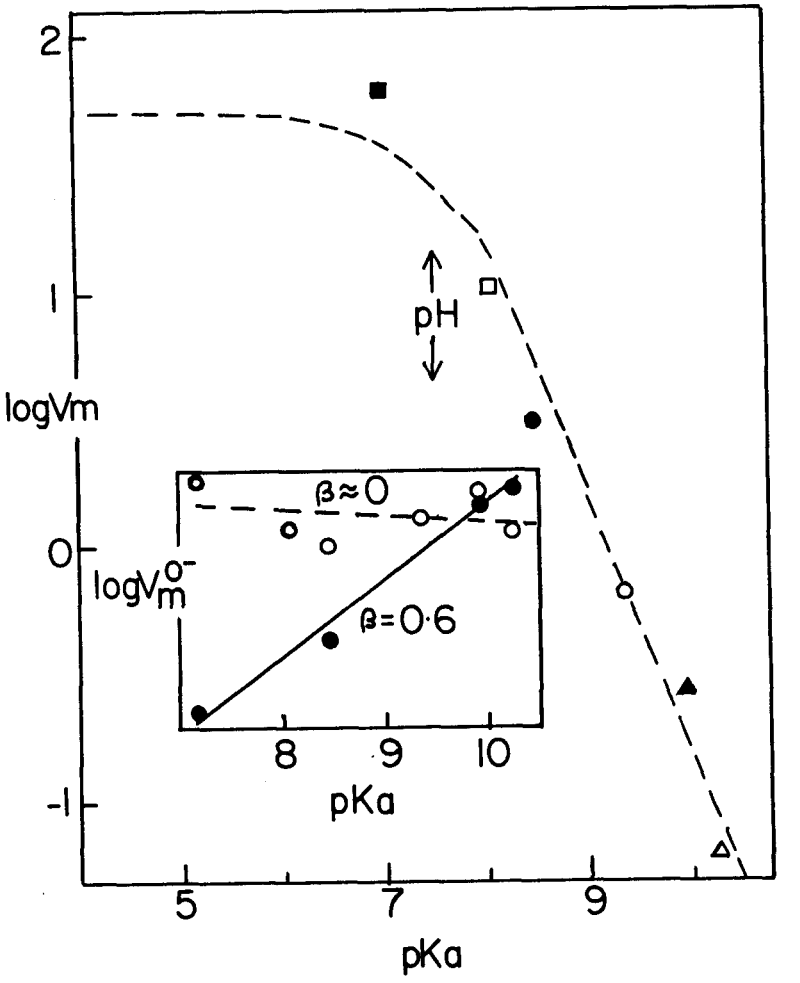


FIGURE 24. Dependence of V_{max} for GT_{2p} catalyzed glucuronidation of a series of substituted phenols on the pKa of the phenol. (■, p-nitrophenol; □, *p*-acetylphenol; ●, *p*-bromophenol; ○, 1-naphthol; ▲, phenol; △, *p*-aminophenol). Data is from Reference 245 and replotted in a more direct format by this author. Dashed line is the theoretical curve calculated for the Brønsted $\beta = 0$ and an experimental pH = 7.5. Inset shows replots of data corrected for the faction of ionization for GT_{2p} (-----) and GT_{1p} (—).[241]

oleoyl lysophosphatidylcholine for a series of substituted phenols was dependent on the acidity of the phenol has been interpreted in terms of the ground state concentration of productive ternary complexes (e.g., [E·phenolate·UDP-glucuronate]),[245] rather than the influence of substituents on the transition state. The data of Magdalou et al.[245] is represented here in the format of a Brønsted plot as shown in Figure 24.

Consideration of the data in terms of a Brønsted plot reveals a correlation between log V_m and the pKa of the nucleophile with a slope of approximately -1 due to the change in the fraction of nucleophile present as the reactive anion (pH > pKa) and $\beta_{nuc} \approx 0$ (Figure 24). Note, that as the pKa of the nucleophile approaches and becomes less than the pH of the experiment V_m should become independent of the pKa, if only the fraction of phenol ionized is relevant to catalysis. The observation[245] that V_m should depend on the mole fraction of the nucleophile (phenolate) in the ternary complex is clear.[246] What is most interesting about the data is that V_m^{o-}, the maximum velocity for the decomposition of the ternary complex containing phenolate anion, is independent of the nucleophilicity of the phenolate

(Figure 24, inset), as suggested by the slope of the Brønsted plot ($\beta_{nuc} \approx 0$). This may suggest an early reactant-like transition state for the reaction.

Isozyme GT_{1p} has been stated to be "dependent only partially on the concentration of phenolate ion" based on a Hammett function for a series of phenols that exhibits a ρ value considerably smaller than expected for phenol ionization.[241] It is perhaps more illuminating to state that the enzyme-catalyzed reaction (in contrast to GT_{2p}) is dependent on the nucleophilicity of the phenol. This is clearly illustrated in the inset of Figure 27 which shows GT_{1p} to have a Brønsted $\beta_{nuc} = 0.6$. Note that the reactivity of the phenolate (V_m^{o-}) increases not decreases[241] with increasing basisity. This can be taken to indicate a somewhat later transition state in the GT_{1p}-catalyzed reaction.

A really exciting observation is that Hammett functions become nonlinear for GT_{2p} in the presence of phospholipids other than oleoyl phosphatidylcholine.[245] Unfortunately, a clear interpretation of this limited data is not possible. Curvature in the Hammett (or Brønsted) plots could be due to (1) a change from an early ($\beta \approx 0$) to a later ($\beta \approx 0.5$ to 1) transition state, (2) a shift in the bound phenols of the pKa to ≤ the pH of the experiment (see Figure 24), (3) an alteration in the solvation and hence nucleophilicity of the phenolates in the active site, or (4) a change in the rate-limiting step for the reaction. The suggestion[245] that such curvature is consistent with a change from a Sn2 to an Sn1 like reaction is perhaps premature. Nevertheless, the fact that phospholipids may alter the transition state for the enzyme-catalyzed reactions is a provocative idea which deserves further investigation.

Not much is known about catalytic residues in the active site of UDP-glucuronosyltransferase. The only information concerning the identity of catalytic residues comes from inhibition studies of isozyme GT_{2p} by 2,3-butanedione.[247] Two arginine residues are apparently modified with this reagent. The rate of inactivation of one of the residues is dependent on both the presence of phospholipid and/or UDP-glucuronate. It has been proposed, based on protection experiments with UDP, glucuronate and UDP-glucuronate that GT_{2p} contains an active site arginine side chain that interacts with the C-6 carboxylate of the substrate.[247]

D. Stereo- and Regioselectivity

The stereo- and regiochemistry of enzyme-catalyzed glucuronidation has been examined in only a limited number of cases. Stereoselective glucuronidation of a number of drugs has been demonstrated. For instance, rat liver UDP-glucuronosyltransferase shows a 1.6-fold preference in k_c/K_m for R-(+)-propranolol as compared to the S-(−)-antipode.[248] The 12 possible positional isomers of hydroxybenzo[*a*]pyrene have been shown to be differentially glucuronidated by two partially purified UDP-glucuronosyltransferases.[249]

Somewhat more information is available on the stereo- and regioselectivity of glucuronidation of dihydrodiols of polycyclic aromatic hydrocarbons. A homogeneous UDP-glucuronosyltransferase from rat liver has been shown to discriminate between the stereochemically distinct hydroxyl groups of *cis*- and *trans*-9,10-dihydroxy-9,10-dihydrophenanthrenes as well as the regiochemically different carbinol groups of the 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes (**36**).[229] The later compounds provide an interesting example of combined stereoselectivity and regiospecificity. As illustrated in Figure 25 the enzyme clearly discriminates kinetically between the four stereoisomers of **36**. Even more striking is the regiospecificity of the glucuronidation in each stereoisomer. The most interesting stereochemical generalization that is evident from Figure 25 is that the enzyme recognizes only hydroxyl groups on the β-face[66] of the molecule. It is interesting to note that the best substrate for the enzyme (4S,5S)-**36** is *not* the one generated by the action of cytochrome P-450$_c$ and epoxide hydrolase (see Figure 17) on benzo[*a*]pyrene. Although the 4R,5R-antipode is a substrate for UDP-glucuronosyltransferase it is a very poor one. It would appear, then, that there is a stereochemical mismatch between dihydrodiol formation and glucuronidation in this particular instance.

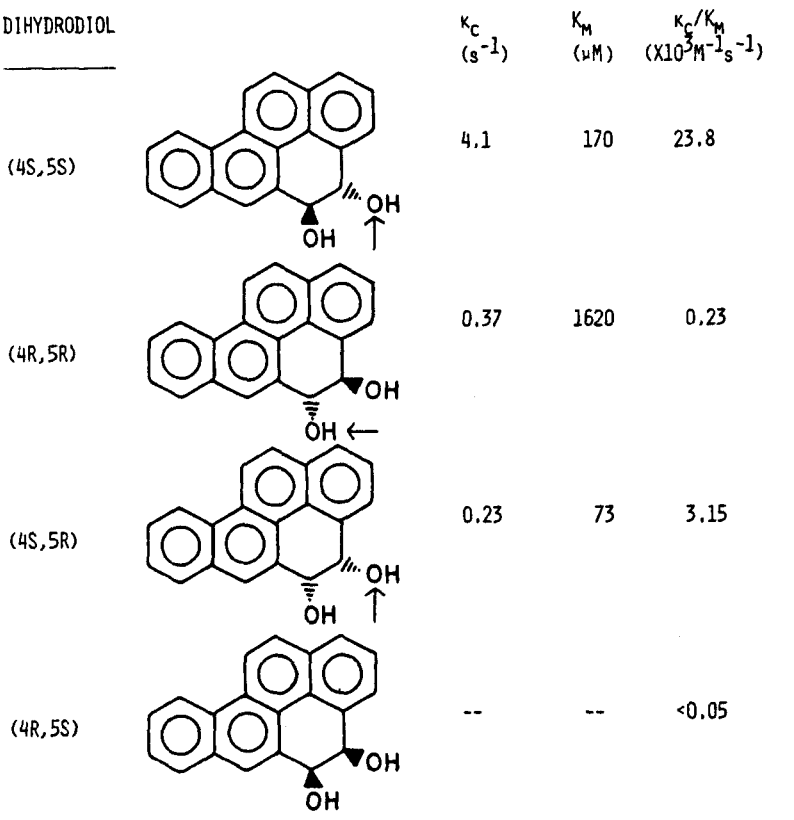


FIGURE 25. Kinetics and regiochemistry of the stereoselective glucuronidation of the four stereoisomeric 4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrenes. Regiospecificity is indicated by arrows. Data from Reference 229.

Trans-dihydrodiols of polycyclic aromatic hydrocarbons exist in solution as rapidly interconverting populations of two conformational isomers,[250] as illustrated for the 9,10-dihydroxy-9,10-dihydrophenanthrenes **47** to **49** in Figure 26. The *cis*-diol, **47**, exists as two conformational enantiomers. The two *trans*-antipodes exist as two pairs of conformational diastereomers with either diaxial or diequatorial hydroxyl groups. It is, therefore, relevant to ascertain which conformational isomers of dihydrodiols are preferred in productive enzyme-substrate complexes. Unfortunately, the kinetic lability of **47** to **49** makes it impossible to obtain this information directly. However, it has been found that the six kinetically *stable*, stereoisomeric conformers of 3,4,5,6-tetramethyl-9,10-dihydroxy-9,10-dihydrophenanthrene can be easily prepared and used for this purpose.[251-253]

The enzyme is capable of distinguishing between the three stereoisomers **47** to **49** (Table 4). Even more interesting is the ability of the enzyme to discriminate between the conformational isomers of **50** to **52.** It is apparent from Table 3 that UDP-glucuronosyltransferase exhibits a clear preference for conformers with diequatorial hydroxyl-groups. Thus, **51E** and **52E** are turned over 30 to >800 times more rapidly than the corresponding conformational diastereromers with diaxial hydroxyl groups. The specificity of the enzyme toward the *cis*-antipodes **50M** and **50P** is also interesting. In this instance only one of the possible diastereomeric products for each enantiomer is formed. It is likely, though not proven, that these two products are from exclusive glucuronidation of the equatorial hydroxyl group in the two stereoisomers.[251,253] This is a good indication that the two diastereomeric glucuronides formed from **47**[249] arise from glucuronidation of the equatorial hydroxyl groups of **47M** and **47P** in the enzyme-substrate complex.

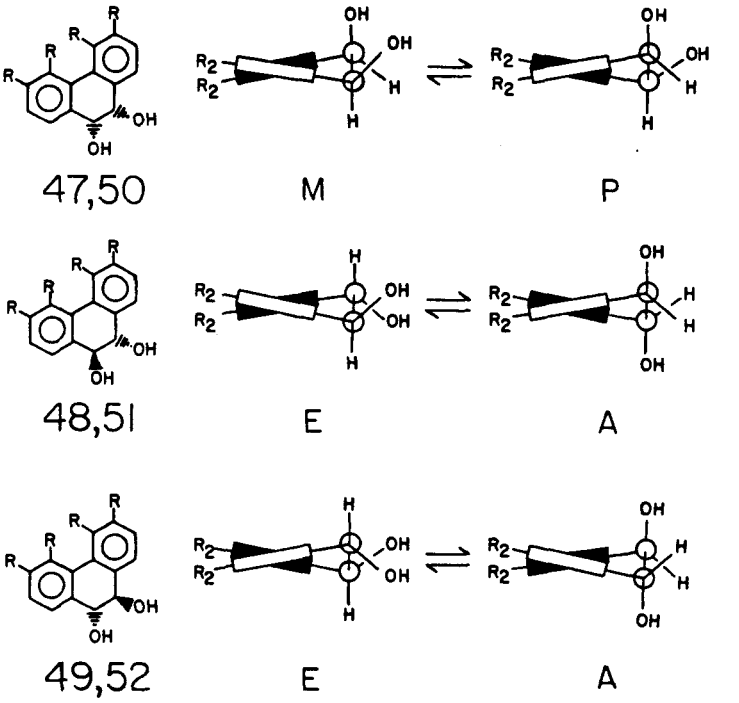


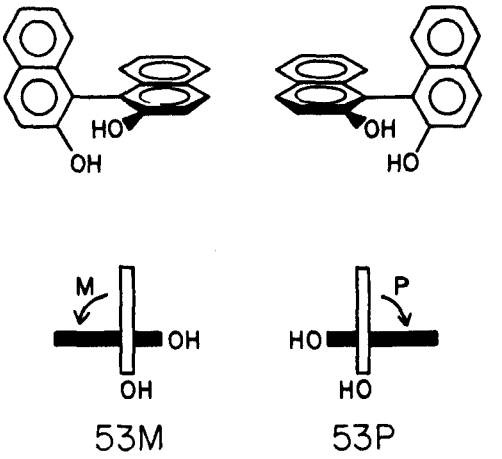
FIGURE 26. Conformational isomers of stereosiomeric 9,10-dihydroxy-9,10-dihydrophenanthrenes and 3,4,5,6-tetramethyl-9,10-dihydroxy phenanthrenes R = H for **47** to **49**. R = CH_3 for **50** to **52**. (From Armstrong, R. N., Lewis, D. A., Ammon, H. L., and Prasad, S. M., *J. Am. Chem. Soc.*, 107, 1057, 1985. With permission.)

Table 3
KINETIC CONSTANTS FOR THE ENZYME-CATALYZED GLUCURONIDATION OF STEREOISOMERIC 9,10-DIHYDROXY-9,10-DIHYDROPHENANTHRENES AND 3,4,5,6-TETRAMETHYL-9,10-DIHYDROXY-9,10-DIHYDROPHENANTHRENES[229,251]

Substrate	Absolute configuration[a]	k_c (sec^{-1})	k_c/K_{mapp} (M^{-1} sec^{-1})
47	R,S	0.037	18,000
48	S,S	1.4	1,100
49	R,R	0.070	4,400
50M	R,S,M	0.0058	—
50P	R,S,P	0.020	—
51A	S,S,P	<0.0005	—
52A	R,R,M	0.0069	—
51E	S,S,M	0.41	980
52E	R,R,P	0.20	910

[a] R and S designate absolute configuration of the carbinol carbons. M and P designate the helicity of the biphenyl system.

The enzyme appears to be insensitive to the helicity of the biphenyl system when one compares, for example, the turnover numbers for **51E** and **52E.** Quite in contrast, the enzyme has been found to exhibit a marked kinetic preference for helical bi-2-naphthols **53M** and **53P.** For example k_c and k_c/K_{mapp} values for **53M** are 0.19 sec^{-1} and $150M^{-1}$ sec^{-1}, respectively. The helical antipode **53P** has a k_c/K_{mapp} of $5.7M^{-1}$ sec^{-1} some 26-fold lower than **53M.** K_{mapp} for **53P** was not measurable for it is considerably above the solubility limit of the substrate. Further study of the stereoselectivity of UDP-glucuronosyltransferase should lead to a better understanding of the active site geometry of the enzyme and its ability to participate in various metabolic reactions.



VII. CONCLUSIONS

It should be apparent from the tremendous progress made in the last decade in elucidating the mechanisms of action of detoxication enzymes that the promise of the future is very bright. That the biological sciences now understand much and are so close to understanding much more about how organisms deal with such a tremendous variety of potential poisons is exciting. It is fair to say that the study of the mechanisms of detoxication enzymes has a lot to offer both the basic science of enzymic catalysis and the biochemistry of xenobiotic metabolism and toxicity. This, it is hoped, has been conveyed here.

ACKNOWLEDGMENTS

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REFERENCES

1. **Jakoby, W. B.,** Detoxication enzymes, in *Enzymatic Basis of Detoxication,* Vol. 1, Jakoby, W. B., Ed., Academic Press, New York, 1980, 2.
2. **Mannervik, B.,** The isoenzymes of glutathione transferase, *Adv. Enzymol.*, 57, 357, 1985.
3. **Satoh, K., Kitahara, A., and Sato, K.,** Identification of heterogeneous and microheterogeneous subunits of glutathione S-transferase in rat liver cytosol, *Arch. Biochem. Biophys.*, 242, 104, 1985.
4. **Tu, C.-P.D. and Reddy, C. C.,** On the multiplicity of rat liver glutathione S-transferase, *J. Biol. Chem.*, 260, 9961, 1985.
5. **Bresnick, E.,** Induction of the enzymes of detoxication, in *Enzymatic Basis of Detoxication,* Vol. 1, Jakoby, W. B., Ed., Academic Press, New York, 1980, 69.
6. **Williams, R. T.,** Species variations in drug biotransformations, in *Fundamentals of Drug Metabolism and Drug Disposition,* La Du, B. N., Mandel, H. G., and Way, E. L., Eds., Williams & Wilkins, Baltimore, 1971, 187.
7. **Eckert, K.-G. and Eyer, P.,** Formation and transport of xenobiotic glutathione-S conjugates in red cells, *Biochem. Pharmacol.*, 35, 325, 1986.
8. **Malaveille, C., Bartsch, H., Barbin, A., Camus, A. M., Montesanto, R., Croisy, A., and Jacquignon, P.,** Mutagenicity of vinyl chloride, chloroethyleneoxide, chloroacetaldehyde and chloroethanol, *Biochem. Biophys. Res. Commun.*, 63, 363, 1975.
9. **van Bladeren, P. J., Breimer, D. D., Rotteveel-Smijs, G. M. T., de Jong, R. A. W., Buijs, W., van der Gen, A., and Mohn, G. R.,** The role of glutathione conjugation in the mutagenicity of 1,2-dibromoethane, *Biochem. Pharmacol.*, 29, 2975, 1980.
10. **Sims, P., Grover, P. L., Swaisland, A., Pal, K., and Hewer, A.,** Metabolic activation of benzo[*a*]pyrene proceeds by a diol-epoxide, *Nature (London)*, 252, 326, 1974.
11. **Levin, W., Buening, M. K., Wood, A. W., Chang, R. L., Kedzierski, B., Thakker, D. R., Boyd, D. R., Gadaginamath, G. S., Armstrong, R. N., Yagi, H., Karle, J. M., Slaga, T. J., Jerina, D. M., and Conney, A. H.,** An enantiomeric interaction in the metabolism and tumorigenicity of (+)- and (−)-benzo-[*a*] pyrene 7,8-oxide, *J. Biol. Chem.*, 255, 9067, 1980.
12. **Armstrong, R. N., Kedzierski, B., Levin, W., and Jerina, D. M.,** Enantioselectivity of microsomal epoxide hydrolase toward arene oxide substrates, *J. Biol. Chem.*, 256, 4726, 1981.
13. **Thakker, D. R., Yagi, H., Akagi, H., Koreeda, M., Lu, A. Y. H., Levin, W., Wood, A. W., Conney, A. H., and Jerina, D. M.,** Metabolism of benzo[*a*]pyrene. VI. Stereoselective metabolism of benzo[*a*]pyrene and benzo[*a*]pyrene 7,8-dihydrodiol to diol epoxides, *Chem. Biol. Interact.*, 16, 281, 1977.
14. **Buening, M. K., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., and Conney, A. H.,** Tumorigenicity of the optical enantiomers of the diastereomeric benzo[*a*]pyrene 7,8-diol-9,10-epoxides in newborn mice: exceptional activity of (+)-7β, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 5358, 1978.
15. **Palcic, M. M. and Klinman, J. P.,** Isotopic probes yield microscopic constants: separation of binding energy from catalytic efficiency in the bovine plasma amine oxidase reaction, *Biochemistry*, 22, 5957, 1983.
16. **Hochman, Y., Zakim, D., and Vessey, D. A.,** A kinetic mechanism for modulation of the activity of microsomal UDP-glucuronosyltransferase by phospholipids, *J. Biol. Chem.*, 256, 4783, 1981.
17. **Jencks, W. P.,** Binding energy, specificity, and enzymic catalysis: the circe effect, *Adv. Enzymol.* 43, 219, 1975.
18. **Kawajiri, K., Gotoh, O., Sogawa, K., Tagashira, Y., Muramatsu, M., and Fujii-Kuriyama, Y.,** Coding nucleotide sequence of 3-methylcholantrhene-inducible cytochrome P-450d cDNA from rat liver, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 1649, 1984.
19. **Rothkopf, G. S., Telakowski-Hopkins, C. A., Stotish, R. L., and Pickett, C. B.,** Multiplicity of glutathione S-transferase genes in the rat and association with a type 2 Alu repetitive element, *Biochemistry*, 25, 993, 1986.
20. **Mackenzie, P. I., Gonzalez, F. J., and Owens, I. S.,** Cloning and characterization of DNA complementary to rat liver UDP-glucuronosyltransferase mRNA, *J. Biol. Chem.*, 259, 12153, 1984.
21. **Adesnik, M. and Atchison, M.,** Genes for cytochrome P-450 and their regulation, *CRC Crit. Rev. Biochem.*, 19, 247, 1986.
22. **Kawabata, T. T., Wick, D. G., Guengerich, F. P., and Baron, J.,** Immunohistochemical localization of carcinogen-metabolizing enzymes within the rat and hamster exocrine pancreas, *Cancer Res.*, 44, 215, 1984.
23. **Thomas, P. E., Reik, L. M., Ryan, D. E., and Levin, W.,** Characterization of nine monoclonal antibodies against rat hepatic cytochrome P-450$_c$. Delineation of at least five spatially distinct epitopes, *J. Biol. Chem.*, 259, 3890, 1984.

24. **Frey, A. B., Waxman, D. J., and Kreibich, G.,** The structure of phenobarbital-induced rat liver cytochrome P-450 isoenzyme PB-4. Production and characterization of site-specific antibodies, *J. Biol. Chem.*, 260, 15253, 1985.
25. **Jakoby, W. B., Bend, J R., and Caldwell, J., Eds.,** *Metabolic Basis of Detoxication. Metabolism of Functional Groups*, Academic Press, New York, 1982.
26. **Jakoby, W. B., Ed.,** *Enzymatic Basis of Detoxication*, Vol. 1 and 2, Academic Press, New York, 1980.
27. **Takikawa, O., Yoshida, R., and Hayaishi, O.,** Monooxygenase activities of dixoygenases. Benzophetamine demethylation and aniline hydroxylation reactions catalyzed by indoleamine 2,3-dioxygenase, *J. Biol. Chem.*, 258, 6808, 1983.
28. **Ortiz de Montellano, P. R. and Catalano, C. E.,** Epoxidation of styrene by hemoglobin and myoglobin. Transfer of oxidizing equivalents to the protein surface, *J. Biol. Chem.*, 260, 9265, 1985.
29. **Barton, H. A. and Marletta, M. A.,** personal communication, 1986.
30. **Starke, D. W., Blisard, K. S., and Mieyal, J. J.,** Substrate specificity of the monooxygenase activity of hemoglobin, *Mol. Pharmacol.*, 25, 467, 1984.
31. **Buhler, D. R. and Mason, H. S.,** Hydroxylation catalyzed by peroxidase, *Arch. Biochem. Biophys.*, 92, 424, 1961.
32. **Dordick, J. S., Klibanov, A. M., and Marletta, M. A.,** Horseradish peroxidase-catalyzed hydroxylations: mechanistic studies, *Biochemistry*, 25, 2946, 1986.
33. **Panthananickal, A., Weller, P., and Marnett, L. J.,** Stereoselectivity of the epoxidation of 7,8-dihydrobenzo[*a*]pyrene by prostaglandin H synthase and cytochrome P-450 determined by the identification of polyguanylic acid adducts, *J. Biol. Chem.*, 258, 4411, 1983.
34. **Ziegler, D. M.,** Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, in *Enzymatic Basis of Detoxication*, Jakoby, W. B., Ed., Academic Press, New York, 1980, 201.
35. **White, R. E. and Coon, M. J.,** Oxygen activation by cytochrome P-450, *Annu. Rev. Biochem.*, 49, 315, 1980.
36. **Guengerich, F. P. and Macdonald, T. L.,** Chemical mechanisms of catalysis by cytochromes P-450: a unified view, *Acc. Chem. Res.*, 17, 9, 1984.
37. **Wislocki, P. G., Miwa, G. T., and Lu, A. Y. H.,** Reactions catalyzed by the cytochrome P-450 system, in *Enzymatic Basis of Detoxication*, Vol. 1, Jakoby, W. B., Ed., Academic Press, New York, 1980, 135.
38. **Ortiz de Montellano, P. R., Ed.,** *Cytochrome P-450: Structure, Mechanism and Biochemistry*, Plenum Press, New York, 1985.
39. **Lichtenberger, F., Nastainczyk, W., and Ullrich, V.,** Cytochrome P-450 as an oxene transferase, *Biochem. Biophys. Res. Commun.*, 70, 939, 1976.
40. **Groves, J. T., Haushalter, R. C., Nakamura, M., Nemo, T. E., and Evans, B. J.,** High-valent iron-prophyrin cmplexes related to peroxidase and cytochrome P-450, *J. Am. Chem. Soc.*, 103, 2884, 1981.
41. **Kato, R., Iwasaki, K., Shiraga, T., and Noguchi, H.,** Evidence for the involvement of cytochrome P-450 in reduction of benzo[*a*]pyrene 4,5-oxide by rat liver microsomes, *Biochem. Biophys. Res. Commun.*, 70, 681, 1976.
42. **Groves, J. T., McClusky, G. A., White, R. E., and Coon, M. J.,** Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450. Evidence for a carbon radical intermediate, *Biochem. Biophys. Res. Commun.*, 81, 154, 1978.
43. **Miwa, G. T., Walsh, J. S., and Lu, A. Y. H.,** Kinetic isotope effects on cytochrome P-450-catalyzed oxidation reactions. The oxidative O-dealkylation of 7-ethoxycoumarin, *J. Biol. Chem.*, 259, 3000, 1984.
44. **Harada, N., Miwa, G. T., Walsh, J. S., and Lu, A. Y. H.,** Kinetic isotope effects on cytochrome P-450-catalyzed oxidation reactions. Evidence for the irreversible formation of an activated oxygen intermediate of cytochrome P-448, *J. Biol. Chem.*, 259, 3005, 1984.
45. **Miwa, G. T., Walsh, J. A., Kedderis, G. L., and Hollenberg, P. F.,** The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450 and peroxidase-catalyzed N-demethylation reactions, *J. Biol. Chem.*, 258, 14445, 1983.
46. **Watanabe, Y., Numata, T., Iyanagi, T., and Oae, S.,** Enzymatic oxidation of alkyl sulfides by cytochrome P-450 and hydroxyl radical, *Bull. Chem. Soc. Jpn.*, 54, 1163, 1981.
47. **Hanzlik, R. P. and Tullman, R. H.,** Suicidal inactivation of cytochrome P-450 by cyclopropylamines. Evidence for cation-radical intermediates, *J. Am. Chem. Soc.*, 104, 2048, 1982.
48. **Macdonald, T. L., Zirui, K., Burka, L. T., Peyman, P., and Guengerich, F. P.,** Mechanism of cytochrome P-450 inhibition by cyclopropylamines, *J. Am. Chem. Soc.*, 104, 2050, 1982.
49. **Heimbrook, D. C., Murray, R. I., Egeberg, K. D., Sligar, S. G., Nee, M. W., and Bruice, T. C.,** Demethylation of *N, N*-dimethylaniline and *p*-cyano-*N,N*-dimethylaniline and their N-oxides by cytochromes P-450$_{LM2}$ and P-450$_{cam}$, *J. Am. Chem. Soc.*, 106, 1514, 1984.
50. **Burka, L. T., Thorsen, A., and Guengerich, F. P.,** Enzymatic monooxygenation of halogen atoms: cytochrome P-450 catalyzed oxidation of iodobenzene by iodosobenzene, *J. Am. Chem. Soc.*, 102, 7615, 1980.

51. **Ortiz de Montellano, P. R., Kunze, K. L., Beilan, H. S., and Wheeler, C.,** Destruction of cytochrome P-450 by vinyl fluoride, fluroxene, and acetylene. Evidence for a radical intermediate in olefin oxidation, *Biochemistry*, 21, 1331, 1982.
52. **Ortiz de Montellano, P. R. and Komives, E. A.,** Branchpoint for heme alkylation and metabolite formation in the oxidation of arylacetylenes by chtochrome P-450, *J. Biol. Chem.*, 260, 3330, 1985.
53. **Groves, J. T. and Watanabe, Y.,** On the mechanism of olefin epoxidation by oxo-iron prophyrins. Direct observation of an intermediate, *J. Am. Chem. Soc.*, 108, 507, 1986.
54. **Jerina, D. M., Daly, J. W., and Witkop, B.,** The role of arene oxide-oxepin systems in the metabolism of aromatic substrates. II. Synthesis of 3,4-toluene-4-^{2}H oxide and subsequent "NIH shift" to 4-hydroxy-toluene-3-^{2}H, *J. Am. Chem. Soc.*, 90, 6523, 1968.
55. **Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S.,** The role of arene oxide-oxipin systems in the metabolism of aromatic substrates. III. Formation of 1,2-naphthalene oxide from naphthalene by liver microsomes, *J. Am. Chem. Soc.*, 90, 6525, 1968.
56. **Daly, J. W., Jerina, D. M., and Witkop, B.,** Arene oxides and the NIH shift: the metabolism, toxicity and carcinogenicity of aromatic compounds, *Experientia*, 28, 1129, 1972.
57. **Boyd, D. R. and Jerina, D. M.,** Arene oxides and oxepins in *Small Ring Heterocycles*, Vol. 42 (Part 3), Hassner, A., Ed., John Wiley & Sons, New York, 1985, 197.
58. **Hanzlik, R. P., Hogberg, K., and Judson, C. M.,** Microsomal hydroxylation of specifically deuterated monosubstituted benzenes. Evidence for direct aromatic hydroxylation, *Biochemistry*, 23, 3048, 1984.
59. **Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., and Muramatsu, M.,** Primary sequence of cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 2793, 1982.
60. **Haniu, M., P.-M., Ryan, D. E., Levin, W., and Shively, J. E.,** Structural analysis of the cysteine-containing peptides from the major 3-methylcholanthrene-induced isozyme of cytochrome P-450 (P-450$_c$) in rat liver microsomes, *Biohemistry*, 23, 2478, 1984.
61. **Haniu, M., Armes, L. G., Yasunobu, K. T., Shastry, B. A., and Gunsalus, I. C.,** Amino acid sequence of the pseudomonas putida cytochrome P-450. II. Cyanogen bromide peptides, acid cleavage peptides and the complete sequence, *J. Biol. Chem.*, 257, 12664, 1982.
62. **Gould, P. V., Gelb, M. H., and Sligar, S. G.,** Interaction of 5-bromocamphor with cytochrome P-450$_{cam}$. Production of 5-ketocamphor from a mixed spin state hemoprotein, *J. Biol. Chem.*, 256, 6686, 1981.
63. **Gelb, M. H., Malkonene, P., and Sligar, S. G.,** Cytochrome P-450$_{cam}$ catalyzed epoxidation of dehydrocamphor, *Biochem. Biophys. Res. Commun.*, 104, 853, 1982.
64. **Lewis, B. A. and Sligar, S. G.,** Structural studies of cytochrome P-450 using small angle X-ray scattering, *J. Biol. Chem.*, 258, 3599, 1983.
65. **Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., and Kraut, J.,** The 2.6Å crystal structure of *Pseudomonal putida* cytochrome P-450, *J. Biol. Chem.*, 260, 16122, 1985.
66. **Rose, I. A., Hanson, K. R., Wilkinson, K. D., and Wimmer, M. J.,** A suggestion for naming faces of ring compounds, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2439, 1980.
67. **Ortiz de Montellano, P. R., Beilan, H. S., and Kunze, K. L.,** *N*-alkylprotoporphyrin IX formation in 3,5-dicarbethoxy-1,4-dihydrocollidine-treated rats, *J. Biol. Chem.*, 256, 6708, 1981.
68. **Ortiz de Montellano, P. R., Kunze, K. L., and Beilan, H. S.,** Chiral orientation of prosthetic heme in the cytochrome P-450 active site, *J. Biol. Chem.*, 258, 45, 1983.
69. **Kunze, K. L., Mangold, B. L. K., Wheeler, C., Beilan, H. S., and Ortiz de Montellano, P. R.,** The cytochrome P-450 active site. Regiospecificity of prosthetic heme alkylation by olefins and acetylenes, *J. Biol. Chem.*, 258, 4202, 1983.
70. **Ortiz de Montellano, P. R., Manogold, B. L. K., Wheeler, C., Kunze, K. L., and Reich, N. O.,** Stereochemistry of cytochrome P-450 catalyzed epoxidation and prosthetic heme alkylation, *J. Biol. Chem.*, 258, 4208, 1983.
71. **Wood, A. W., Ryan, D. E., Thomas, P. E., and Levin, W.,** Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes, *J. Biol. Chem.*, 258, 8839, 1983.
72. **Waxman, D. J., Ko, A., and Walsh, C.,** Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver, *J. Biol. Chem.*, 258, 11937, 1983.
73. **Fasco, M. J., Vatsis, K. P., Kaminsky, L. S., and Coon, M. J.,** Regioselective and stereoselective hydroxylation of R and S warfarin by different forms of purified cytochrome P-450 from rabbit liver, *J. Biol. Chem.*, 253, 7813, 1978.
74. **Kaminsky, L. S., Fasco, M. J., and Guengerich, F. P.,** Comparison of different forms of purified cytochrome P-450 from rat liver by immunological inhibition of regio- and stereoselective metabolism of warfarin, *J. Biol. Chem.*, 255, 85, 1980.

75. **White, R. E., McCarthy, M.-B., Egeberg, K. D., and Sligar, S. G.,** Regioselectivity in the cytochromes P-450: control by protein constraints and by chemical reactivities, *Arch. Biochem. Biophys.*, 228, 493, 1984.
76. **Waxman, D. J., Light, D. R., and Walsh, C.,** Chiral sulfoxidations catalyzed by rat liver cytochromes P-450, *Biochemistry*, 21, 2499, 1982.
77. **Light, D. R., Waxman, D. J., and Walsh, C.,** Studies on the chirality of sulfoxidation catalyzed by bacterial flavoenzyme cyclohexanone monooxygenase and hog liver flavin adenine dinucleotide containing monooxygenase, *Biochemistry*, 21, 2490, 1982.
78. **Armstrong, R. N., Levin, W., Ryan, D. E., Thomas, P. E., Mah, H. D., and Jerina, D. M.,** Stereoselectivity of rat liver cytochrome P-450$_c$ on formation of benzo[*a*]pyrene 4,5-oxide, *Biochem. Biophys. Res. Commun.*, 100, 1077, 1981.
79. **Jerina, D. M., Michaud, D. P., Feldman, R. J., Armstrong, R. N., Vyas, K. P., Thakker, D. R., Yagi, H., Thomas, P. E., Ryan, D. E., and Levin, W.,** Stereochemical modeling of the catalytic site of cytochrome P-450$_c$, in *Microsomes, Drug Oxidations and Drug Toxicity*, Sato, R. and Kato, R., Eds., Interscience, New York, 1982, 195.
80. **van Bladeren, P. J., Armstrong, R. N., Cobb, D., Thakker, D. R., Ryan, D. E., Thomas, P. E., Sharma, N. D., Boyd, D. R., Levin, W., and Jerina, D. M.,** Stereoselective formation of benz[*a*]anthracene (+)-(5S,6R)-oxide and (+)-(8R,9S)-oxide by a highly purified and reconstituted system containing cytochrome P-450$_c$, *Biochem. Biophys. Res. Commun.*, 106, 602, 1982.
81. **Yagi, H. and Jerina, D. M.,** Absolute configuration of the *trans*-9,10-dihydrodiol metabolite of the carcinogen benzo[*a*]pyrene, *J. Am. Chem. Soc.*, 104, 4026, 1982.
82. **van Bladeren, P. J., Vyas, K. P., Sayer, J. M., Ryan, D. E., Thomas, P. E., Levin, W., and Jerina, D. M.,** Stereoselectivity of cytochrome P-450$_c$ in the formation of naphthalene and anthracene 1,2-oxides, *J. Biol. Chem.*, 259, 8966, 1984.
83. **Balani, S. K., Yeh, H. J. C., Ryan, D. E., Thomas, P. E., Levin, W., and Jerina, D. M.,** Absolute configuration of the 5,6-oxide formed from 7,12-dimethylbenz[*a*]anthracene by cytochrome P-450$_c$, *Biochem. Biophys. Res. Commun.*, 130, 610, 1985.
84. **van Bladeren, P. J., Sayer, J. M., Ryan, D. E., Thomas, P. E., Levin, W., and Jerina, D. M.,** Differential stereoselectivity of cytochromes P-450$_b$ and P-450$_c$ in the formation of naphthalene and anthracene 1,2-oxides. The role of epoxide hydrolase in determining the enantiomer composition of the 1,2-dihydrodiols formed, *J. Biol. Chem.*, 260, 10226, 1985.
85. **Panthananickal, A., Weller, P., and Marnett, L. J.,** Stereoselectivity of the epoxidation of 7,8-dihydrobenzo[*a*]pyrene by prostaglandin H synthase and cytochrome P-450 determined by the identification of polyguanylic acid adducts, *J. Biol. Chem.*, 258, 4411, 1983.
86. **Lu, A. Y. H., Ryan, D., Jerina, D. M., Daly, J. W., and Levin, W.,** Liver microsomal epoxide hydrase. Solubilization, purification and characterization, *J. Biol. Chem.*, 250, 8283, 1975.
87. **Halpert, J., Glaumann, H., and Ingelman-Sundberg, M.,** Isolation and incorporation of rabbit liver epoxide hydrase into phospholipid vesicles, *J. Biol. Chem.*, 254, 7437, 1979.
88. **Guengerich, F. P., Wang, P., Mitchell, M. B., and Mason, P. S.,** Rat and human liver microsomal epoxide hydratase. Purification and evidence for the existence of multiple forms, *J. Biol. Chem.*, 254, 12248, 1979.
89. **Lu, A. Y. H., Thomas, P. E., Ryan, D. E., Jerina, D. M., and Levin, W.,** Purification of human liver microsomal epoxide hydrolase. Differences in the properties of the human and rat enzymes, *J. Biol. Chem.*, 254, 5878, 1979.
90. **Ota, K. and Hammock, B. D.,** Cytosolic and microsomal epoxide hydrolases: Differential properties in mammalian liver, *Science*, 207, 1479, 1980.
91. **Wang, P., Meijer, J., and Guengerich, F. P.,** Purification of human liver cytosolic epoxide hydrolase and comparison to the microsomal enzyme, *Biochemistry*, 21, 5769, 1982.
92. **Meijer, J. and Depierre, J. W.,** Properties of cytosolic epoxide hydrolase purified from the liver of untreated and clofibrate-treated mice. Purification procedure and physicochemical characterization of the pure enzymes, *Eur. J. Biochem.*, 148, 421, 1985.
93. **McGee, J. and Fitzpatrick, F.,** Enzymatic hydration of leukotriene A_4. Purification and characterization of a novel epoxide hydrolase from human erythrocytes, *J. Biol. Chem.*, 260, 12832, 1985.
94. **Bulleid, N. J., Graham, A. B., and Craft, J. A.,** Microsomal epoxide hydrolase of rat liver. Purification and characterization of enzyme fractions with different chromatographic characteristics, *Biochem. J.*, 233, 607, 1986.
95. **Guenthner, T. M. and Oesch, F.,** Identification and characterization of a new epoxide hydrolase from mouse liver microsomes, *J. Biol. Chem.*, 258, 15054, 1983.
96. **Levin, W., Michaud, D. P., Thomas, P. E., and Jerina, D. M.,** Distinct rat hepatic microsomal epoxide hydrolases catalyze the hydration of cholesterol 5,6α-oxide and certain xenobiotic alkene and arene oxides, *Arch. Biochem. Biophys.*, 220, 485, 1983.

97. **Sevanian, A. and Mcleod, L. L.,** Catalytic properties and inhibition of hepatic cholesterol-epoxide hydrolase, *J. Biol. Chem.*, 261, 54, 1986.
98. **Oesch, F.,** Microsomal epoxide hydrolase, in *Enzymatic Basis of Detoxication*, Vol. 2, Jakoby, W. B. Ed., Academic Press, New York, 1980, 277.
99. **Lu, A. Y. H. and Miwa, G. T.,** Molecular properties and biological functions of microsomal epoxide hydrase, *Annu. Rev. Pharmacol. Toxicol.* 20, 513, 1980.
100. **Seidegård, J. and DePierre, J. W.,** Microsomal epoxide hydrolase properties, regulation and function, *Biochim. Biophys. Acta*, 695, 251, 1983.
101. **DuBois, G. C., Apella, E., Armstrong, R., Levin, W., Lu, A. Y. H., and Jerina, D. M.,** Hepatic microsomal epoxide hydrolase. Chemical evidence for a single polypeptide chain, *J. Biol. Chem.*, 254, 6240, 1979.
102. **Heinemann, F. S. and Ozols, J.,** The covalent structure of hepatic microsomal epoxide hydrolase. I. Isolation and characterization of the cyanogen bromide fragments, *J. Biol. Chem.*, 259, 791, 1984.
103. **Heinemann, F. S. and Ozols, J.,** The covalent structure of hepatic microsomal epoxide hydrolase. II. The complete amino acid sequence, *J. Biol. Chem.*, 259, 797, 1984.
104. **Armstrong, R. N., Levin, W., and Jerina, D. M.,** unpublished results, 1980.
105. **Hanzlik, R. P., Edelman, M., Michaely, W. J., and Scott, G.,** Enzymatic hydration of [^{18}O] epoxides. Role of nucleophilic mechanisms, *J. Am. Chem. Soc.*, 98, 1952, 1976.
106. **Armstrong, R. N., Levin, W., and Jerina, D. M.,** Hepatic microsomal epoxide hydrolase. Mechanistic studies of the hydration of K-region arene oxides, *J. Biol. Chem.*, 255, 4698, 1980.
107. **Bellucci, G., Berti, G., Ferretti, M., Marioni, F., and Re, F.,** The epoxide hydrolase catalyzed hydrolysis of *trans*-3-bromo-1,2-epoxycyclohexane. A direct proof for a general base catalyzed mechanism of the enzymatic hydration, *Biochem. Biophys. Res. Commun.*, 102, 838, 1981.
108. **Hanzlik, R. P., Heideman, S., and Smith, D.,** Regioselectivity in enzymatic hydration of *cis*-1,2-disubstituted [^{18}O]-epoxides, *Biochem. Biophys. Res. Commun.*, 82, 310, 1978.
109. **Prestwich, G. D., Lucarelli, I., Park, S.-K., Loury, D. N., Moody, D. E., and Hammock, B. D.,** Cyclopropyl oxiranes: reversible inhibitors of cytosolic and microsomal epoxide hydrolase, *Arch. Biochem. Biophys.*, 237, 361, 1985.
110. **DuBois, G. C., Apella, E., Levin, W., Lu, A. Y. H., and Jerina, D. M.,** Hepatic microsomal epoxide hydrolase. Involvement of a histidine at the active site suggests a nucleophilic mechanism, *J. Biol. Chem.*, 253, 2932, 1978.
111. **Dansette, P. M., Makedonska, V. B., and Jerina, D. M.,** Mechanism of catalysis for the hydration of substituted styrene oxides by hepatic epoxide hydrolase, *Arch. Biochem. Biophys.*, 187, 290, 1978.
112. **Jencks, W. P.,** *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York, 1969.
113. **Bruice, P. Y., Bruice, T. C., Dansette, P. M., Selander, H. G., Yagi, H., and Jerina, D. M.,** Comparison of the mechanisms of solvolysis and rearrangement of K-region vs. non-K-region arene oxides of phenanthrene. Comparative solvolytic rate constants of K-region and non-K-region arene oxides, *J. Am. Chem. Soc.*, 98, 2965, 1976.
114. **Whalen, D. L., Ross, A. M., Dansette, P. M., and Jerina, D. M.,** Specific effects of chloride ion in the hydrolysis of a K-region arene oxide, *J. Am. Chem. Soc.*, 99, 5672, 1977.
115. **Prestwich, G. D., Kuo, J.-W., Park, S.-K., Loury, D. N., and Hammock, B. D.,** Inhibition of epoxide metabolism by α,β-epoxyketones and isosteric analogs, *Arch. Biochem. Biophys.*, 242, 11, 1985.
116. **Hanzlik, R.P., Jacober, S.P., Hamrick, K.J., Moon, J.B., Hogberg, K., and Judson, C.M.** Use of stable isotopes for mechanistic studies of cytochrome P-450 and epoxide hydrolase,, *2nd Int. Symp. on the Synthesis and Applications of Isotopically Labeled Compounds*, Muccino, R., Ed., Elsevier, New York, 1986, 53.
117. **Hanzlik, R. P. and Westkaemper, R. B.,** Mechanistic studies of epoxide hydrolase utilizing a continuous spectrophotometric assay, *Arch. Biochem. Biophys.*, 208, 195, 1981.
118. **Watabe, T. and Akamatsu, K.,** Stereoselective hydrolysis of *cis*- and *trans*-stilbene oxides by hepatic microsomal epoxide hydrolase, *Biochem. Biophys. Res. Commun.*, 44, 199, 1971.
119. **Sayer, J. M., Yagi, H., vanBladeren, P. J., Levin, W., and Jerina, D. M.,** Stereoselectivity of microsomal epoxide hydrolase toward diol epoxides and tetrahydroperoxides derived from benz[*a*]anthracene, *J. Biol. Chem.*, 260, 1630, 1985.
120. **Bellucci, G., Berti, G., Ingrosso, G., and Mastrorilli, E.,** Stereoselectivity in the epoxide hydrase catalyzed hydrolysis of the stereoisomeric 4-*tert*-butyl-1,2-epoxycyclohexanes, *J. Org. Chem.*, 45, 299, 1980.
121. **Gatelani, G. and Mastrorilli, E.,** Acid-catalyzed and enzymatic hydrolysis of *trans*- and *cis*-2-methyl-3,4-epoxytetrahydropyron, *J. Chem., Soc. Perkin Trans.*, 1, 2717, 1983.
122. **Bellucci, G., Berti, G., Ferretti, M., Mastroroilli, E., and Silvestri, L.,** Enantioselectivity of the microsomal epoxide hydrolase catalyzed hydrolysis of *trans*-4,5-dimethyl-1,2-epoxycyclohexane, *J. Org. Chem.*, 50, 1471, 1985.

123. **Jakoby, W. B.,** The glutathione S-transferases: a group of multifunctional detoxification proteins, *Adv. Enzymol.*, 46, 383, 1978.
124. **Jakoby, W. B. and Habig, W. H.,** Glutathione transferases in *Enzymatic Basis of Detoxication*, Vol. 2, Jakoby, W. B., Ed., Academic Press, New York, 1980, 63.
125. **Chasseaud, L. F.,** The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents, *Adv. Cancer Res.*, 29, 175, 1979.
126. **Aris, I. M., Ohmi, N., Bargava, M., and Listowsky, I.,** Ligandin: an adventure in liverland, *Mol. Cell. Biochem.*, 29, 71, 1980.
127. **Homma, H., Maruyama, H., Niitsu, Y., and Listowsky, I.,** A. subclass of glutathione S-transferases as intracellular high-capacity and high-affinity steroid-binding proteins, *Biochem. J.*, 235, 763, 1986.
128. **Senjo, M., Ishibashi, T., and Imai, Y.,** Purification and characterization of cytosolic liver protein facilitating heme transport into apocytochrome b_5 from mitochondria. Evidence for identifying the heme transfer protein as belonging to a group of glutathione S-transferases, *J. Biol. Chem.*, 260, 9191, 1985.
129. **Vander Jagt, D. L., Hunsaker, L. A., Garcia, K. B., and Royer, R. E.,** Isolation and characterization of multiple glutathione S-transferases from human liver. Evidence for unique heme-binding sites, *J. Biol. Chem.*, 260, 11603, 1985.
130. **Hanson-Painton, O., Griffin, M. J., and Tang, J.,** Evidence for cytosolic benzo[*a*]pyrene carrier proteins which functions in cytochrome P-450 oxidation in rat liver, *Biochem. Biophys. Res. Commun.*, 101, 1364, 1981.
131. **Boyer, T. D., Zakin, D., and Vessey, D. A.,** Do the soluble glutathione S-transferases have direct access to membrane-bound substrates, *Biochem. Pharmacol.*, 32, 29, 1983.
132. **Federici, G., Dillio, C., Sacchetta, P., Polidoro, G., and Bannister, J. V.,** The isolation, characterization and kinetics of glutathione S-transferase from human platelets, *Int. J. Biochem.*, 17, 653, 1985.
133. **Awasthi, Y. C., Singh, S. V., Das, M., and Mukhtar, H.,** Differential activities of rat and human lung glutathione S-transferase isoenzymes towards benzo[*a*]pyrene epoxides, *Biochem. Biophys. Res. Commun.*, 133, 863, 1985.
134. **Mozer, T. J., Tiemeier, D. C., and Jaworski, E. G.,** Purification and characterization of corn glutathione S-transferase, *Biochemistry*, 22, 1068, 1983.
135. **Morgenstern, R., DePierre, J. W., and Jornvall, H.,** Microsomal glutathone transferase. Primary structure, *J. Biol. Chem.*, 260, 13976, 1985.
136. **Jakoby, W. B., Ketterer, B., and Mannervik, B.,** Glutathione transferases: nomenclature, *Biochem. Pharmacol.*, 33, 2539, 1984.
137. **Kalinyak, J. E. and Taylor, J. M.,** Rat glutathione S-transferase. Cloning of double-stranded cDNA and induction of its mRNA, *J. Biol. Chem.*, 257, 523, 1982.
138. **Frey, A. B., Friedberg, T., Oesch, F., and Kreibich, G.,** Studies on the subunit composition of rat liver glutathione S-transferase, *J. Biol. Chem.*, 258, 11321, 1983.
139. **Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J.-F., Argenbright, L., and Lu, A. Y. H.,** Rat liver glutathione S-transferases. Complete nucleotide sequence of a glutathione S-transferase mRNA and the regulation of the Ya, Yb, and Yc mRNAs by 3-methylcholanthrene and phenobarbital, *J. Biol. Chem.*, 259, 5182, 1984.
140. **Lai, H.-C.J., Li, N.-q., Weiss, M. J., Reddy, C. C., and Tu, C.-P.D.,** The nucleotide sequence of a rat liver glutathione S-transferase subunit cDNA clone, *J. Biol. Chem.*, 259, 5536, 1984.
141. **Tu, C.-P.D., Lai, H.-C.J., Li, N.-q., Weiss, M. J., Reddy, C. C.,** The Yc and Ya subunits of rat liver glutathione S-transferase are the products of separate genes, *J. Biol. Chem.*, 259, 9434, 1984.
142. **Telakowski-Hopkins, C. A., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., and Pickett, C. B.,** Rat liver glutathione S-transferases. Construction of a cDNA clone complementary to a Yc mRNA and prediction of the complete amino acid sequence of a Yc subunit, *J. Biol. Chem.*, 260, 5820, 1985.
143. **Ding, G.J.-F., Lu, A. Y. H., and Pickett, C. B.,** Rat liver glutathione S-transferases. Nucleotide sequence analysis of a Ybl cDNA clone and prediction of the complete amino acid sequence of the Ybl subunit, *J. Biol. Chem.*, 260, 13268, 1985.
144. **Habig, W. H., Pabst, M. J., and Jakoby, W. B.,** Glutathione S-transferases, the first step in mercapturic acid formation, *J. Biol. Chem.*, 249, 7130, 1974.
145. **deBethizy, J. D. and Rickert, D. E.,** Sequential enzyme-catalyzed metabolism of 4-nitrotoluene to S-(4-nitrobenzyl)glutathione, *Biochem. Biophys. Res. Commun.*, 114, 500, 1983.
146. **Hutson, D. H.,** Some observations on the chemical and stereochemical specificity of the de-alkylation of organophosphorus esters by a hepatic glutathione transferase, *Chem. Biol. Interact.*, 16, 315, 1977.
147. **Aldrige, W. N., Grasdalen, H., Aarstad, K., Street, B. W., and Norkov, T.,** Trialkyl phosphorothioates and glutathione S-transferases, *Chem. Biol. Interact.*, 54, 243, 1985.
148. **Keen, J. H., Habig, W. H., and Jakoby, W. B.,** Mechanism for the several activities of the glutathione S-transferases, *J. Biol. Chem.*, 251, 6183, 1976.
149. **Keen, J. H. and Jakoby, W. B.,** Glutathione transferases. Catalysis of nucleophilic reactions of glutathione, *J. Biol. Chem.*, 253, 5654, 1978.

150. **Fjellstedt, T. A., Allen, R. H., Duncan, B. K., and Jakoby, W. B.,** Enzymatic conjugation of epoxides with glutathione, *J. Biol. Chem.*, 248, 3702, 1973.
151. **Jerina, D. M. and Bend, J. R.,** Glutathione S-transferases, in *Biological Reactive Intermediates*, Jallow, D. J., Kocsis, J. J., Snyder, R., and Vainio, H., Eds., Plenum Press, New York, 1977, 207.
152. **Cobb, D., Boehlert, C., Lewis, D., and Armstrong, R. N.,** Stereoselectivity of isozyme C of glutathione S-transferase toward arene and azaarene oxides, *Biochemistry*, 22, 805, 1983.
153. **Dohn, D. R., Quebbemann, A. J., Borch, R. F., and Anders, M. W.,** Enzymatic reaction of chlorotrifluoroethene with glutathione: ^{19}F NMR evidence for stereochemical control of the reaction, *Biochemistry*, 24, 5137, 1985.
154. **Prohaska, J. R. and Ganther, H. E.,** Glutathione peroxidase activity of glutathione S-transferase purified from rat liver, *Biochem. Biophys. Res. Commun.*, 76, 437, 1977.
155. **Morgenstern, R., DePierre, J. W., Lind, C., Guthenberg, C., Mannervik, B., and Ernster, L.,** Benzo[*a*]pyrene quinones can be generated by lipid peroxidation and are conjugated with glutathione by glutathione S-transferase B from rat liver, *Biochem. Biophys. Res. Commun.*, 99, 682, 1981.
156. **Koga, N., Inskeep, P. B., Harris, T. M., and Guengerich, F. P.,** S-[2-(N^7-Guanyl)ethyl]glutathione, the major DNA adduct formed from 1,2-dibromoethane, *Biochemistry*, 25, 2191, 1986.
157. **van Bladeren, P. J., Breimer, D. D., Rotteveel-Smijs, G. M. T., deKnijff, P., Mohn, G. R., Buijs, W., van Meeteren-Walchli, B., and van der Gen, A.,** The relation between structure of vicinal dihalogen compounds and their mutagenic activation via conjugation to glutathione, *Carcinogenesis*, 2, 499, 1981.
158. **Buijs, W., van der Gen, A., Mohn, G.R., and Breimer, D.D.,** The direct mutagenic activity of alpha, omega-dihalogenoalkanes in *Salmonella typhimurium:* strong correlation between chemical properties and mutagenic activity, *Mutat. Res.*, 141, 11, 1984.
159. **Marchand, D. H. and Abdel-Monem, M. M.,** Glutathione S-transferases catalyzed conjugation of 1,4-dusubstituted butanes with glutathione in vitro, *Biochem. Biophys. Res. Commun.*, 128, 360, 1985.
160. **Alin, P., Danielson, U. H., and Mannervik, B.,** 4-Hydroxyalk-2-enals are substrates for glutathione transferase, *FEBS Lett.*, 179, 267, 1985.
161. **Benson, A. M., Talalay, P., Keen, J. H., and Jakoby, W. B.,** Relationship between the soluble glutathione-dependent Δ^5-3-keto-steroid isomerase and the glutathione S-transferases of the liver, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 158, 1977.
162. **Bach, M. K., Brashler, J. R., and Morton, D. R., Jr.,** Solubilization and characterization of the leukotriene C_4 synthetase of rat basophil leukemia cells: a novel, particulate glutathione S-transferase, *Arch. Biochem. Biophys.*, 230, 455, 1984.
163. **Soderstrom, M., Mannervik, B., Orning, L., and Hammarstrom, S.,** Leukotriene C_4 formation catalyzed by three distinct forms of human cytosolic glutathione transferase, *Biochem. Biophys. Res. Commun.*, 128, 265, 1985.
164. **Duffel, M. W. and Jakoby, W. B.,** Cysteine S-conjugate *N*-acetyltransferase from rat kidney microsomes, *Mol. Pharmacol.*, 21, 444, 1982.
165. **Stevens, J. and Jakoby, W. B.,** Cysteine conjugate β-lyase, *Mol. Pharmacol.*, 23, 761, 1983.
166. **Pabst, M. J., Habig, W. H., and Jakoby, W. B.,** Glutathione S-transferase A. A novel kinetic mechanism in which the major reaction pathway depends on the substrate concentration, *J. Biol. Chem.*, 249, 7140, 1974.
167. **Jakobson, I., Askelöf, P., Warholm, M., and Mannervik, B.,** A steady-state-kinetic random mechanism for glutathione S-transferase A from rat liver: a model involving kinetically significant enzyme-product complexes in the forward reaction, *Eur. J. Biochem.*, 77, 253, 1977.
168. **Jakobson, I., Warholm, M., and Mannervik, B.,** Multiple inhibition of glutathione S-transferase A from rat liver by glutathione derivatives: kinetic analysis supporting a steady-state random sequential mechanism, *Biochem. J.*, 177, 861, 1979.
169. **Jakobson, I., Warholm, M., and Mannervik, B.,** The binding of substrates and a product of the enzymatic reaction to glutathione S-transferase A. *J. Biol. Chem.*, 254, 7085, 1979.
170. **Chen, W.-J., Boehlert, C. C., Rider, K., and Armstrong, R. N.,** Synthesis and characterization of the oxygen and desthio analogues of glutathione as dead-end inhibitors of glutathione S-transferase, *Biochem. Biophys. Res. Commun.*, 128, 233, 1985.
171. **Schramm, V. L., McCluskey, R., Emig, F. A., and Litwack, G.,** Kinetic studies and active site-binding properties of glutathione S-transferase using spin-labeled glutathione, a product analogue, *J. Biol. Chem.*, 259, 714, 1984.
172. **Mangold, J. B. and Abdel-Monem, M. M.,** Stereoselectivity of the glutathione S-transferase conjugation of aralkyl halides, *Biochem. Biophys. Res. Commun.*, 96, 333, 1980.
173. **Mangold, J. B. and Abdel-Monem, M. M.,** Stereochemical aspects of conjugation reactions catalyzed by rat liver glutathione S-transferase isozymes, *J. Med. Chem.*, 26, 66, 1983.
174. **Vander Jagt, D. L., Wilson, S. P., Dean, V. L., and Simons, P. C.,** Bilirubin binding to rat liver ligandins (glutathione S-transferase A and B). Relationship between bilirubin binding and transferase activity, *J. Biol. Chem.*, 257, 1997, 1982.

175. **Vander Jagt, D. L., Dean, V. L., Wilson, S. P., and Royer, R. E.,** Regulation of the glutathione S-transferase activity of bilirubin transport protein (Ligandin) from human liver. Enzymic memory involving protein-protein interactions, *J. Biol. Chem.*, 258, 5689, 1983.
176. **Boyer, T. D., Vessey, D. A., Holcomb, C., and Saley, N.,** Studies of the relationship between the catalytic activity and binding of non-substrate ligands by the glutathione S-transferases, *Biochem. J.*, 217, 179, 1984.
177. **Chen, W.-J. and Armstrong, R. N.,** unpublished results, 1986.
178. **Sugimoto, M., Kuhlenkamp, J., Ookhtens, M., Aw, T. Y., Reeve, J., Jr., and Kaplowitz, N.,** Gamma-glutamylcysteine: a substrate for glutathione S-transferases, *Biochem. Pharmacol.*, 34, 3643, 1985.
179. **Yamada, T. and Kaplowitz, N.,** Propylthiouracil, A substrate for the glutathione S-transferases that competes with glutathione, *J. Biol. Chem.*, 255, 3508, 1980.
180. **Habig, W. H., Jakoby, W. B., Guthenberg, C., Mannervik, B., and Vauder Jagt, D. L.,** 2-Propyl-thiouracil does not replace glutathione for the glutathione transferases, *J. Biol. Chem.*, 259, 7409, 1984.
181. **Chen, W.-J., Lee, D. Y., and Armstrong, R. N.,** 4N-(malonyl-D-cysteinyl)-L-2,4-diaminobutyrate: the end-group modified retro-inverso isomer of glutathione, *J. Org. Chem.*, 51, 2848, 1986.
182. **Wright, W. B.,** The crystal structure of glutathione, *Acta Cryst.*, 11, 632, 1958.
183. **Epp, O., Ladenstein, R., and Wendel, A.,** The refined structure of the selenoenzyme glutathione peroxidase at 0.2 nm resolution, *Eur. J. Biochem.*, 133, 51, 1983.
184. **Pai, E. F. and Schulz, G. E.,** The catalytic mechanism of glutathione reductase as derived from X-ray diffraction analysis of reaction intermediates, *J. Biol. Chem.*, 258, 1752, 1983.
185. **Homma, H. and Listowsky, I.,** Identification of Yb-glutathione-S-transferase as a major rat liver protein labeled with dexamethasone 21-methane-sulfonate, *Proc. Natl. Acad. Sci. U.S.A.*, 82, 7165, 1985.
186. **Hernandez, O., Walker, M., Cox, R. H., Foureman, G. L., Smith, B. R., and Bend, J. R.,** Regiospecificity and stereospecificity in the enzymatic conjugation of glutathione with (±)-benzo[*a*]pyrene 4,5-oxide, *Biochem. Biophys. Res. Commun.*, 96, 1494, 1980.
187. **Boehlert, C. C. and Armstrong, R. N.,** Investigation of the kinetic and stereochemical recognition of arene and azaarene oxides by isozymes A_2 and C_2 of glutathione S-transferase, *Biochem. Biophys. Res. Commun.*, 121, 980, 1984.
188. **Watabe, T., Ozawa, N., Hiratsuka, A., Saito, Y., and Tsurumori, T.,** Regiospecific glutathione conjugation of alkylarylethlene oxides by hepatic glutathione S-transferase, *Biochem. Pharmacol.*, 33, 2687, 1984.
189. **Watabe, T., Hiratsuka, A., and Tsurumori, T.,** Regiospecific and diastereoselective inactivation of mutagenic 9,10-dihydrobenzo[*a*]pyrene 7,8-oxide by hepatic cytosolic glutathione S-transferase, *Biochem. Pharmacol*, 33, 4051, 1984.
190. **Watabe, T., Hiratsuka, A., and Tsurumori, T.,** Enantioselectivity in glutathione conjugation of 1,2-epoxy-1,2,3,4-tetrahydronaphthalene by hepatic glutathione S-transferase, *Biochem. Biophys. Res. Commun.*, 130, 65, 1985.
191. **Danielson, U. H. and Mannervik, B.,** Kinetic independence of the subunits of cytosolic glutathione transferase from the rat, *Biochem. J.*, 231, 263, 1985.
192. **Jakoby, W. B., Sekura, R. D., Lyon, E. S., Marcus, C. J., and Wang, J.-L.,** Sulfotransferases, in *Enzymatic Basis of Detoxication*, Vol. 2, Jakoby, W. B., Ed., Academic Press, New York, 1980, 199.
193. **Jakoby, W. B., Duffel, M. W., Lyon, E. S., and Ramaswamy, S.,** Sulfotransferases active with xenobiotics — comment on mechanism, in *Progress in Drug Metabolism*, Vol. 8, Bridges, J. W. and Chasseaud, L. F., Eds., Taylor & Francis, London, 1984, 11.
194. **Lyon, E. S. and Jakoby, W. B.,** The identity of alcohol sulfotransferases with hydroxysteroid sulfotransferases, *Arch. Biochem. Biophys.*, 202, 474, 1980.
195. **Marcus, C. J., Sekura, R. D., and Jakoby, W. B.,** A hydroxysteroid sulfotransferase from rat liver, *Anal. Biochem.*, 107, 296, 1980.
196. **Singer, S. S. and Bruns, L.,** Enzymatic sulfation of steroids. XI. The extensive purification and some properties of hepatic sulfotransferase III from female rats, *Can. J. Biochem.*, 58, 660, 1980.
197. **Sekura, R. D. and Jakoby, W. B.,** Phenol sulfotransferases, *J. Biol. Chem.*, 254, 5658, 1979.
198. **Sekura, R. D. and Jakoby, W. B.,** Aryl sulfotransferase IV from rat liver, *Arch. Biochem. Biophys.*, 211, 352, 1981.
199. **Borchardt, R. T. and Schasteen, C. S.,** Phenol sulfotransferase. I. Purification of a rat liver enzyme by affinity chromatography, *Biochim. Biophys. Acta*, 708, 272, 1982.
200. **Watabe, T.,** Metabolic activation of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 7-methylbenz[*a*]anthracene (7-MBA) by rat liver P-450 and sulfotransferase, *J. Toxicol. Sci.*, 8, 119, 1983.
201. **Watabe, T., Fujieda, T., Hiratsuka, A., Ishizuka, T., Hakamata, Y., and Ogura, K.,** The carcinogen, 7-hydroxymethyl-12-methylbenz[*a*]anthracene is activated and covalently binds to DNA via a sulfate ester, *Biochem. Pharmacol*, 34, 3002, 1985.

202. **Watabe, T., Hiratsuka, A., Ogura, K., and Endoh, K.,** A reactive hydroxymethyl sulfate ester formed regioselectively from the carcinogen 7,12-dihydroxymethylbenz[*a*]anthracene, by rat liver sulfotransferase, *Biochem. Biophys. Res. Commun.*, 131, 694, 1985.
203. **Duffel, M. W. and Jakoby, W. B.,** On the mechanism of aryl sulfotransferase, *J. Biol. Chem.*, 256, 11123, 1981.
204. **Borchardt, R. T., Schasteen, C. S., and Wu, S.-E.,** Phenol sulfotransferase. II. Inactivation by phenylglyoxal, *N*-ethylmaleimide, and ribonucleotide 2',3'-dialdehydes, *Biochim. Biophys. Acta*, 708, 280, 1982.
205. **Cummings, J., Graham, A. B., and Wood, G. C.,** Kinetic studies of latent microsomal UDP-glucuronosyltransferases. Kinetics of glucuronidation in intact and perturbant-treated membranes, *Biochim. Biophys. Acta*, 771, 127, 1984.
206. **Antoine, B., Magdalou, J., and Siest, G.,** Kinetic properties of UDP-glucuronosyltransferase(s) in different membranes of rat liver cells, *Xenobiotica*, 14, 575, 1984.
207. **Boutin, J. A., Antoine, B., Batt, A.-M., and Siest, G.,** Heterogeneity of hepatic microsomal UDP-glucuronosyltransferase(s) activities: comparison between human and mammalian species activities, *Chem. Biol. Interact.*, 52, 173, 1984.
208. **Boutin, J. A., Thomassin, J., Siest, G., and Cartier, A.,** Heterogeneity of hepatic microsomal UDP-glucoronosyltransferase activities. Conjugations of phenolic and monoterpenoid aglycones in control and induced rats and guinea pigs, *Biochem. Pharmacol.*, 34, 2235, 1985.
209. **Peters, W. H. M., Jansen, P. L. M., and Nauta, H.,** The molecular weights of UDP-glucuronosyltransferase determined with radiation inactivation analysis. A molecular model of bilirubin UDP-glucuronosylytransferase, *J. Biol. Chem.*, 259, 11701, 1984.
210. **Mackenzie, P. I., Gonzalez, F. J., and Owens, I. S.,** Cell-free translation of mouse liver mRNA coding for two forms of UDPglucuronosyltransferase, *Arch. Biochem. Biophys.*, 230, 676, 1984.
211. **Kasper, C. B. and Henton, D.,** Glucoronidation, in *Enzymatic Basis of Detoxication*, Vol. 2, Jakoby, W. B., Ed., Academic Press, New York, 1980, 4.
212. **Chowdhury, J. R. and Arias, I. M.,** Dismutation of bilirubin monoglucuronide, in *Enzymatic Basis of Detoxication*, Vol. 2, Jakoby, W. B., Ed., Academic Press, New York, 1980, 37.
213. **Gorski, J. P. and Kasper, C. B.,** Purification and properties of microsomal UDP-glucuronosyltransferase from rat liver, *J. Biol. Chem.*, 252, 1336, 1977.
214. **Burchell, B.,** Substrate specificity and properties of uridine diphosphate glucuronosyltransferase purified to apparent homogeneity from phenobarbital treated rat liver, *Biochem. J.*, 173, 749, 1978.
215. **Burchell, B.,** Purification of mouse liver udpglucuronosyltransferase, *Med. Biol.*, 57, 265, 1979.
216. **Burchell, B.,** Isolation and purification of bilirubin UDP-glucuronyltransferase from rat liver, *FEBS Lett.*, 111, 131, 1980.
217. **Weatherill, P. J. and Burchell, B.,** The separation and purification of rat liver UDP-glucuronosyltransferase activities towards testosterone and oestrone, *Biochem. J.*, 189, 377, 1980.
218. **Matern, H., Matern, S., and Gerok, W.,** Isolation and characterization of rat liver microsomal UDP-glucuronosyltransferase activity toward chenodeoxycholic acid and testosterone as a single form of enzyme, *J. Biol. Chem.*, 257, 7422, 1982.
219. **Falany, C. N. and Tephly, T. R.,** Separation, purification and characterization of three isoenzymes of UDP-glucuronosyltransferase from rat liver microsomes, *Arch. Biochem. Biophys.*, 227, 248, 1983.
220. **Magdalou, J., Balland, W., Thirion, C., and Siest, G.,** Effects of membrane perturbants on UDP-glucuronosyltransferase activity in rat-liver microsomes. Circular dichroism studies, *Chem. Biol. Interact*, 27, 255, 1979.
221. **Singh, O. M. P., Graham, A. B., and Wood, G. C.,** The phospholipid dependence of UDP-glucuronosyltransferase: conformation/reactivity studies with purified enzyme, *Biochem. Biophys. Res. Commun.*, 107, 345, 1982.
222. **Mackenzie, P. I., Gonzakz, F. J., and Owens, I. S.,** Cloning and characterization of DNA complementary to rat liver UDP-glucuronosyltransferase mRNA, *J. Biol. Chem.*, 259, 12153, 1984.
223. **Mackenzie, P. I.,** Rat liver UDP-glucuronosyltransferase. Sequence and expression of a cDNA encoding a phenobarbital-inducible form, *J. Biol. Chem.*, 261, 6119, 1986.
224. **Boyland, E. and Levi, A. A.,** Metabolism of polycyclic compounds. II. Production of dihydroxydihydroanthraceneglycuronic acid from anthracene, *J. Chem. Soc*, 728, 1936.
225. **Fahl, W. E., Shen, A. L., and Jefcoate, C. R.,** UDP-glucuronosyltransferase and the conjugation of benzo[*a*]pyrene metabolites to DNA, *Biochem. Biophys. Res. Commun.*, 85, 891, 1978.
226. **Batt, A. M., Mackenzie, P., Hanninen, O., and Vainio, H.,** Glucuronidation of 3-hydroxybenzo[*a*]pyrene in liver microsomes, *Med. Biol.*, 57, 281, 1979.
227. **Hirakawa, T., Nemoto, N., Yamada, M-A., and Takayama, S.,** Metabolism of benzo[*a*]pyrene and the related activities in hamster embryo cells, *Chem. Biol. Interact.*, 25, 189, 1979.
228. **Bansal, S. K., Zaleski, J., and Gessner, T.,** Glucuronidation of oxygenated benzo[*a*]pyrene derivatives by UDP-glucuronosyltransferase of nuclear envelope, *Biochem. Biophys. Res. Commun.*, 98, 131, 1981.

229. **Lewis, D. A. and Armstrong, R. N.,** Stereoselectivity and regioselectivity of uridine 5′-diphosphoglucuronosyltransferase toward vicinal dihydrodiols of polycyclic aromatic hydrocarbons, *Biochemistry*, 22, 6297, 1983.
230. **Lind, C.,** Formation of benzo[*a*]pyrene-3,6-quinol mono- and diglucuronides in rat liver microsomes, *Arch. Biochem. Biophys.*, 240, 226, 1985.
231. **Lilienblum, W., Bock-Hennig, B. S., and Bock, K. W.,** Protection against toxic redox cycles between benzo[a]pyrene 3,6-quinone and its quinol by 3-methylcholanthrene-inducible formation of the quinol mono- and diglucuronide, *Mol. Pharmacol.*, 27, 451, 1985.
232. **Nemoto, N. and Gelboin, H. V.,** Enzymatic conjugation of benzo[*a*]pyrene oxides, phenols, and dihydrodiols with UDP-glucuronic acid, *Biochem. Pharmacol.*, 25, 1221, 1976.
233. **Chaudhuri, N. K., Servando, O. A., Manniello, M. J., Luders, R. C., Chao, D. K., and Bartlett, M. F.,** Metabolism of tripelennamine in man, *Drug Metab. Dispos.*, 4, 372, 1976.
Metabolism of tripelennamine in man, *Drug Metab. Dispos.*, 4, 372, 1976.
234. **Richter, W. J., Alt, K. O., Dieterle, W., Faigle, J. W., Kriemler, H. P., Mory, H., and Winkler, T.,** C-Glucuronides, a novel type of drug metabolite, *Helv. Chim. Acta*, 58, 2512, 1975.
235. **Blanckaert, N., Gollan, J., and Schmid, R.,** Bilirubin diglucoronide synthesis by a UDP-glucuronic acid-dependent enzyme system in rat liver microsomes, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 2037, 1979.
236. **Gordon, E. R., Sommerer, U., and Guresky, C. A.,** The hepatic microsomal formation of bilirubin diglucuronide, *J. Biol. Chem.*, 258, 15028, 1983.
237. **Gordon, E. R., Meier, P. J., Goresky, C. A., and Boyer, J. L.,** Mechanism and subcellular site of bilirubin diglucuronide formation in rat liver, *J. Biol. Chem.*, 259, 5500, 1984.
238. **Whitmer, D., Ziurys, J. C., and Gollan, J. L.,** Hepatic microsomal glucuronidation of bilirubin in unilamellar liposomal membranes. Implications for intracellular transport of lipophilic substrates, *J. Biol. Chem.*, 259, 11969, 1984.
239. **Kirkpatrick, R. B., Falany, C. N., and Tephly, T. R.,** Glucuronidation of bile acids by rat liver 3-OH androgen UDP-glucuronosyltransferase, *J. Biol. Chem.*, 259, 6176, 1984.
240. **Zakim, D. and Vessey, D. A.,** Regulation of microsomal UDP-glucuronosyltransferase by metal ions. Differential effects of Mn^{2+} on forward and reverse reactions, *Eur. J. Biochem.*, 64, 459, 1976.
241. **Hochman, Y. and Zakim, D.,** A comparison of the kinetic properties of two different forms of microsomal UDP-glucuronosyltransferase, *J. Biol. Chem.*, 258, 4143, 1983.
242. **Vessey, D. A. and Zakim, D.,** Regulation of microsomal enzymes by phospholipids. V. Kinetic studies of hepatic uridine diphosphate-glucuronyltransferase, *J. Biol. Chem.*, 247, 3023, 1972.
243. **Hochman, Y. and Zakim, D.,** Studies of the catalytic mechanism of microsomal UDP-glucuronyltransferase, α-glucuronidase activity and its stimulation by phospholipids, *J. Biol. Chem.*, 259, 5521, 1984.
244. **Lewis, D. A. and Armstrong, R. N.,** unpublished results, 1985.
245. **Magdalou, J., Hochman, Y., and Zakim, D.,** Factors modulating the catalytic specificity of a pure form of UDP-glucuronosyltransferase, *J. Biol. Chem.*, 257, 13624, 1982.
246. **Dansette, P. M., Makedonska, V. B., and Jerina, D. M.,** *Arch. Biochem. Biophys.*, 187, 83, 1978.
247. **Zakim, D., Hochman, Y., and Kenney, W. C.,** Evidence for an active site arginine in UDP-glucuronyltransferase, *J. Biol. Chem.*, 258, 6430, 1983.
248. **Thompson, J. A., Hull, J. E., and Norris, K. J.,** Glucuronidation of propranolol and 4′-hydroxypropanolol. Substrate specificity and stereoselectivity of rat liver microsomal glucuronyltransferase, *Drug. Metab. Dispos.*, 9, 466, 1981.
249. **Owens, I. S. and Mackenzie, P. I.,** A comparison of the isoelectric points of mouse liver UDP-glucuronosyltransferase enzymes conjugating the twelve benzo[*a*]pyrene phenols, *Biochem. Biophys. Res. Commun.*, 109, 1075, 1982.
250. **Cobb, D.I., Lewis, D. A., and Armstrong, R. N.,** Solvent dependence of the conformation and chiroptical properties of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and its monoglucuronides, *J. Org. Chem.*, 48, 4139, 1983.
251. **Armstrong, R. N., Lewis, D. A., Ammon, H. L., and Prasad, S. M.,** Kinetically stable conformers of 3,4,5,6-tetramethyl-9,10-dihydroxy-9,10-dihydrophenanthrene as probes of the conformer specificity of UDPglucuronosyltransferase, *J. Am. Chem. Soc.*, 107, 1057, 1985.
252. **Armstrong, R. N. and Lewis, D. A.,** Pseudorotation barriers in *cis*-4,5-dimethyl and *cis*-3,4,5,6-tetramethyl-9,10-dihydroxy-9,10-dihydrophenanthrene: measurement of the buttressing effect, *J. Org. Chem.*, 50, 907, 1985.
253. **Armstrong, R. N., Lewis, D. A., Darnow, J. N., and Ammon, H. L.,** Sterically hindered aromatic hydrocarbons as stereochemical probes of xenobiotic metabolizing enzymes, in *Mechanisms of Enzymatic Reactions: Stereochemistry*, Frey, P. A., Ed., Elsevier, New York, 1986, 267.