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REVIEW ARTICLE

Metabolically Oxygenated Compounds: Formation, Conjugation, and Possible Biological Implications

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Keyphrases □ Metabolism—introduction of oxygen in biotransformations, major conjugation reactions, aspects in mutagenicity and carcinogenicity, review □ Oxygenation in drug metabolism—role in biotransformations, major conjugation reactions, aspects in mutagenicity and carcinogenicity, review □ Mutagenicity and carcinogenicity—implications of metabolically oxygenated compounds, review

The development of drug metabolism into an essential aspect of new drug discovery has gone through a number of interesting phases. Originally, it was considered to be the domain of a few individuals who were more or less happily puttering about. The data being gathered were treated as interesting, but their implications made little or no impact on the larger scientific community concerned with the discovery, evaluation, and development of therapeutic entities. It borders on the incredible that, for many years, an almost complete lack of appreciation of the value of drug metabolism studies existed among medicinal chemists, pharmaceutical scientists, and the medical profession in general.

Today, it is inconceivable that any serious development of a new therapeutic agent would not include extensive drug metabolism studies which influence every step of a drug's history from its synthesis and early animal pharmacology through toxicology, formulation development, and human clinical trials. Perhaps the pendulum has swung too far and now drug metabolism is being regarded as the means of answering every therapeutic or toxicological puzzle, which, in fact, it cannot hope to do.

Drug metabolism is a term that is sorely in need of definition. We are all aware of the old story of the four blindfolded men standing about an elephant. One has his hand placed on the end of the elephant's trunk, another holds a tusk, one man's hands are placed around a leg, and the fourth holds the tail. Each man is then asked to describe an elephant. The reader can use his or her own imagination as to the variety of descriptions of an elephant that would be forthcoming. Unfortunately, all too often, the answer to the question of what is drug metabolism reflects the interest of the person to whom the query is posed. A pharmaceutical scientist couches an answer in terms of bioavailability and biopharmaceutics, the medicinal chemist thinks of biotransformation and active metabolites, toxicologists and pharmacologists refer to selective toxicity and species differences, biochemists mention events at the enzyme level, and more mathematically inclined individuals respond in terms of rate constants, half-lives, and compartmental models. It is all of these and more. Drug metabolism is that branch of science devoted to studying all aspects of the fate of foreign molecules in biological systems under *in vivo* or *in vitro* conditions.

Since the investigations described as "drug metab-

olism studies" frequently do not include either drug substances *per se* (*e.g.*, pesticides and the influences of adjuvants) or biotransformation of the parent compound, perhaps a new term should be considered. "Xenobionics," which loosely translated from the Greek means "the fate of strangers in a biological system," is perhaps more generally appropriate than drug metabolism.

It is not possible to present all aspects of such a multifaceted area in one review article. A number of recent texts, one of which is particularly outstanding (1), include discussions of particular matters such as absorption, protein binding, transfer of drugs across membranes, and routes of excretion. The present article will be confined to the introduction of oxygen in selected cases of biotransformations and the major conjugation reactions of these compounds that have been published recently. Some aspects of the possible implication of highly reactive metabolites in mutagenicity and carcinogenicity will be discussed.

DETOXICATION

The relationship of enzymic or hydrolytic biotransformations and the resulting physiological consequences of the exposure of biological systems to foreign chemical agents are no longer open to discussion; they have been demonstrated in numerous studies. Unfortunately, there is a tendency to extrapolate findings inappropriately and to make generalizations that are not quite justified.

Biotransformation of drugs into their metabolites is often referred to as "detoxication" (2) and is considered by some to be a relatively nonspecific mechanism developed by animals for eliminating foreign substances. The continued habit of calling biotransformation detoxication is unfortunate in view of the fact that it requires involving an almost supernatural power to suggest that any structural modification taking place *in vivo* will automatically reduce the toxic potential of a substance. Indeed, when one considers the concept of an inactive prodrug being converted into a therapeutically active molecule, the term detoxication seems even more inappropriate today.

An example illustrative of the effect of metabolism on the toxicity of two closely related compounds is the case of sulfadiazine and sulfamethazine. Both compounds are conjugated to the corresponding N^4-acetyl derivatives. In the mouse, the LD_{50} of sulfadiazine is 1.6 g/kg; N^4-acetylsulfadiazine is more toxic, 0.6 g/kg. With sulfamethazine (LD_{50} of 0.9 g/kg), the opposite is true; the LD_{50} of N^4-acetylsulfamethazine is greater, 1.3 g/kg (2).

Another frequently stated generalization is that biotransformation increases the aqueous solubility of compounds. This statement also must be accepted cautiously. When *o*-chlorophenylacetic acid is metabolized to *o*-chlorophenylaceturic acid, the metabolite has 1.5 times the aqueous solubility of the parent compound. On the other hand, when *p*-chlorophenylacetic acid is converted to its aceturic metabolite, the solubility of the conjugate is only one-half that of the parent compound. The aqueous solubility of metabolites can have important therapeutic consequences. Sulfamethazine and sulfathiazole have roughly the same aqueous solubility. Both compounds are metabolized to acetyl conjugates, which are excreted as such. The acetyl metabolite of sulfamethazine is more water soluble than the parent compound. In the sulfathiazole case, however, the acetyl metabolite is only one-tenth as soluble as the parent compound. The occurrence of kidney blockage caused by crystalline deposits within the kidney is rare with sulfamethazine but very frequent with sulfathiazole (2).

These cases are cited to point out that adherence to generalizations about the probable physicochemical, therapeutic, or toxicological consequences of biotransformations must be undertaken with considerable reservation. This does not mean that they cannot be used as the basis of "educated guesses." It is *usually* true that metabolic alterations *do* lead to more polar substances which are less lipophilic, more water soluble, and more acidic, leading to an accelerated termination of pharmacological activity and more rapid excretion by the renal tubule. The importance of these factors in the elimination of drugs from the body is exemplified by pentobarbital. According to one researcher (3), if biotransformations were not present, pentobarbital would exert its pharmacological effects in the body for nearly a century because of its high lipid solubility.

The hydroxylation of compounds and the subsequent conjugation of these products probably can be considered as being among the most important events that occur among the myriad of biotransformation reactions.

Aside from the implications of these transformations, insofar as they affect the excretion of foreign compounds from the animal because of altered hydrophilicity and/or acidity, the possibility of serious physiological consequences of some reactions of oxygen with foreign molecules are alarming. Although imperfectly understood, there can be little doubt that the carcinogenicity of many compounds is due to highly reactive transitory molecular forms which may occur during the process of biotransformation (4–6).

ALIPHATIC HYDROXYLATION

The hydroxylation of many aliphatic compounds has been reported, including fatty acids (7), alkanes (8, 9), substituted barbituric acids (10), and alkylamines (11). The aliphatic chains are most commonly oxidized at a terminal methyl group to form the corresponding primary alcohol. Usually, this primary alcohol undergoes further biotransformation to the aldehyde and then the acid. However, hydroxylation is not confined exclusively to terminal methyl groups. In addition to the oxidation at this position, hydroxylation at the penultimate carbon atom ($\omega - 1$) is also frequently encountered (7, 12–14). Metabolism of the fatty acids seems to involve hydroxylation at both the ultimate and penultimate carbon atoms.

Stearic acid has been reported (7) as being such a case; in this instance, terminal methyl oxidation is favored 3:1 over methylene group oxidation.

Hydroxylation of benzylic carbon atoms is also well known and the initial product, as in the case of purely aliphatic systems, is a primary alcohol (15–18). As with aliphatic compounds, the final product is often a carboxylic acid. Hydroxylation is not confined to methyl substituents but also is known to occur with ethyl, propyl, or butyl substituents. The stereospecific formation of *S*-(+)-1-indanol from indane has been reported (18).

Systematic studies (19, 20) of *o*-, *m*-, and *p*-methylbenzenes suggest that steric factors play an important role in determining whether the metabolic pathway will proceed by benzylic oxidation or an alternative pathway. *para*-Substituted methylbenzenes undergo benzylic oxidation, as with *trans*-2-(*p*-tolyl)cyclohexanol (20), whereas the corresponding *ortho*-component does not undergo benzylic oxidation but is hydroxylated in the cyclohexyl ring instead. In studies with isomeric methylanisoles (19), *p*-methylanisole underwent benzylic oxidation; *o*-methylanisole afforded the *p*-hydroxy analog.

Under *in vitro* conditions, cyclohexanol is formed (21) from cyclohexane. The position at which oxygenation of the cyclohexane ring occurs seems to be under the influence of stereochemical factors, wherein the bulk of the substituent groups plays a major role in the selection of the sites of attachment. Thus, when methylcyclohexane was studied (22), approximately equal amounts of 3- and 4-hydroxylated products were formed. On the other hand, when *trans*-2-(*o*-tolyl)cyclohexanol was studied, axial hydroxylation took place at the ring position opposite the bulky tolyl substituent as the principal reaction (20).

Particularly interesting studies were reported where the disposition of decalin and tetralin was investigated. *trans*-Decalin was metabolized to (±)-*trans*-*cis*-2-decalol containing an equatorial hydroxyl group, whereas *cis*-decalin yielded (±)-*cis*-*cis*-2-decalol with an axial hydroxyl (23). Tetralin was converted largely to α-tetralol, with β-tetralol being a minor product (24, 25). When ethinamate (1-ethynylcyclohexanol carbamate) (26) or 1-cyclohexanol (27) undergoes hydroxylation, it is at the 3- or 4-position of the alicyclic ring. The major metabolites found in the urine contain the hydroxyl moiety in the 4-equatorial or 3-axial orientation. Relative amounts of 3- and 4-hydroxylated products and the stereochemical nature of the metabolite in studies with acetohexamide {1-[(*p*-acetylphenyl)sulfonyl]-3-cyclohexylurea} were shown to be a function of species differences.

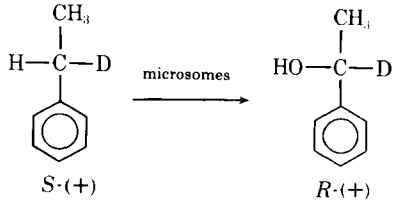
MECHANISMS OF HYDROXYLATION

The hydroxylation of carbon atoms has been exhaustively investigated. The oxygen entering the molecule is derived from molecular oxygen and not water, as one might intuitively expect. That the oxygen source is O_2 rather than —OH has been confirmed by a number of researchers. These studies include the hydroxylation of phenethylamine by dopamine-β-hydroxylase (28). These workers also employed phenylalanine hydroxylase, enzyme hydroxylation of steroids (29), and the benzylic hydroxylation of ethylbenzene in microsomal preparations (30). McMahon *et al.* (30) also demonstrated an isotope effect in the reaction, which indicates the rate-limiting role of the insertion step; the benzylic hydroxylation to yield methylphenylcarbinol had a K_H/K_D ratio of 1.8, which the corresponding benzylic hydroxylation of the oral antidiabetic agent, tolbutamide, did not have (15).

Early work with microbial systems (31, 32) showed that aliphatic hydroxylation of steroids in microbiological systems proceeded without inversion at the 11-position. In the rat, hydroxylation of cholesterol-7α-^{3}H resulted in the formation of the 7α-metabolite without loss of tritium (33). Additional examples were reported (34, 35), and it appears that monooxygenase catalyzes the hydroxylation of aliphatic hydrocarbons stereospecifically with retention of configuration. An interesting case is the fate of ethylbenzene *in vitro* when the system is composed of normal rat liver microsomes or those derived from rats treated with phenobarbital. In the normal case, ethylbenzene is oxidized *in vitro* to a mixture which is four parts *R*-(+)- to one part *S*-(−)-methylphenylcarbinol (36). The stereospecificity of this reaction was greatly reduced when the microsomes of rats previously dosed with phenobarbital were used (30, 36). Unequivocal evidence, demonstrating a case of hydroxylation in the presence of microsomes proceeding with retention of configuration, was also obtained (30, 36). Incubation of *S*-(+)-α-^{2}H-ethylbenzene with normal rat microsomes resulted in a product that was 92% *R*-(+)-α-^{2}H-methylphenylcarbinol, with 14% of the deuterium having been lost (Scheme I).

AROMATIC OXIDATION

The susceptibility of various aromatic rings to attack by oxygen during biotransformation is reminiscent of the reactivity of such compounds in the synthetic reactions of aromatic systems undergoing electrophilic substitution. Thus, aromatic rings that contain electron-donating groups are described as being "*ortho*–*para* directing" by chemists engaged in organic synthesis, whereas groups that are electron withdrawing are called "*meta* directing." The presence of groups (*e.g.*, —NH_2, —CH_3, and —OCH_3) (37, 38) that increase the electron density of the aromatic ring cause it to undergo metabolism more readily to



Scheme I

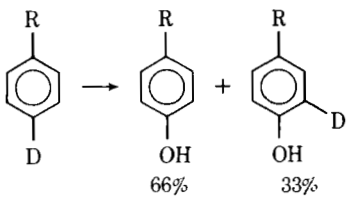
para-phenols. On the other hand, substituents that tend to decrease the electron density of the ring (*e.g.*, $-NO_2$, $-CONH_2$, and $-COOH$) make the compound more resistant to hydroxylation (37).

As with almost all other biotransformations, steric factors play an important role in aromatic hydroxylations. There is a pronounced tendency for such substitutions to take place in the *para*-position if it is available (37, 39–42). The ratio of *ortho*- to *para*-hydroxylation should be 2:1 if only electron density is a factor in determining the point of entry of oxygen into the molecule. In any particular instance, this ratio should be a constant if only one enzyme system is involved. The ratio of *ortho*- to *para*-hydroxylation products is species dependent and can be altered by enzyme induction. This latter observation strongly implies that several P-450 cytochromes can be present within a species. Even in the presence of a strong *meta*-directing group such as is found in nitrobenzene (37, 38), significant *para*-hydroxylation was noted. The entry of substituents into the *para*-position, even when it is a less favorable position in terms of electron density, is most probably ascribable to steric factors.

When more than one substituent is present in the aromatic substrate as in di- or trisubstituted compounds, the position where hydroxylation will most probably take place appears to be reasonably predictable on the basis of the electron-donating characteristics of the individual substituents, keeping in mind that *para*- rather than *ortho*-substitution is preferred (43–49). When the aromatic system contains more than one ring, whether it be aromatic, heterocyclic, or alicyclic, projection of the possible or probable site of hydroxylation is not simple because both electronic and steric factors interact. Some of the many papers dealing with these systems include skatole (50), zoxazolamine (51), and tricyclic compounds acting on the central nervous system (CNS) (52–54). The metabolism of β-blocking agents was recently reviewed (55), and an older review (2) is especially thorough.

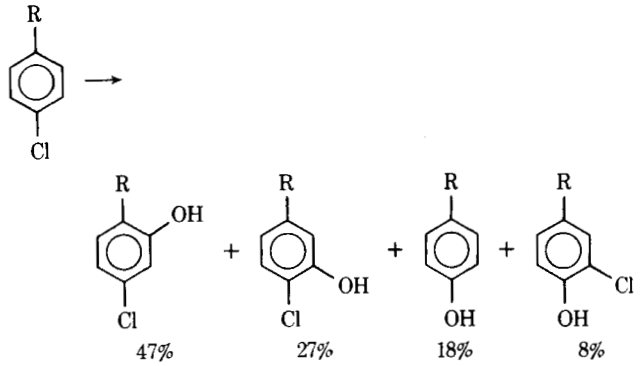
As was the case with aliphatic compounds undergoing hydroxylation, molecular oxygen is also involved when aromatic compounds are metabolized (28, 56). A number of studies have been conducted with substrates containing deuterium labels; in no instance could any isotope effect on the rate of hydroxylation be demonstrated when ^2H was inserted in place of ^1H (57–59).

An important feature of many carbon–oxygen bond formation steps is the "NIH shift," and an understanding of this phenomenon is necessary for the explanation of some seemingly strange metabolic changes. Briefly stated, the NIH shift is the phenomenon whereby the intramolecular migration of a ring substituent takes place during aromatic hydroxylation (Scheme II) (60–64).



R = $NHCOCH_3$

Scheme II



R = $NHCOCH_3$

Scheme III

The fate of deuterium or tritium labels has been studied in a number of cases. The label is largely retained if it is originally introduced in a position adjacent to the carbon atom undergoing hydroxylation.

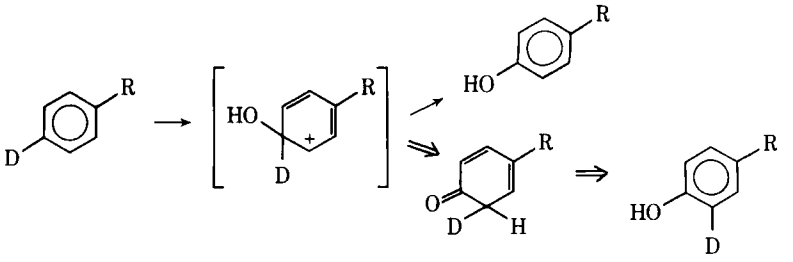
A series of investigations established that the NIH shift is a general reaction associated with the hydroxylation of aromatic compounds by animal, bacterial, fungal, and plant systems. The only reported exception is the aerobic hydroxylation of acetanilide-4-^2H and anisole-4-^2H with peroxidase in the presence of dihydroxyfumaric acid, which serves as a reducing agent. Free radical hydroxylation of aromatic compounds does not lead to migration of substituents (65). In the case of the peroxidase system, it may be presumed that the lack of migration is a reflection of the free radical nature of this hydroxylase system.

The migration of a number of substituents from positions undergoing hydroxylation has been demonstrated. Halogen or methyl groups migrate from the *para*- to the *meta*-position when *para*-substituted phenylalanines are subjected to hydroxylation with phenylalanine hydroxylase (61, 66, 67). When *p*-chloroacetanilide was exposed to liver microsomes from rats treated with the enzyme-inducing agent benzpyrene (68), a variety of products was obtained (Scheme III). In studies of the oxidative hydroxylation of *p*-methylacetanilide or *p*-methylanisole in liver microsomal systems, it was not possible to demonstrate migration of the methyl group (69); instead, hydroxylation of the methyl group ($-CH_2OH$) was found.

Although the examples cited have involved *in vitro* systems, the NIH shift has been demonstrated under *in vivo* conditions with 2-[(*p*-chlorophenyl)thiazol-4-yl]acetic acid (70).

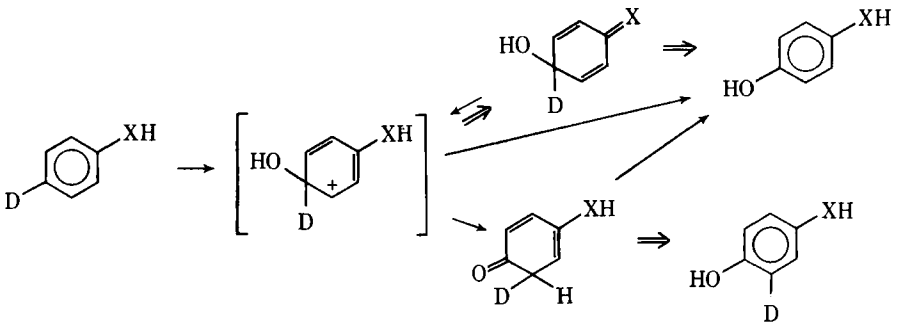
A very incisive and reasonable explanation of the mechanism of the NIH shift and of factors that influence it has been suggested (71); the observed data have been interpreted in terms of cationoid intermediates (Scheme IV). The investigators classified the

Group I



R =	OCH_3	CH_3	C_6H_5	$C{\equiv}N$	$CONH_2$	NO_2	Cl	Br	F	$NCH_3SO_2C_6H_5$
% D retained	60	54	64	41	42	40	54	40	47	53

Group II



X =	O	$NSO_2C_6H_5$	NH	NCOH	$NCOC_6H_5$	$NCOCH_3$
% D retained	0	1	6	19	21	30

Scheme IV

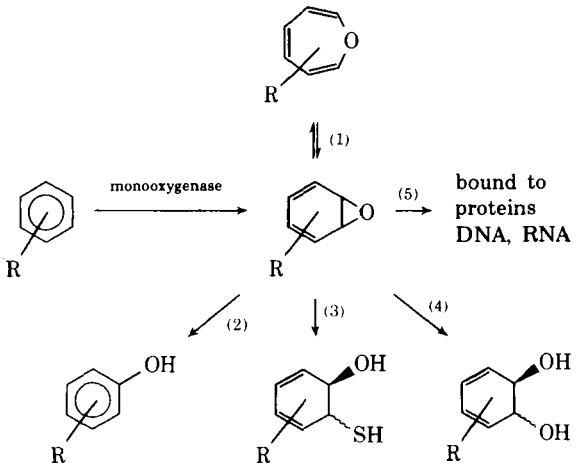
substrates as Group I or II. Group I is comprised of those substrates with substituents that do not have a labile proton adjacent to the aromatic system and have high retention values. The Group II substrates can form a stable cationoid by losing a proton from a heteroatom adjacent to the aromatic ring; these substrates show low retentions. The degree of retention is influenced by the acidity of the substituent and by pH. Retention in Group II substrates decreases as the ability of the substituent to donate electrons is increased. A recent publication (72) amplified the suggested mechanisms to include the formation of *ortho*-isomers in the metabolism of chlorobenzene.

The highly unstable nature of some oxygenated metabolites make their detection very difficult. As early as 1950, the formation of arene oxides, which are compounds arising from the epoxidation of a double bond in an aromatic ring system, was suggested and speculations were made as to their possible role in chemical carcinogenesis. The question as to whether arene oxides could be formed under biological conditions was not answered until much later (73, 74). Shortly thereafter, evidence showed that the carcinogenicity of polycyclic hydrocarbons having planar configurations was probably due to these reactive intermediates (75). The arene oxide intermediates rearrange nonenzymatically to phenols, which subsequently are converted to either vicinal diols by epoxide hydrases or to glutathione conjugates by glutathione *S*-epoxide conjugases. In addition, the glutathione *S*-epoxide conjugation seems to proceed without enzymic participation (76). The glutathione *S*-epoxide transferases are found in the cytoplasm (76), whereas the epoxide hydrases have been shown to be present in endoplasmic reticulum (77). The hydrases are inducible (77), whereas the glutathione *S*-transferases are not (78). Surprisingly, a number of investigators have been unable to demonstrate that hydration of arene oxides can take place without the participation of enzymes (79, 80).

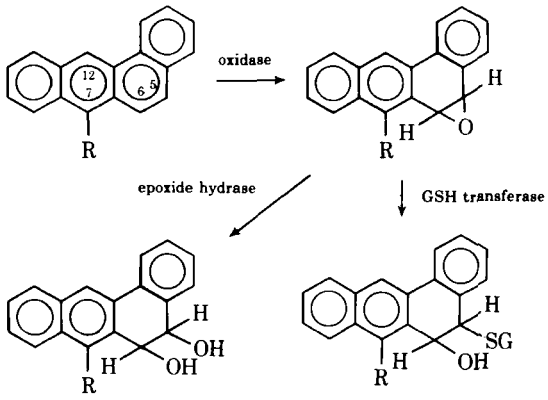
Arene oxides have been demonstrated as being microsomal metabolites of several benzanthracenes (81–83), pyrene, and benzo[*a*]pyrene (84). Also, naphthalene oxide was prepared in a reconstituted P-450 system (85).

The action of monooxygenases is not confined to aromatic double bonds. A related situation also has been found with alkene oxides when epoxidation of olefinic bonds has been reported. These epoxides have been found among a number of insecticides (86–89), allyl-substituted barbituric acids (90, 91), and the 10,11-epoxide of carbamazepine isolated from human urine (92), as well as a variety of other compounds. The alkene epoxides are far more stable than the arene oxides and are not as subject to enzymatic attack or spontaneous alteration. Frequently, vicinal glycols, but not epoxides, are found as products of oxidative metabolism of olefins (93–99). Theoretically, glycols could be formed by the direct dihydroxylation of an olefinic bond or by means of an intermediate epoxide. Cyclohexene, indene, and styrene form intermediate epoxides (100), although the glycol is the isolated product. The presence of intermediate epoxides was demonstrated in the case of octenes (101).

A discussion of some mechanistic considerations of



Scheme V



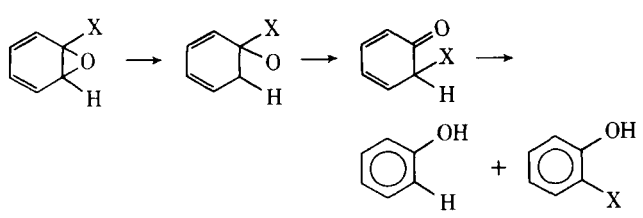
R = CH_2OH
GSH = glutathione

Scheme VII

oxirane formation was published (78). A review of those proposals, shown in Scheme V, offers an appreciation of the unusual nature of the oxirane system and why it may have great importance in the toxicity of chemical agents.

The aromatic substrate is acted upon by a monooxygenase to yield a corresponding arene oxide. The arene oxide, once formed, has a multiple number of paths available to follow. Pathway 1 shows that it may undergo carbon–carbon bond scission to form the seven-membered oxepin ring system. The equilibrium between the oxepin and arene oxide forms is a valence bond tautomerism and the equilibrium may be greatly displaced in either direction. Naphthalene 2,3-oxide appears to exist only in the oxepin form (102), whereas naphthalene 1,2-oxide has been shown to exist only as the oxide (103, 104). The failure of naphthalene 1,2-oxide to enter into an equilibrium with its tautomeric oxepin form is thought to be due to the fact that such a step would cause the aromatic ring to lose its aromaticity. Oxepins that are not converted to the arene oxide do not isomerize to the phenol. To date, only those arene oxides that at least partly exist in the oxide form have been demonstrated to be metabolic intermediates. The isomerization of the oxide (Pathway 2) is an internal spontaneous reaction (105–107) which occurs under basic or neutral conditions and pH < 6. This isomerization of the arene oxide to the phenol is frequently accompanied by the NIH shift previously discussed (108–111). The sequence shown in Scheme VI has been proposed (110) to take account of the role of the NIH shift in the arene–phenol conversion.

The third pathway (Pathway 3) open to the arene



NIH shift

Scheme VI

oxide is conjugation with glutathione by direct coupling without the involvement of enzymes or through catalysis by glutathione *S*-epoxide transferase (74, 112–114). The glutathione conjugates undergo conversion to premercapturic acids, which in the presence of acid are dehydrated to mercapturic acids (115).

The arene oxides can undergo hydration (Pathway 4) under the influence of epoxide hydrases to form *trans*-1,2-diols. These diols may then conjugate with glucuronic acid or undergo enzymatic dehydrogenation to form catechols (116, 117).

The remaining pathway of Scheme V (Pathway 5) is the covalent binding of an arene oxide structure to the macromolecules of biological systems. A number of investigators (107, 114, 118) described the formation of covalent bonds between the arene oxides and nucleophilic substances such as methanol, azide, and sulfhydryl compounds. Aromatic hydrocarbons do not bind with the nucleophilic centers present in DNA or RNA under *in vitro* conditions if NADPH is excluded from a liver microsome system, but they do bind readily if all cofactors of the system are present (119, 120). Pretreatment of the rats with an enzyme-inducing agent (120) known to stimulate the liver microsomal system results in considerable enhancement of the binding to DNA by the hydrocarbon when exposed to these microsomes. These findings strongly suggest, but do not prove, that the moiety that binds to the DNA of the system or tissue may be the arene oxide.

Other work tends to reinforce the likelihood that this is the case. In the absence of a microsomal system, firm binding of arene oxides to DNA, RNA, and histone was found under *in vitro* conditions (121). The same study demonstrated that the parent aromatic hydrocarbons and diols did not bind to these macromolecules. Cell culture studies in systems having the capability of causing biotransformations by oxidative pathways demonstrated that the parent hydrocarbons, their arene oxides, and the phenols and diols derived from them all firmly bound to DNA, RNA, and other proteins present in the medium; however, the arene oxides underwent covalent bonding to a much greater extent than did the other components (122, 123). It has been demonstrated

glucose-1-phosphate + uridine triphosphate $\xrightarrow{\text{pyrophosphorylase}}$ uridine diphosphate glucose + pyrophosphate

uridine diphosphate glucose + $2NAD^+ + H_2O \xrightarrow{\text{uridine diphosphate glucose dehydrogenase}}$ uridine diphosphate glucuronic acid + $2NADH + 2H^+$

Scheme VIII

that both alkene and arene oxides are active as alkylating agents (124, 125). The latter study is particularly interesting in that "K-region" (126) activity is involved (Scheme VII).

CONJUGATION REACTIONS

Conjugation with glucuronic acid is a common phenomenon in the metabolism of foreign compounds in mammalian species. Although this review is primarily concerned with the introduction of oxygen as a biotransformation step, glucuronide conjugation will be discussed on a somewhat broader basis.

The mechanism of glucuronide formation involves the reaction of the compound with D-glucuronic acid (127). For the condensation to proceed, glucuronic acid is activated by biosynthesis of uridine diphosphate glucuronic acid. The synthetic sequence is initiated by the formation of glucose-1-phosphate, as shown in Scheme VIII. The formation of uridine diphosphate glucuronic acid from uridine diphosphate glucose is under the mediation of a dehydrogenase present in the supernatant fraction of liver preparations.

The condensation of uridine diphosphate glucuronic acid with the compound undergoing conjugation (RZH) takes place upon catalysis of the reaction by a solubilized microsomal enzyme (128), glucuronyl transferase, which is found primarily in the liver. Glucuronyl transferase is also present in other body tissues. The reaction proceeds according to Scheme IX. The C^1 atom of glucuronic acid is present in the α-configuration in uridine diphosphate glucuronic acid but appears in the β-configuration in the conjugates formed.

Glucuronic acid forms conjugates with the hydroxyl groups of primary, secondary, or tertiary aliphatic compounds, phenols, enolic compounds, and hydroxylamines. The carboxyl group of straight-chain aliphatic, heterocyclic, carbocyclic, and arylalkyl acids form such conjugates. Aromatic amines, carboxyamides, and sulfonamides have also been reported as reacting with glucuronic acid (129).

Alcohols and phenols tend to form "ether-type" glucuronides. Many aromatic and aliphatic carboxylic compounds form "ester-type" glucuronides. Aromatic amines form *N*-glucuronides enzymatically through glucuronyl transferase, but some compounds apparently form these conjugates by nonenzymatic reactions (130).

MERCAPTURIC ACID SYNTHESIS

Conjugation with glutathione to form mercapturates is probably much more widespread in animals than in humans (131, 132). Hydrocarbons, aliphatic or acylhalogen compounds, nitro compounds, aromatic amines, sulfonate esters, carbamic esters, *etc.*, have all been reported to undergo this type of conjugation *in vivo*. Thus, the acetylcystyl residue of glutathione has been shown to replace H, halogen, $-NO_2$, $-SO_3CH_3$, $-NH_2$, and $-SO_2NH_2$ groups from a wide variety of structures.

uridine diphosphate glucuronic acid + RZH $\xrightarrow{\text{glucuronyl transferase}}$ RZ-glucuronic acid + uridine diphosphate

$$Z = O, \overset{O}{\overset{\|}{C}}O, NH, \text{or } S$$

Scheme IX

The reactive position of the drug molecule reacts with glutathione under mediation of glutathione *S*-transferase and glutathionkinase, which are present in the soluble fraction of liver, kidneys, and heart. Some compounds seem to react with glutathione in the absence of enzyme involvement (133). The initial complex undergoes transpeptidization to lose glutamate followed by peptidase cleavage of glycine. The final step of mercapturic acid synthesis requires *N*-acetylation.

Normally stable cyclic hydrocarbons have been shown to be excreted as mercapturates. In contrast to the displacement reactions already mentioned, the reaction of glutathione with aromatic hydrocarbons does not result from the displacement of an atom, in this case, hydrogen. In this instance, it is thought that an addition reaction involving an intermediate epoxide structure takes place (Scheme VII).

SULFATE CONJUGATION

This form of conjugation is quantitatively less important than those involving glucuronic acid, probably because the body pool of sulfate is relatively small. Primarily aliphatic alcohols and phenols react to form "ethereal sulfates" (129, 131). During the reaction, the phenol reacts with 3′-phosphoadenosine-5′-phosphosulfate in the presence of a sulfokinase to yield the sulfate and 3′-phosphoadenosine-5′-phosphate. "*N*-Sulfates," or sulfamates, are formed from aromatic amines in the rat, rabbit, and guinea pig. Conjugation takes place in soluble fractions of the liver (132, 133).

Morphine is treated similarly by the cat (134, 135) as are codeine, norcodeine, and normorphine (136, 137). Although the cat acetylates sulfadimethoxine reasonably well (6%), it forms no glucuronide with this compound (138).

Since biotransformations other than conjugation reactions frequently take place, a situation often exists where a series of competing reactions occurs simultaneously. It is not rare for excretion of unchanged drug, conjugation of the parent substance, molecular alteration of the parent, conjugation of the daughter compound, and excretion of these various entities to take place at the same time. This phenomenon is demonstrated by an experimental anti-inflammatory agent, I (139).

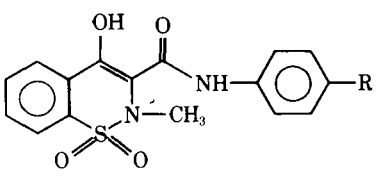
Table I—Conjugation of Phenylacetic Acid

Species (Number, Sex)	Route of Administration and Dose, mg/kg	Metabolite, % in 24-hr Urine[a]			
		1	2	3	4
Human (2M)	po 1	—	<0.05	91	7
Rhesus monkey (2F)	ip 80	41	0.9	24	9
Cynomolgus monkey (1F, 1M)	ip 80	9	0.6	46	2
Green monkey (1M)	im 25	10	0.4	69	3.5
Red-bellied monkey (1M)	im 8	3.4	0.5	39	1.2
Mona monkey (1F)	im 8	6.8	0.2	4.8	3.2
Mangabey (1M)	im 8	43.5	0.4	24.5	5.9
Drill (1F)	im 8	34	0.2	14.6	3.6
Baboon (1M)	im 2	5	0.1	85	10
Squirrel monkey (1F, 2M)	im 50	2.9	1.1	52	12
Capuchin (2F)	ip 80	1.2	5.3	14	10
Marmoset (2M)	im 80	3.5	0.6	56	0.3
Bushbaby (1M)	ip 80	—	60	—	9
Slow loris (1M)	ip 80	16.2	56.7	—	8.1
Dog (3F)	ip 80	0	76	—	3
Cat (3F)	ip 80	0.5	74	—	0.5
Ferret (3F)	ip 80	3	41	—	30
Rabbit (3F)	ip 80	2	82	—	0.4
Rat (3F)	ip 80	0	94	—	1
Mouse (8F)	ip 80	17	30	—	3.5
Hamster (3F)	ip 80	44	40	—	0.9
Guinea pig	ip 80	3.4	59	—	0.3
Vampire bat (3F)	ip 80	—	75	—	—
Pigeon (3F)	po 80	2	43	—	28
Chicken (3F)	po 80	4	4	(Metabolite 5, 51%)	

[a] Metabolites: 1, phenylacetic acid; 2, phenylacetylglycine; 3, L-(—)-phenylacetylglutamine; 4, phenylacetyltaurine; and 5, L-(+)-diphenylacetylornithine.

Several phenomena are to be noted. Of special interest is that the parent compound (I), 4-hydroxy-2-methyl-2*H*-1,2-benzothiazin-3-carboxanilide 1,1-dioxide, has a shorter half-life (21 hr) in humans than does its hydroxy metabolite (37 hr), which is in contradiction to the generalization that increased hydrophilicity speeds the rate of elimination. In the dog, a more normal pattern is seen, with the half-life of the administered compound being 30 hr and that of the hydroxy metabolite being only 14 hr. The rat, however, presents an entirely different case; here the half-lives of the parent and metabolite are essentially the same. After a 300-mg daily dose, a human eliminated 30% of the administered drug in the form of the conjugate of the parent compound, no free drug was found, and 70% was eliminated as the hydroxy metabolite and its conjugate. The dog, after receiving 10 mg/kg daily, excreted 10% of the dose as free drug and 70% as the hydroxy metabolite and its conjugate. Rats and monkeys afforded values between those observed for humans and dogs.

In addition to the unusual half-life observations alluded to earlier, this paper forcefully demonstrates how species differences can markedly affect the validity of animal toxicity and pharmacological efficacy data when extrapolation to humans is undertaken. The hydroxy metabolite (II) was tested for anti-inflammatory activity (140) and was classified as non-



I: R = H
II: R = OH

active. Obviously, testing the parent compound in a species that excretes a substantial portion of the drug unchanged would make the compound appear much more potent than with a species that conjugates it rapidly or converts it to its primary biotransformation product.

It is of interest to see how phenylacetic acid is excreted and conjugated by animals (Table I). In one study (141), 25 species were given phenylacetic acid and urine was then collected for 24 hr. The urine samples were investigated as to the percent of unchanged drug excreted; the glycine, glutamine, taurine, and ornithine conjugates were individually determined. Table I shows that humans excrete the glutamine conjugate, a small amount of the taurine metabolite, and no unchanged compound. Except for significant amounts of unchanged material being eliminated by the kidney, old world monkeys follow the same pattern. New world monkeys, however, excrete little unchanged phenylacetic acid but more of the taurine conjugate. The nonprimates do not form glutamine conjugates nor, with the exception of the ferret and pigeon, do they form large amounts of the taurine conjugate. Only the chicken is reported as excreting the ornithine conjugate (51%).

Table II shows some data from this study (141) and data from another experiment conducted by the same laboratory (142). *p*-Chlorophenylacetic and *p*-nitrophenylacetic acids were studied in some of the same species. The *p*-chloro substituent apparently had little effect on altering the metabolic pattern as was seen with phenylacetic acid in humans, rhesus monkey, and rats. With the capuchin monkey, which had eliminated virtually no unchanged phenylacetic acid, 24% of the administered dose appeared in urine following dosing with *p*-chlorophenylacetic acid. The *p*-nitrophenylacetic acid was largely eliminated un-

Table II—Species Differences in Conjugation of Phenylacetic Acids[a]

	Species	Unchanged	Glycine Conjugate	Glutamine Conjugate
Phenylacetic acid	Human	<2	~0	91
	Rhesus monkey	41	1	24
	Capuchin	1	5	14
	Rat	0	94	1
p-Chlorophenylacetic acid	Human	5	0	90
	Rhesus monkey	27	<1	38
	Capuchin	24	<1	14
	Rat	3	92	0
p-Nitrophenylacetic acid	Human	87	0	0
	Rhesus monkey	80	0	0
	Rat	28	61	0

[a] Values are percent of administered material excreted in urine.

changed by humans (87%) and rhesus monkey (80%), with neither the glycine nor glutamine conjugates having been formed. The rat excreted 28% unchanged and 61% as the glycine conjugate. Both humans and rhesus monkey excreted approximately 7% as a conjugate which, upon acid hydrolysis, afforded *p*-nitrophenylacetic acid. The investigators had available to them *p*-acetamidophenylacetic acid, but it did not appear to be a metabolite formed by any of these species. It would seem that the strong electron-withdrawal properties of a nitro group in the *para*-position reduces electron density sufficiently to impart a considerable degree of chemical inertness to the molecule.

Several other major studies designed to investigate species differences involving conjugation reactions have appeared recently. One (143) reports on the sulfate and glucuronide formation of phenol or its metabolite, hydroquinone, in 19 species (Table III). Another (138) reports on N^4-acetylation and N^1-glucuronide formation after exposing 18 species to sulfadimethoxine [N^1-(2,6-dimethoxy-4-pyrimidinyl)sulfanilamide]. The N^1-glucuronide was the primary urinary metabolite in humans, rhesus monkey, squirrel monkey, baboon, capuchin, bushbaby, slow loris, and tree shrew. It was a minor metabolite in dog, rat, mouse, guinea pig, Indian fruit bat, and hen. However, the N^4-acetyl compound, which was excreted to some extent by all species except the dog, was the major urinary metabolite in the green monkey, guinea pig, and rabbit.

The ferret, which has been suggested by some individuals to be a good biotransformation model for humans, certainly does not resemble humans, especially insofar as conjugation reactions are concerned. In the case of phenylacetic acid, humans excrete 91% of the dose as the glutamine conjugate, whereas the ferret does not excrete this metabolite at all but does eliminate roughly equal amounts of the glycine and taurine forms. Similar differences appear with phenol and sulfadimethazine.

NITROGEN

The hydroxylation reactions previously described have been confined to events involving the formation

Table III—Conjugation of Phenol[a]

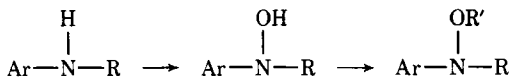
Species	Sulfate	Glucuronide	*p*-Hydroxyphenyl Sulfate	*p*-Hydroxyphenyl Glucuronide
Human	77	16	1	Trace
Rhesus monkey	60, 70	40, 30	—	—
Squirrel monkey	7	68	—	25
Capuchin	14	65	—	21
Ferret	28	40	30	—
Cat	87	—	13	—
Dog	33, 68	24, 12	43, 20	—
Pig	—	100	—	—
Hedgehog	63, 86	20, 10	17, 4	—
Fruitbat	9, 11	91, 89	—	—
Rabbit	45	46	9	—
Chicken	78	22	—	—
Rat	54	42	1	2
Mouse	46	35	5	1
Jerboa	61	26	12	1
Gerbil	42	35	19, tr	1
Hamster	27, 24	44	1	28, 27
Lemming	35	39	10	15
Guinea pig	13, 22	82	tr, tr	5, 5

[a] Data are expressed as percent of orally administered dose excreted in urine in 24 hr. All animals were given 25 mg/kg, except humans (0.01), rhesus monkey (50), pigs (21), and hedgehogs (20).

of a C—O bond. The introduction of oxygen into arylamines at the nitrogen atom has been studied extensively because of the association of these products with the development of malignancies in laboratory animals. The usual cause of the reaction is that a hydrogen atom of a primary or secondary arylamine is replaced by a hydroxy group; this metabolite then is further metabolized by conjugation with glucuronic or sulfuric acid (Scheme X). *N*-Hydroxylation reactions lead to the formation of highly reactive intermediates, which are frequently more toxic than the parent compounds, particularly in the area of chemical carcinogenicity. Only in recent years has an understanding of this reaction come about; this long delay is probably due to the fact that, as in the case of arene oxides, many of these *N*-hydroxy metabolites are quite unstable and difficult to isolate.

The conversion of arylamines to *N*-hydroxy metabolites under *in vivo* conditions was unambiguously demonstrated in 1959. The development of a new assay procedure (144) made it possible to assay arylhydroxylamines and arylnitroso compounds in tissues, blood, and urine. The formation of methemoglobin was studied in animals treated with aromatic amines, and a number of *in vitro* studies were undertaken in a series of experiments (145–150) wherein a variety of substituted aniline compounds was investigated. In compounds where groups that decreased ring hydroxylation were introduced into aniline, it was demonstrated that a corresponding increase in *N*-hydroxylation frequently occurred. Importantly, it was demonstrated that biochemical oxidation of *N*-alkylanilino compounds resulted in the formation of nitrosobenzene analogs.

The carcinogenic properties of *N*-2-fluorenylacetamide were intensively investigated. When rats were given single doses of *N*-2-fluorenylacetamide, no metabolites indicating *N*-hydroxylation were recovered but several ring-hydroxylated metabolites



Scheme X

were identified (151). Multiple exposures of rats to *N*-2-fluorenylacetamide administered in the diet led to the isolation of a previously unreported glucuronide (152). Enzymic hydrolysis, followed by exhaustive characterization of the metabolite, showed it to be an *N*-hydroxy derivative. This important breakthrough was vigorously pursued, and it was soon established that this metabolite was capable of producing all the effects, both physiological and pathological, of the parent compound when given to rats (153). The carcinogenicity study with the *N*-hydroxy metabolite of *N*-2-fluorenylacetamide produced the same tumors as did *N*-2-fluorenylacetamide and showed the metabolite to be more potent than the parent compound. Tumors were also formed at the site of injection of the *N*-hydroxy metabolite. Metabolites of *N*-2-fluorenylacetamide that had undergone ring hydroxylation reactions were shown to be either very weakly carcinogenic or inactive.

The *in vitro* *N*-hydroxylation of a series of *para*-substituted anilines was studied (154). These compounds all underwent *N*-hydroxylation more rapidly than did aniline itself. Reactivity was as follows: *p*-aminopropiophenone > *p*-phenetidine > *p*-chloroaniline > *p*-toluidine > aniline. When using the cat, it was shown that methemoglobin formation varied directly with reactivity. In another series of experiments by the same laboratory (155), aromatic amines known to be chemical carcinogens were investigated by measuring the generation of nitroso derivatives by rat liver microsomes. The formation of nitroso derivatives was in the order: 4-aminodiphenyl > 2-aminofluorene > 2-naphthylamine ≈ 4-aminostilbene ≈ aniline. As in the previous study, methemoglobin formation in the cat followed the pattern of reactivity in the microsomal system.

The presence of *N*-hydroxylating enzymes has been reported in many organs of several species. These tissues include the liver, lung, kidneys, brain, small intestine, and bladder, and the species include the rat, dog, guinea pig, rabbit, pig, cat, and hamster.

When the lung or liver of cats was perfused with a series of alkylanilines, the level of the amines was lowered and an increased level of nitroso compounds was noted. *N*-Alkylanilines yielded both aniline and nitrosobenzene (149). 2,4-Dichlorophenol reduced aniline formation but increased nitrosobenzene synthesis. The ability to synthesize nitrosobenzene was *N*-methylaniline > *N*-ethylaniline > *N*-butylaniline > aniline > *N,N*-dimethylaniline when rat liver microsomes were used. In a similar series of experiments (153, 156), no inhibition of nitrosobenzene formation could be demonstrated with proadifen hydrochloride[1], α,α-dipyridyl, cyanide, hydrogen peroxide, ascorbic acid, or catalase.

[1] SK&F 525-A.

Dogs pretreated with phenobarbital excrete much more 2-naphthylhydroxylamine after exposure to 2-naphthylamine than do normal controls (157). Phenobarbital treatment also strongly enhanced the microsomal activity in rabbits (158). Investigations (159–161) of the effect of enzyme-inducing agents on metabolic parameters such as the *N*-hydroxylation of *p*-chloroaniline, aniline, and *N*-alkylaniline; the C-hydroxylation of the *N*-alkylanilines; and effects on *N*- and *O*-dealkylation were all studied. *N*-Hydroxylation of *p*-chloroaniline was greatly increased with liver microsomes whose host had undergone stimulation with methylcholanthrene, some increase followed phenobarbital induction, but no discernible change followed exposure to chlorophenothane (DDT). A contrasting pattern was noted for ring hydroxylation, *N*-dealkylation of *p*-chloroaniline, and the formation of nitrosobenzene from *N*-alkylaniline. In the latter instance, all three inducers led to a reduced yield.

Although the question of N—O bonds and some of their physiological implications have been approached from the viewpoint of a biochemical synthetic reaction, it must be borne in mind that *N*-hydroxylamines can also be generated by reductive reactions. The hematopoietic consequences of nitrobenzene exposure have been documented in humans and animals, and fatalities have been reported after treatment with chloramphenicol. Nitrofurans have been implicated as chemical carcinogens. The experimental evidence gathered to date suggests that aromatic organic nitro compounds undergo conversion to arylamines by being first reduced to nitroso compounds and then to arylhydroxylamines. Such a reductive pathway need not be confined to the enzymatic characteristics of the host but may be produced by the microbiological components of the GI system (162).

Administration of toxic levels of nitrobenzene to cats resulted in the detection of nitrosobenzene in the blood (163, 164). The urine of rabbits treated with *m*-dinitrobenzene yielded *m*-nitrosonitrobenzene (165). Kinetic analysis of the reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid by rat liver microsomes clearly demonstrated that a hydroxylamino derivative was an intermediate step in the reductive sequence (166).

The known carcinogen, 4-nitroquinoline *N*-oxide, also undergoes biotransformation to the corresponding hydroxylamino compound, with the amine being the final product (167–171). In addition to the liver, this reductive sequence was also reported to occur in the subcutaneous tissue of the rat (172). A number of microorganisms have shown ability to reduce this compound to a hydroxylamino intermediate. *Pseudomonas aeruginosa* and *Escherichia coli* produce further reduction. Reduction in bacterial systems has been shown to proceed through sequences that include the intermediate hydroxylamine compound, which is reduced to the 4-aminoquinoline *N*-oxide and, in some cases, ultimately to 4-aminoquinoline. A considerable literature exists on the carcinogenic and mutagenic aspects of this compound (172–175).

The ability of these intermediates to react with DNA and other macromolecules has been extensively documented (176–181).

A number of investigations have been undertaken to elaborate those aspects of chemical structures or functional groups that are thought to be implicated in chemical carcinogenesis; mutagenic aspects, other than carcinogenicity, also have received attention. Thus, nitro compounds (182), epoxides (183), aromatic amines (184), and alkylating agents (185) have been studied in considerable detail.

Although many early studies designed to demonstrate the carcinogenicity of arene oxides and similar compounds under *in vivo* conditions did not permit any firm conclusions to be drawn (186–189), the most generally advanced explanation is that these highly energetic intermediates are too reactive to undergo any physical migration and must be generated *in situ*. A very comprehensive and systematic investigation (75) lends support to this hypothesis. In this study, K-region (126) epoxides of benz[*a*]anthracene, dibenz[*a,h*]anthracene, and 3-methylcholanthrene were studied in cell culture employing mouse prostate cells (G-23 line) and were evaluated in terms of toxicity and ability to produce malignant transformations (190). Comparisons were made using these epoxides, the parent hydrocarbons, and the corresponding K-region dihydrols. In this transformation assay, 3-methylcholanthrene was found to be weakly active, but benz[*a*]anthracene, dibenz[*a,h*]anthracene, and the *cis*- and *trans*-dihydrates of the three hydrocarbons were completely devoid of carcinogenic activity. The K-region phenol of 3-methylcholanthrene was moderately active. The K-region epoxides of the hydrocarbons were very active. One epoxide, the 8,9-epoxide of benz[*a*]anthracene, which is a non-K-region epoxide, was only weakly active if active at all. However, the situation is much more complex because the K-region epoxides of phenanthrene and chrysene, noncarcinogenic hydrocarbons, did not exhibit any transformations of this cell line. To complicate the question further, the K-region epoxide of 7-methylbenzanthracene was not as active a carcinogen as its parent hydrocarbon (although both compounds are only very weakly active).

Studies of the binding of epoxides to RNA, DNA, and the protein constituents of normal or transformed cells have not demonstrated a definitive relationship between the binding observed and cell transformation or toxicity (123). The mechanism of these malignant transformations still awaits elucidation (75). It seems that epoxide formation is essential in some cases, but recent papers (191, 192) suggested that certain hydrocarbons may be activated by metabolism of methyl groups through the formation of carbonium ions. Bromomethyl derivatives of benzanthracene and dimethylbenzanthracene were prepared as carbonium-ion-generating model compounds. The latter compound was found to be carcinogenic (192), but it was reported (75) that neither 7-bromomethylbenzanthracene nor 7-bromomethyl-12-methylbenzanthracene was active in the G-23 mouse prostate cell line. It is possible that metabolic activation is not a prerequisite for certain methylated hydrocarbons to transform cells.

An alternative possibility is that the intermediate metabolites forming these compounds are so reactive that they never survive long enough to reach this biochemical target within the cell. However, tissue culture studies (192) using Chinese hamster cells have shown that 7-methylbenz[*a*]anthracene and the bromomethyl compounds discussed earlier are mutagenic in that system. This makes the hypothesis less tenable. Also, the epoxides of chrysene and phenanthrene, which are highly toxic but do not transform mouse prostate cells, do transform hamster embryo cells.

A study of the metabolic disposition of 7-hydroxymethylbenz[*a*]anthracene 5,6-oxide in rat liver preparations was published (125). 7-Hydroxymethylbenz[*a*]anthracene 5,6-oxide was converted by rat liver microsomal preparations or rat liver homogenates into *trans*-5,6-dihydro-5,6-dihydroxy-7-hydroxymethylbenz[*a*]anthracene and into a glutathione conjugate by rat liver homogenates or by rat liver soluble fraction in the presence of glutathione. Sometimes trace amounts of 5-hydroxy-7-hydroxymethylbenz[*a*]anthracene was formed in these reactions, but it is felt that such a substance was probably the result of a nonenzymatic reaction. The 7-hydroxymethylbenz[*a*]anthracene was metabolized to a number of products, including the corresponding *trans*-5,6-dihydrol, when rat liver microsomes were used, but this was not seen when rat liver homogenate was employed in the incubation. No *cis*-dihydrol could be detected. When the glutathione conjugates of K-region epoxides are treated with acid, the unoxidized parent hydrocarbon is regenerated. However, when a non-K-region glutathione conjugate is treated similarly, the corresponding phenol is formed. In assessing the possible carcinogenicity of the 7-hydroxymethylbenz[*a*]anthracene 5,6-oxide in the 4-(*p*-nitrobenzyl)pyridine procedure, it was shown to be a less efficient alkylating agent than 7-methylbenz[*a*]anthracene 5,6-oxide which, in turn, is less efficient than the unoxidized parent hydrocarbon.

BIOTRANSFORMATION AND CARCINOGENICITY

Much of the preceding discussion has centered on the concepts of "drug" and "prodrug" or "carcinogens" and "procarcinogens" and "direct"- or "indirect"-acting carcinogens without making any special attempt to allude to them as such.

The distribution of the drug-metabolizing enzymes has not been vigorously pursued by most investigators of drug metabolism; efforts have been largely focused on the liver as the primary site of biotransformation. Recent studies (193–197) demonstrated that polycyclic hydrocarbon hydroxylase activity, at least, is widely distributed throughout the animal. The hydroxylase activity in the proximal position of the small intestine of the rat is about equivalent to that of the liver. Hydroxylase activity has also been demonstrated in the forestomach, the glandular stomach, the large intestine, the lung, the sebaceous glands of

the skin, the distal and proximal sections of the convoluted tubules of the kidney, and the adrenal of the rat. Hydroxylase activity increases in all tissues, except the adrenal, upon exposure of the rat to various enzyme inducers. Benzpyrene hydroxylase activity has been shown in the proximal small intestine of the baboon, bull, dog, guinea pig, hamster, human, monkey, mouse, and rabbit.

In early studies of the role of drug-metabolizing enzymes in the biochemical aspects of oncology, it was shown that administration of known microsomal enzyme inducers would protect animals from the carcinogenic effects of aminoazo compounds and *N*-2-fluorenylacetamide (197–199). Later studies also demonstrated protection against polycyclic hydrocarbons (200–203).

A provocative consideration of these findings and their implications was presented (204). This analysis suggested that the portals of entry into the animal, *i.e.*, the GI tract, the lungs, and the skin, act as a first line of defense to prevent the exposure of the internal organs to contact with the unaltered form of the foreign compounds and that "detoxification" here would result in a protective effect. The liver is considered a very potent second line of defense. Presumably, the microsomal enzymes of the kidney would be a third line of defense in their role of facilitating renal excretion. This hypothesis, however, does not appear to take into account that certain polycyclic hydrocarbons require metabolic activation to K-region epoxides before their carcinogenic effects can be demonstrated.

There is little doubt that there has been a continually increasing exposure of the population to airborne polycyclic hydrocarbons from fuel combustion and cigarette smoke and that these agents are probably the most widely distributed carcinogens present in our environment. Protection against pulmonary adenoma formation was achieved when β-naphthoflavone, an enzyme inducer, was given with orally administered 7,12-dimethylbenz[*a*]anthracene (203). Flavones are widely distributed in plants, and this finding implies that diet may have a hithertofore unappreciated role in carcinogenicity studies.

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