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Arene Oxides: A New Aspect of Drug Metabolism

Metabolic formation of arene oxides explains many toxic and carcinogenic properties of aromatic hydrocarbons.

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The metabolic conversion of aromatic compounds to phenols in mammals has long been considered a means for the detoxification and excretion of a variety of foreign substances, including many drugs and environmental pollutants. Recent investigations into the mechanisms by which hepatic monooxygenase enzymes catalyze the formation of phenols have, however, revealed that highly reactive arene oxides, compounds in which a formal aromatic double bond has undergone epoxidation, are intermediates in the reaction. Arene oxides, in addition to isomerizing to phenols, react readily with a variety of nucleophiles, including such cellular macromolecules as DNA, RNA, and protein. Thus, arene oxides have become prime candidates for the "bioactivated intermediates" responsible for the binding of aromatic compounds to biopolymers within the cell. Since the toxic, carcinogenic, and mutagenic effects of aromatic compounds often correlate with the extent of this binding, arene oxides are now strongly implicated as causative agents in producing these effects. Clearly, the extent of binding will depend both on the rate of formation of arene oxides and on the rates of isomerization to phenols, hydration, and conjugation with glutathione. In this article we review and interpret the current knowledge of arene oxides and their relevancy to the cytotoxicity and carcinogenicity of aromatic compounds.

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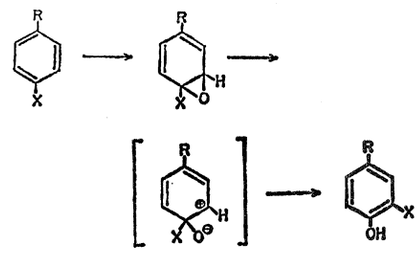
Arene Oxides as Metabolic Intermediates

The migration and retention of substituents that occur during the monooxygenase-catalyzed formation of phenols from most aromatic substrates has been termed the "NIH shift" (*1*). The occurrence of this shift suggested that the "aromatic hydroxylations" are actually epoxidations and that the intermediate arene oxides undergo a ready isomerization to phenols with a concomitant migration and retention of substituents (Fig. 1). A wide variety of substituents including isotopes of hydrogen, halogens, and alkyl groups have been observed to migrate in this way, and monooxygenases from plants, microorganisms, and animals catalyze the initial epoxidations (*2*, *3*). When the NIH shift was discovered in 1966, practically nothing was known of the chemistry or biochemistry of arene oxides. Boyland (*4*) in 1950 had suggested that arene oxides might be intermediates in the formation of *trans*-dihydrodiols and glutathione conjugates from polycyclic aromatic substrates in mammals, and later provided evidence for the conversion of stable K-region oxides of phenanthrene, benzo[*a*]anthracene, and dibenzo[*a*,*h*]anthracene to dihydrodiols and glutathione conjugates (*5*, *6*).

The relevancy of arene oxides to the metabolism of aromatic substrates in higher organisms was firmly established in the following studies. Investigations with benzene oxide, a typical arene oxide, and homogenates of mammalian liver (*7*) revealed that under physiological conditions arene oxides undergo spontaneous rearrangement to form phenols and react nonenzymatically with the thiol group of glutathione. The microsomal fraction of liver was found to contain an epoxide hydrase that converted arene oxides to *trans*-dihydrodiols, while the soluble fraction contained a glutathione-*S*-epoxide transferase that catalyzed the addition of glutathione (Fig. 2) (*8*). The products formed in vitro from benzene oxide were consonant with all the metabolites of benzene formed in vivo. The low rate of metabolism of benzene in vitro and the instability of benzene oxide appeared primarily responsible for the inability to demonstrate directly the formation of benzene with liver homogenates.

Naphthalene, in contrast to benzene, is readily metabolized to oxidized products by liver homogenates. In addition, the expected intermediate, naphthalene 1,2-oxide, is significantly more stable than benzene oxide (*9*). Naphthalene 1,2-oxide was indeed found to be the obligatory intermediate in the conversion of naphthalene in vitro to 1-naphthol, 2-naphthol, *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, and a glutathione conjugate (Fig. 3) (*10*). Isolation of the enzymatically formed intermediate by countercurrent distribution and thin-layer chromatography, followed by isomerization to a mixture of 1- and 2-naphthols, established its structure as the unstable naphthalene 1,2-oxide; other possible monooxygenated derivatives of naphthalene exist as stable annulenes or oxepins

Fig. 1. The NIH shift during the monooxygenase-catalyzed formation of phenols (*2*). A meta-hydroxylated product could be formed by the alternate opening of the oxirane ring.



[Fig. 3, structures **3** to **5**; for a review of the chemistry of arene oxides and oxepins, see (*11*)]. The intermediacy of naphthalene 1,2-oxide was compatible with the formation of all the mammalian metabolites of naphthalene. Naphthalene 1,2-oxide isomerizes to 1-naphthol and 2-naphthol in a ratio of 10 : 1; the same ratio of naphthols is found after microsomal metabolism of naphthalene. The migration and retention of deuterium observed during the rearrangement of [1-^{2}H]- and [2-^{2}H]-naphthalene 1,2-oxides to 1-naphthol is consonant with that observed during the metabolism of [1-^{2}H]- and [2-^{2}H]-naphthalenes to 1-naphthol (Fig. 4) (*12*).

Further examples of the NIH shift during isomerization of substituted arene oxides have been reported (*13*, *14*). The *trans*-dihydrodiol formed with microsomes from synthetic naphthalene 1,2-oxide was identical with that derived from naphthalene (*15*), although a minor difference in optical purity was suggestive of a low degree of asymmetric synthesis in the formation of naphthalene 1,2-oxide from naphthalene by hepatic microsomes (*16*). Finally, the glutathione addition product, formed from naphthalene when microsomes, glutathione, and glutathione-*S*-epoxide transferase were present, was identical to that formed from glutathione and synthetic naphthalene 1,2-oxide. The formation of naphthols and naphthalene dihydrodiols from naphthalene decreased in proportion to an increase in the formation of the glutathione conjugate when increasing amounts of glutathione were added to microsomal incubations. Similarly, in the absence of glutathione, inhibition of microsomal epoxide hydrase with a competitive substrate, styrene oxide, blocked the formation of the dihydrodiol from naphthalene and resulted in a stoichiometric increase in naphthols.

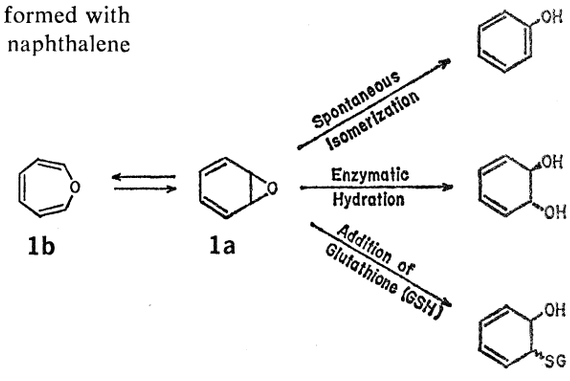


Fig. 2. Hepatic metabolism of benzene oxide (**1a**); the equilibrium with oxepin (**1b**) is shown (*8*).

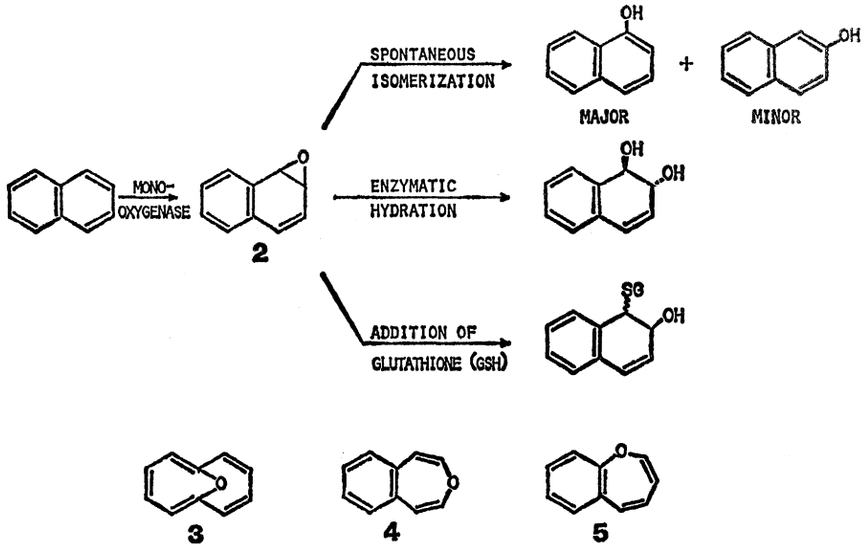


Fig. 3. Hepatic metabolism of naphthalene by way of the unstable intermediate naphthalene 1,2-oxide (**2**).

As a result of the preceding studies, it became evident that several criteria could be used to indicate the formation of an arene oxide during the metabolism of an aromatic hydrocarbon: (i) the occurrence of the NIH shift during the formation of a phenol; (ii) the formation in vitro of glutathione conjugates such as those shown in Figs. 2 and 3, or the appearance of precursors of mercapturic acids in the urine; and (iii) the formation of *trans*-dihydrodiols, catechols, and their conjugates. Catechol metabolites of xenobiotic aromatic compounds are generally formed by way of dehydrogenation of intermediate *trans*-dihydrodiols rather than through sequential "hydroxylations" of the aromatic ring (*17*). The ubiquitous nature of the NIH shift (*2*, *3*) suggests that most phenolic metabolites are derived from intermediate arene oxides.

The major pathways for oxidative metabolism of xenobiotic compounds in mammals (alkyl hydroxylation, *N*- and *S*-oxidation, and epoxidation) are catalyzed primarily by a class of nonspecific monoxygenases known as cytochrome P-450. Cytochrome P-450 activity is localized in the endoplasmic reticulum (microsomal fraction) of cells from liver, lung, kidney, intestine, and skin. Highest activities of P-450 are found in liver. Treatment of animals with polycyclic hydrocarbons induces the formation of a spectrally distinct class of enzymes known as cytochrome P-448 [for pertinent reviews on these monoxygenases, see (*18*)]. The reactivity of different substrates with these nonspecific mammalian monoxygenases varies considerably. In addition, the activities and specificities of cytochrome monoxygenases differ widely in different tissues, in the same tissue from different species, and in tissues of animals previously treated with inducing agents such as phenobarbital, polycyclic hydrocarbons, or other foreign substances. Such differences are reflected in differing ratios and amounts of oxi-

dized products formed on metabolism of a particular compound. Relatively little is known of the electronic and steric factors responsible for this wide variation.

For metabolism of the aromatic ring, electron-donating substituents appear to enhance the rate of enzymatic oxidation, while the bulk of substituents appears to direct oxidative attack away from hindered 1- and 2-positions [see (*2, 14, 19*)]. For example, the phenolic metabolites produced from toluene indicate that only two arene oxides, 3-methyl- (**6**) and 4-methylbenzene oxide (**7**), are formed as intermediates (Fig. 5) (*14*). The labile oxides, **6** and **7**, isomerize rapidly to ortho- and para-cresol, respectively. None of the meta-isomer is formed. Aromatic compounds with an oxidizable substituent, such as the methyl group in toluene, are often metabolized to a large extent at such moieties (see benzyl alcohol formation, Fig. 5) rather than by way of arene oxide formation at the ring. The relative extent of formation of different oxides and of other metabolites from an aromatic substrate will, of course, depend on the biological system.

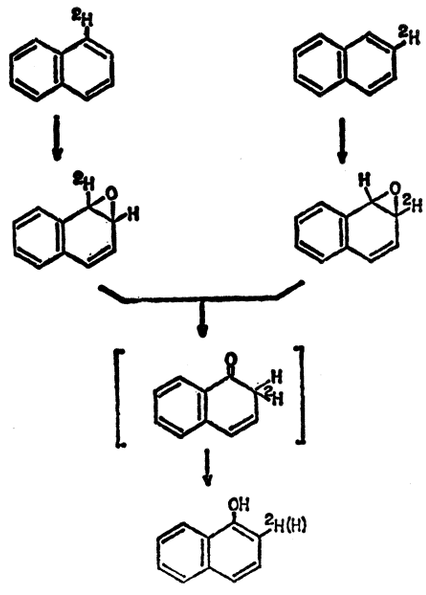


Fig. 4. The NIH shift during conversion of naphthalene to 1-naphthol.

Chemical Reactivity of Arene Oxides

Studies of the properties of arene oxides indicate that the steady-state concentration of a particular arene oxide in a biological system depends not only on the rate at which it is formed and subsequently metabolized, but also on the occurrence of two nonenzymatic reactions: (i) spontaneous isomerization of the arene oxide to a phenol and (ii) the addition of glutathione, a nucleophile whose concentration in liver is about 6 millimolar. Thus, a thorough understanding of the mechanisms by which arene oxides rearrange to form phenols and undergo reactions with nucleophiles is of key importance to an understanding of their disposition in biological systems. The kinetics and mechanisms of isomerization to phenols have been examined for more than a dozen arene oxides (*9, 20–24*). In general, two distinct pathways are operative: an acid-catalyzed (k_H) reaction and a spontaneous, *p*H-independent (k_0), reaction (Fig. 6). Rapid spontaneous isomerization (k_0) is probably the most characteristic feature of arene oxide chemistry. Thus, in water at *p*H 7 (1*M* KCl, 30°C) naphthalene 1,2-oxide has a half-life of only 4 minutes and benzene oxide a half-life of less than 2 minutes. Detailed investigations (*9, 20*) of the mechanisms of isomerization have indicated that carbonium ions (Fig. 6) are formed during the rate-determining steps in these reactions. The proportions of different phenols that will be formed on isomerization of an arene oxide metabolite can often be predicted by a consideration of the relative stabilization of such transient carbonium ion intermediates (*14*). In certain instances, these carbonium ions are readily trapped by reaction with solvent molecules (*21, 23*) as shown for 1,4-dimethylbenzene oxide (Fig. 7). This facile solvolysis is indicative of how readily arene oxides should be able to bind even to very weak nucleophilic sites within the cell.

Comparison of relative rates of isomerization for a series of substituted benzene oxides (*9*) has indicated that a Hammet relationship applies—that is, electron-withdrawing substituents stabilize arene oxides, while electron-donating substituents have a destabilizing effect. Thus, it is not surprising that aromatic compounds with electron-donating substituents such as anisoles, alkylbenzenes, or acetanilides form only phenols in vivo, while less reactive compounds such as halobenzenes or nitrobenzene form phenols, dihydrodiols, and premercapturic acids; the intermediate arene oxides formed from the less reactive compounds are sufficiently stable to undergo subsequent enzymatic reactions. The stability of a particular arene oxide will also influence the extent of its nonenzymatic reaction with intracellular nucleophiles. In this regard, it should be emphasized that the K-region oxides of polycyclic aromatic hydrocarbons are much more stable than the non-K-region oxides (*24*). In the case of one such K-region oxide, phenanthrene 9,10-oxide, spontaneous isomerization to the phenol (k_0) could not be detected (*20*). Relative rates of isomerization have recently been reported for K-region oxides of six polycyclic hydrocarbons (*25*).

A number of nucleophiles, in addition to glutathione (*7, 10*), react with arene oxides (*25–28*). These include other sulfhydryl compounds, alcohols, azide, methyllithium, and *p*-nitrobenzylpyridine. In general, softer and more polarizable nucleophiles such as those with sulfhydryl groups are the most effective. The order of reactivity of arene oxides toward glutathione indicates that strong electron-withdrawing groups facilitate the addition reaction (*29*), a result more compatible with nucleophilic opening of the oxide rather than trapping of an intermediate carbonium ion. With deuterium-labeled

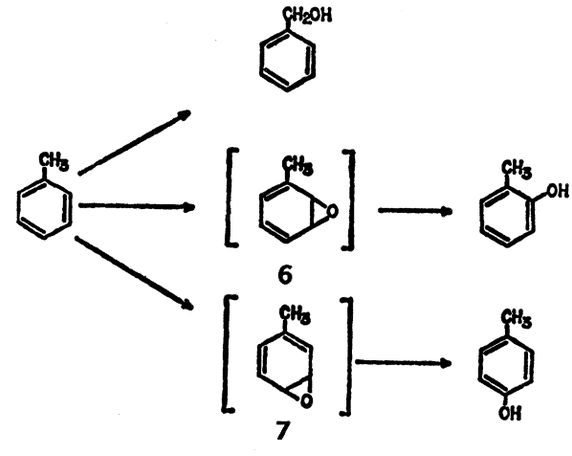


Fig. 5. The hepatic metabolism of toluene, showing the formation of benzyl alcohol and labile oxides (**6**, **7**). The latter compounds are proposed as intermediates in the formation of ortho- and para-cresols.

benzene oxide, *cis*- and *trans*-1,4- and 1,6-additions of nucleophiles, as well as the expected *trans*-1,2-additions, have been demonstrated (*28*). Attack by intracellular nucleophiles at sites remote from the epoxide ring in a polycyclic arene oxide must, therefore, be considered possible. The complexity of the reactions of arene oxides with simple nucleophiles indicates that reactions with DNA, RNA, and protein will in all likelihood proceed by many pathways and that considerable and careful effort will be required to delineate these pathways.

Enzymatic Reactions of Arene Oxides

Epoxide hydrases. With many hydrocarbons, epoxide hydrase plays a major role in further transformation of the metabolically formed arene oxides. Indeed, with naphthalene, the major product in vivo and in vitro is the *trans*-1,2-dihydroxy-1,2-dihydronaphthalene. Total activities and probably specificities of epoxide hydrases differ greatly among tissues and between species (*30, 31*). These differences will affect the concentrations of metabolically generated arene oxides. A relatively nonspecific epoxide hydrase has been purified and extensively studied (*30–34*). A different extent of purification and a lack of cross-inhibition with different substrates provided evidence for the presence of a less stable microsomal hydrase that is highly active in the conversion of benzene oxide to the *trans*-dihydrodiol (*31, 33, 34*). Further studies must be done to establish the number and specificities of the microsomal hydrase enzymes. Epoxide hydrases are inducible by treatment of animals with phenobarbital and, to a lesser extent, by treatment with the polycyclic hydrocarbon, 3-methylcholanthrene (*30, 31*). However, the induction of hydrases and monoxygenases by these agents does not occur in parallel and is not under common genetic control (*35, 36*). An assay for epoxide hydrase with 3-methylcholanthrene 11,12-oxide was recently reported (*37*).

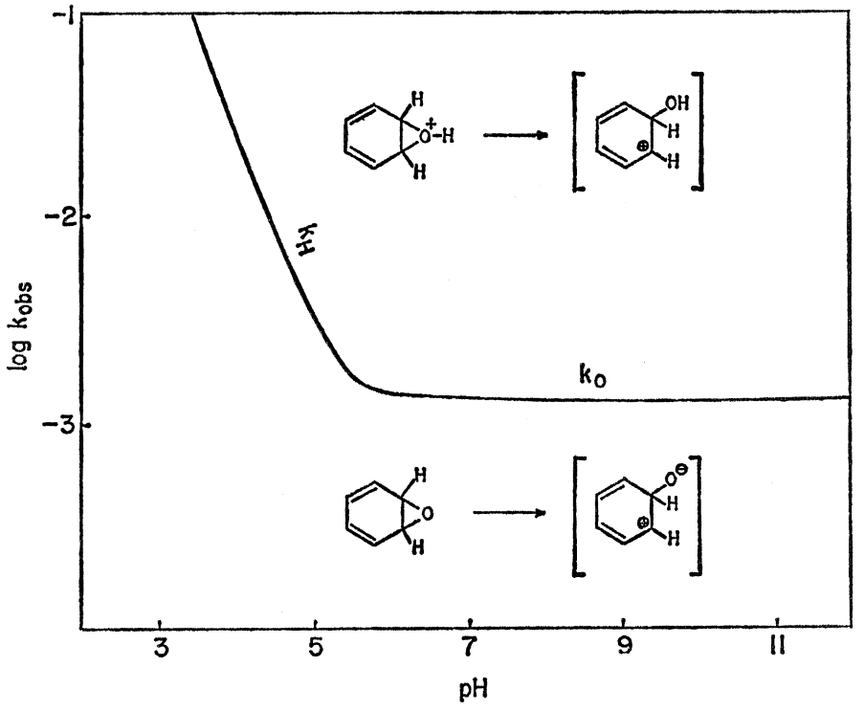


Fig. 6. Rate of isomerization of benzene oxide as a function of *p*H (*9, 20*). Proposed intermediates formed in the rate-limiting step for acid-catalyzed (k_H) and spontaneous (k_o) isomerizations are shown.

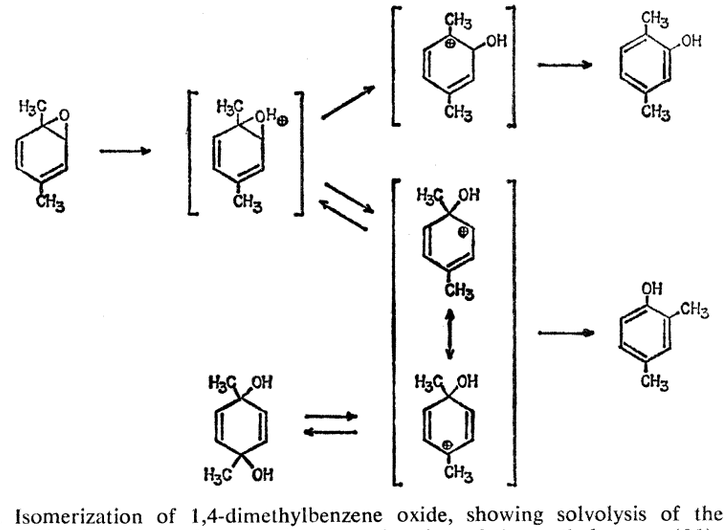


Fig. 7. Isomerization of 1,4-dimethylbenzene oxide, showing solvolysis of the intermediate carbonium ion and mechanism for migration of the methyl group (*21*).

Relative activities of microsomal hydrases toward benzene oxide, naphthalene 1,2-oxide, and phenanthrene 9,10-oxide (*33*), and data on the hydration of benzo[*a*]anthracene 5,6-oxide (*25*), benzo[*a*]pyrene 7,8-oxide (*38*), benzo[*a*]pyrene 9,10-oxide (*38*), 7,12-dimethylbenzo[*a*]anthracene 5,6-oxide (*39*), benzo[*a*]anthracene 8,9-oxide (*40*), dibenzo[*a,c*]anthracene 10,11-oxide (*41*), and 7-hydroxymethylbenzo[*a*]anthracene 5,6-oxide (*42*) by hepatic microsomal hydrases have been reported. With hamster embryo cells, benzo[*a*]anthracene 5,6-oxide appeared more readily converted to a dihydrodiol than was dibenzo[*a,h*]anthracene 5,6-oxide (*43*); the latter compound had previously been shown to be a poor substrate for hepatic epoxide hydrases (*44*). Careful quantitative studies with epoxide hydrases on a wide range of arene oxides have yet to be done.

Even more important than total activities and specificities of epoxide hydrases may be their degree of association with the monoxygenases that produce a specific arene oxide. Monoxygenase activity toward naphthalene, and hydrase activity toward naphthalene 1,2-oxide have been retained in preparations solubilized from hepatic microsomes of phenobarbital- and 3-methylcholanthrene–treated rats (*45*), suggesting a close association of the two enzymes in the microsomal membrane. The relative proportions of naphthol and *trans*-dihydrodiols formed from naphthalene with preparations from rats previously treated with either phenobarbital or 3-methylcholanthrene suggests a much closer association of

monoxygenase and hydrase in the preparations from rats treated with 3-methylcholanthrene (*45, 46*). Lack of complete equilibration of oxide formed from radioactive naphthalene with carrier naphthalene 1,2-oxide (*46*) provides further evidence for a very close association of the monoxygenase and hydrase enzymes involved in the metabolism of naphthalene with hepatic microsomes. Such close association of monoxygenase and hydrase enzymes may or may not pertain to the metabolism of other aromatic substrates.

Glutathione-S-epoxide transferases. The conjugation of arene oxides with glutathione is a major metabolic pathway that has significant effects on the steady-state concentration of arene oxides in tissues. Thus, in vitro, glutathione markedly decreases the formation of dihydrodiol from naphthalene by reacting spontaneously with the intermediate oxide (*10, 46*). Glutathione reacts with arene oxides both nonenzymatically and through catalysis by soluble glutathione-*S*-epoxide transferases. Glutathione transferase enzymes have been purified from the liver of rats with either simple epoxides or with *p*-nitrobenzylchloride as substrates (*47*), and from the liver of sheep with naphthalene 1,2-oxide as substrate (*48*). Two of the three different glutathione transferases isolated from rat liver with *p*-nitrobenzylchloride as substrate (*47*) were active toward naphthalene 1,2-oxide (*48*). It is remarkable that the highly active glutathione-*S*-epoxide transferase from sheep was virtually inactive toward three of the arene oxides of benzo[*a*]pyrene (*29*). Epoxide hydrase may, therefore, be more important than glutathione in detoxifying the arene oxides of this carcinogenic hydrocarbon.

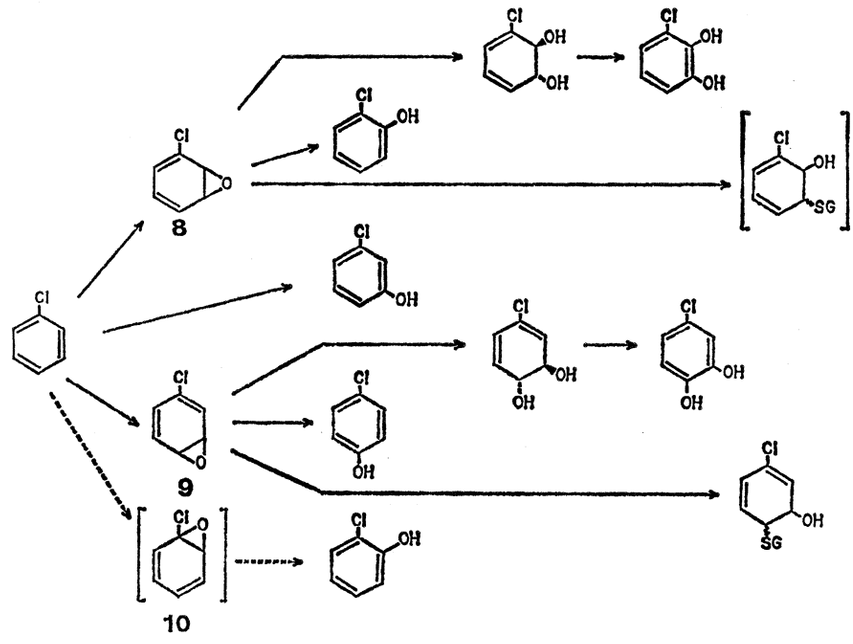


Fig. 8. Mammalian metabolism of chlorobenzene (*50*) to phenols, dihydrodiols, catechols, and glutathione conjugates. Oxides are proposed as intermediates in the formation of 2- and 4-chlorophenol, while the formation of 3-chlorophenol is proposed to occur directly. The structure of the glutathione conjugate derived from **8** is a tentative formulation. Evidence for formation of the oxide **10** has not been obtained. On isomerization, it would be expected to yield 2-chlorophenol.

Oxide Formation from Aromatic Hydrocarbons

A variety of indirect evidence, such as the NIH shift accompanying the formation of phenols (*2*) and the isolation of urinary dihydrodiols, catechols, and premercapturic acids (*49*), implicates arene oxides as intermediates in the metabolism of benzene and substituted benzenes in mammalian systems. However, a direct demonstration of an arene oxide in the metabolism of benzene and its derivatives has not been reported. In the case of benzene, the low rates at which it was metabolized in vitro hindered such studies (*7*), while the instability of intermediate arene oxides derived from alkylbenzenes precluded any possibility of their isolation (*14*).

Halobenzenes are, however, metabolized to a variety of products which are strongly suggestive of the intermediacy of relatively stable arene oxides (Fig. 8) (*50*). Two of these oxides (**8**, **9**) have now been synthesized and their isomerization to phenols examined: **8** isomerizes exclusively to 2-chlorophenol while **9** isomerizes exclusively to 4-chlorophenol (*51*). Both oxides react with glutathione and are converted to *trans*-dihydrodiols. The formation of 3-halophenols from chlorobenzene, bromobenzene, and fluorobenzene (*52, 53*) does not appear explicable on the basis of arene oxide intermediates, and thus such metabolites may represent examples of the direct enzymatic formation of a phenol. The magnitude of the isotope effects observed during the formation of metaphenols from deuterated nitrobenzene and phenylmethylsulfone was also incompatible with the intermediacy of arene oxides (*53*). Such "meta-hydroxylations" would, therefore, not be expected to be accompanied by the NIH shift. The relative importance of direct metabolic pathways from hydrocarbon to phenols requires further investigation, particularly with polycyclic hydrocarbons, where a variety of phenols can be formed by alternative pathways from one substrate.

Arene oxide formation from polycyclic hydrocarbons. A thorough investigation in 1968 of the metabolism of naphthalene in vitro (*10*) established arene oxides as the principal route to phenols from aromatic substrates and prompted a search for direct evidence of arene oxides being formed as intermediates in the metabolism of carcinogenic polycyclic hydrocarbons. Direct evidence has now been provided that K-region oxides are formed from phenanthrene (*54*), benzo[*a*]anthracene (*54*), dibenzo[*a,h*]anthracene (*54, 55*), pyrene (*56*), benzo[*a*]pyrene in the 4,5-position (*56, 57*), 7,12-dimethylbenzo[*a*]anthracene, and other substituted benzo[*a*]anthracenes (*58*) (Fig. 9, structures **11** to **16**, respectively). In all cases, microsomes from rat or hamster liver were employed and various inhibitors of epoxide hydrase [see (*34*)] were included during brief incubation periods at 25° to 30°C. In one instance (*55*), a brief heat treatment was employed to "deactivate" epoxide hydrase. It is unlikely that the hydrase can be selectively deactivated in this way because it is thermally a very stable enzyme (*30*). Instead, it is more likely that this brief heat treatment disrupted a close association between monoxygenase and hy-

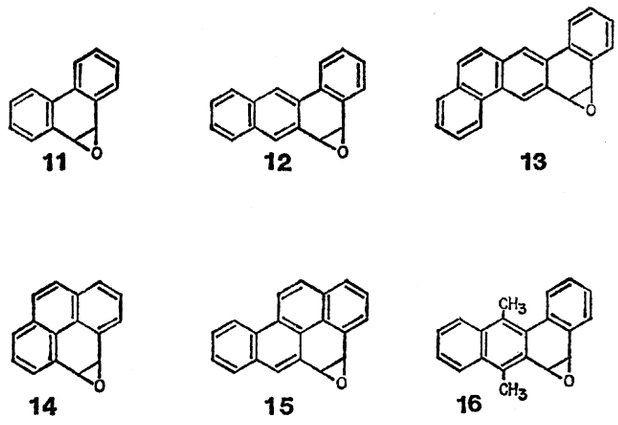


Fig. 9. K-region oxides that have been identified as metabolites of polycyclic hydrocarbons. Naphthalene 1,2-oxide (Fig. 3) has also been identified as such a metabolite.

drase (*45, 46*). The radioactive polycyclic hydrocarbons used in these studies were metabolized to products with chromatographic properties consonant with those expected of arene oxides, that is, products with R_F values intermediate between those of the nonpolar parent hydrocarbon and those of the more polar phenol, quinone, and dihydrodiol metabolites. The suspected oxide metabolites were acid-labile and could be isomerized to phenols. In some instances, partial conversion of the metabolites to dihydrodiols or glutathione conjugates was effected. Formation and metabolism of benzo[*a*]anthracene 5,6-oxide has now been studied with lung preparations (*59*).

To demonstrate the formation of a specific arene oxide from a polycyclic hydrocarbon is a difficult task which is compounded by the fact that suitable reference compounds are usually not available. For example, in the case of benzo[*a*]pyrene, only 6 of the 12 possible phenols, only 1 of the *trans*-dihydrodiols, and only 3 of the 6 probable arene oxides are adequately described in the literature. With most polycyclic hydrocarbons, the spectrum of products seen after metabolism in vitro (*60, 61*) indicates that a variety of intermediate oxides have been formed. Virtually nothing is known of the relative stability of these isomeric oxides except that the K-region oxides are considerably more stable than the non-K-region oxides (*24*), nor is anything known about the relative activity of these isomeric oxides toward enzymatic hydration or about the degree of association of monoxygenase and hydrase enzymes relevant to the formation and hydration of each arene oxide. Although it appears certain that arene oxides have been detected in the studies we have cited (*54–58*), the amount and spectrum of oxides formed in vitro and in vivo must remain in question until careful studies with complete sets of reference compounds are conducted.

Benzene Oxides as Mediators of Tissue Necrosis

While the hepatotoxicity of simple benzene compounds has been known for many years, only recently has evidence been obtained to explain the mechanism of these toxic effects. It has now been demonstrated that the toxicity is caused by aromatic compounds such as halobenzenes being metabolized to arene oxides which covalently bind to hepatic protein (*62*). When radioactive bromobenzene was administered to rats, massive centrolobular necrosis resulted and the necrotic regions contained significant amounts of covalently bound radioactivity (*62*). Prior treatment of the animals with phenobarbital, to induce microsomal monoxygenases, enhanced both the bromobenzene-elicited necrosis and the hepatic binding (*62, 63*). Conversely, prior treatment of the animals with inhibitors of oxidative drug metabolism, such as β-diethylaminoethyldiphenylpropyl acetate (SKF 525-A) or piperonyl butoxide, substantially reduced both the bromobenzene-elicited necrosis and the hepatic binding (*63–65*). The macromolecules to which the radioactivity became bound after the administration of bromobenzene were mainly proteins (*63, 66*).

An important factor controlling the time course and extent of liver necrosis, induced by radioactive bromobenzene, as well as binding of radioactivity to liver protein, was the amount of glutathione present in the liver. Prior treatment of animals with diethyl maleate, an agent which depletes glutathione in liver, enhanced both hepatic binding and the extent of liver necrosis elicited by bromobenzene (*63*). Conversely, prior administration of cysteine, a precursor of glutathione, retarded necrosis. Binding of radioactivity was low until a critical dose, 1 to 2 millimoles per kilogram of body weight, of radioactive bromobenzene was administered; only at this dose did the amount of hepatic glutathione become depleted (*63, 67*). A protective role for glutathione with respect to halobenzene-elicited liver necrosis is, thus, clearly established.

Attempts to establish whether or not epoxide hydrase also plays a protective role with respect to halobenzene-elicited necrosis produced results that were inconclusive (*31*). In rats, prior administration of cyclohexene oxide, a relatively potent inhibitor of epoxide hydrase, did inhibit formation of dihydrodiol-derived metabolites from chlorobenzene and, in addition, greatly reduced glutathione concentrations during the first few hours. The inhibition of pathways for subsequent metabolism of toxic chlorobenzene oxides would have been expected to result in exacerbation of hepatotoxicity. Instead, necrosis was completely prevented, perhaps by a relatively small cyclohexene oxide–elicited reduction in the initial rate of conversion of chlorobenzene to metabolites.

Prior treatment of animals with the inducing agent, 3-methylcholanthrene, would be expected to enhance bromobenzene-elicited necrosis, as was the case with the inducing agent, phenobarbital. Instead, prior treatment of rats with 3-methylcholanthrene decreases both the binding and the necrosis elicited by bromobenzene (*68*). In the mouse, prior treatment with 3-methylcholanthrene has little effect on either binding or necrosis (*68*). The overall rate of metabolism of bromobenzene is not markedly increased in the rat after the administration of 3-methylcholanthrene, but the proportions of various metabolites change, there being significant increases in dihydrodiols and catechols (*64*). This might be the consequence of the induction of a more tightly coupled monoxygenase-hydrase system (*45, 46*). Prior treatment of the animals with 3-methylcholanthrene also greatly increases the relative amount of urinary 2-chlorophenol (*64*). The lack of necrosis under those conditions suggests that the 3-chlorobenzene oxide (**8**), formed as an intermediate to 2-chlorophenol, is either not as cytotoxic or is not as stable toward isomerization as the 4-chlorobenzene oxide (**9**) that

is intermediate to 4-chlorophenol. Further studies are obviously necessary to fully delineate the mechanisms involved in halobenzene-elicited hepatic necrosis.

A variety of other compounds including phenylbutazone, diphenylhydantoin, 2-acetylaminofluorene, acetaminophen (4-hydroxyacetanilide), acetanilide, and carbon tetrachloride have been shown to be bioactivated before they become bound to hepatic macromolecules (*69, 70*). Although such bioactivation and subsequent binding are probably responsible for the toxic effects of these substances, it is clear that the mechanisms involved cannot in all cases be dependent on the formation of arene oxide intermediates. The hepatotoxicity elicited by carbon tetrachloride appears to be dependent on bioactivation that perhaps results in the formation of free radicals (*71*), while 2-acetylaminofluorene, acetanilide, and acetaminophen must be first converted to *N*-hydroxy derivatives [see (*70, 72, 73*) and references therein]. Carbon tetrachloride can, in addition, in low dosages cause inactivation of cytochrome P-450, thereby protecting against the metabolism-linked hepatotoxicity of a subsequent higher dose (*74*). This effect is reminiscent of the destruction of cytochrome P-450 by bioactivated intermediates derived from *N*-allyl compounds such as secobarbital and allyisopropylacetamide (*75*). These examples illustrate the diverse nature of bioactivation which, as in the formation of arene oxides, can result in cytotoxic effects.

Metabolically induced binding of foreign compounds and subsequent necrosis is not limited to the liver. Renal necrosis, for example, results from the administration of halobenzenes (*76*). Whether or not the bioactivated intermediates are generated in situ at such extrahepatic sites warrants further investigation, because if they were, the induction of hepatic enzymes might prevent extrahepatic necrosis by rapidly reducing the plasma concentrations of the parent hydrocarbon.

Polycyclic Hydrocarbon Oxides as Mediators of Carcinogenicity

A variety of chemicals have been implicated as causative agents in carcinogenesis. Each of the chemical carcinogens that has been thoroughly studied has been found to bind covalently with DNA, RNA, and protein of the target tissue (*77*). Certain carcinogenic compounds, such as β-propiolactone, succinic anhydride, uracil mustard, dimethylsulfate, and 1,2,7,8-diepoxyoctane, are presumed to act by direct acylation or alkylation of certain intracellular components while others, such as aromatic amines and azo compounds, nitrosamines, urethanes, carbon tetrachloride, safrole, cycasin, aflatoxins, griseoflavin, and polycyclic aromatic hydrocarbons, appear to require bioactivation in order to react with macromolecules (*73*).

Polycyclic aromatic hydrocarbons do not covalently bind to biopolymers without prior "activation." The magnitude of the weak physical binding which occurs between the hydrocarbons and macromolecules does not correlate with the carcinogenic efficacy of the hydrocarbon (*78*). Polycyclic hydrocarbons become bioactivated by drug-metabolizing enzymes, both in vivo and with hepatic microsomes in vitro, and this bioactivation results in their binding covalently to DNA, RNA, and protein (*79, 80*). Covalent binding alone, however, cannot be considered a sufficient criterion for carcinogenesis; benzo[*a,c*]anthracene and the [*a,h*] isomer bind equally well to mouse skin, yet only the latter compound is carcinogenic (*81*).

The role of metabolism in the cytotoxicity or carcinogenicity of polycyclic hydrocarbons is not clearly defined. There is a correlation between the degree of cytotoxicity of polycyclic hydrocarbons toward cells in culture and the total activity of monoxygenases (*82*). Furthermore, such toxicity is blocked by 7,8-benzoflavone, an inhibitor of these enzymes (*83*). The flavone also blocks the bone marrow damage elicited by 7,12-dimethylbenzo[*a*]anthracene (*84*). Prior induction of monoxygenases, however, unexpectedly decreases the extent of adrenal necrosis caused by 7,12-dimethylbenzo[*a*]anthracene (*85*). Similarly, prior induction of monoxygenases (*86*) decreases the carcinogenic effects of 7,12-dimethylbenzo-[*a*]anthracene or benzo[*a*]pyrene (*87*). Possible genetic relationships between the inducibility of enzymes which metabolize polycyclic hydrocarbons and the susceptibility of mouse strains to induction of tumors by benzo[*a*]pyrene (*35*) and 3-methylcholanthrene (*88*) have been investigated. Only in the latter case has a positive correlation been observed. The inhibition of monoxygenases with 7,8-benzoflavone markedly inhibited tumor formation elicited by 7,12-dimethylbenzo[*a*]anthracene but had no effect on or enhanced the carcinogenic effect of benzo[*a*]pyrene (*79*).

Such apparently anomalous results may be resolved when more is known about the effect of prior treatments or inhibitors on the specific bioactivation pathways and about the way in which these pathways are coupled to the relevant deactivation pathways. With polycyclic hydrocarbons, the specific bioactivation is presumably a monoxygenase-catalyzed conversion to one or more arene oxides, while the deactivation pathways include isomerization of the intermediate arene oxides to phenols, hydration to dihydrodiols, conjugation with glutathione, and further oxidation. Competing pathways may be of great importance. Although the proposal that arene oxides are the bioactivated intermediates responsible for the binding and the cytotoxic and carcinogenic effects of polycyclic hydrocarbons is attractive, the possibility that metabolites other than arene oxides are active agents in carcinogenesis should certainly not be excluded.

Early studies did not implicate arene oxides as the ultimate carcinogens formed from aromatic hydrocarbons. Thus, the 5,6- (K-region) oxide of 7-methylbenzo[*a*]anthracene was markedly less active as a carcinogen than the parent hydrocarbon when the effects of the two compounds on subcutaneous injection or topical application in rat and mouse were compared (*89*). Similarly, the K-region oxides of phenanthrene, benzo[*a*]anthracene, dibenzo[*a,h*]anthracene, chrysene, and 3-methylcholanthrene were all relatively inactive (*90*). These negative results, however, may be the consequence of transport and inactivation phenomena which lead to deactivation of these oxides in the intact organism before the critical target macromolecule is reached.

The development of cultured cell systems (*91*) in which cell transformation represents a reliable and rapid method of estimating activity of chemical carcinogens (*92*) has allowed a reevaluation of the effects of arene oxides (Table 1). In primary and secondary cultures of hamster embryo cells, K-region oxides of benzo[*a*]anthracene, dibenzo[*a,h*]anthracene, and 3-methylcholanthrene are more active than the parent hydrocarbons or corresponding dihydrodiols and phenols in inducing cell transformations (*93*). In a clone of C3H mouse ventral prostate cells, which shows a very low degree of spontaneous transformation, the K-region oxides of benzo[*a*]anthracene

Table 1. Transformations of cultured cells in vitro elicited by polycyclic hydrocarbons and metabolites (*93*). The compounds BP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; and DBA, dibenzo[*a*]anthracene, were added in amounts shown in parentheses. The numbers are proportional to the extent of cell transformation.

Agent	Hamster embryo cells			C3H mouse ventral cells	
	BP series (2.5 μg/ml)	3-MC series (2.5 μg/ml)	DBA series 5 μg/ml)	BP series (1.0 μg/ml)	3-MC series (1.5 μg/ml)
None	0.0	0.4	0.4	0.0	0.0
Hydrocarbon	0.2	1.2	0.8	0.0	0.1
K-region oxide	6.9	3.8	2.3	1.0	2.2
K-region diol	1.2	0.9	1.2		
K-region phenols	1.1	0.2	Toxic		

and 3-methylcholanthrene are also more active than the parent hydrocarbons (*93*). In the mouse cell line, dihydrodiols and phenols are relatively inactive as are the K-region oxides of the noncarcinogenic hydrocarbons, phenanthrene and chrysene (*94*). The K-region oxides of phenanthrene and chrysene are, however, active in the hamster cell system (*95*). The hamster system seems more sensitive to various carcinogenic agents: 7-bromomethyl-12-methylbenzo[*a*]anthracene causes transformation in hamster cells, but not in the mouse cells (*95, 96*). It is interesting that the K-region oxide of 7,12-dimethylbenzo[*a*]-anthracene is less active in causing cell transformation than the parent hydrocarbon (*96*). Inhibition of monoxygenases by 7,8-benzoflavone increased the transformation elicited by the K-region oxide of 3-methylcholanthrene (*97*), perhaps by preventing further metabolism. In addition to eliciting transformations in cultured cells, arene oxides have been shown to be mutagenic to mammalian cell cultures, *Drosophila*, bacteriophage, and strains of *Salmonella typhimurium* (*98*).

Although these studies with various cell types have been highly informative, questions such as whether or not K-region oxides, non-K-region oxides, or subsequent metabolites are the ultimate carcinogens or mutagens remain to be answered, perhaps through careful parallel comparisons of biochemical formation and disposition of hydrocarbon metabolites and their biological effects. One of the biochemical parameters of relevance is the nature and extent of binding of metabolically activated polycyclic hydrocarbons and of their various derivatives to biopolymers. The binding in vitro of polycyclic hydrocarbons, K-region arene oxides, phenols, and dihydrodiols to DNA, RNA, and proteins has been studied extensively (*57, 61, 99–101*). In general, the oxides were found, as expected, to be substantially more reactive and, as has been the case with other alkylating agents (*40, 81*), purine bases, especially guanine, were most susceptible to attack (*100–102*). However, no correlation is apparent between the degree of covalent binding of K-region oxides and the carcinogenicity of the parent hydrocarbons. A phenolic metabolite, 6-hydroxybenzo[*a*]pyrene, binds covalently to DNA in vitro (*103*). It is of interest that the binding of the 7,8-*trans*-dihydrodiol of benzo[*a*]pyrene to DNA is greatly enhanced when active hepatic microsomes are present (*61*). This result suggests that certain dihydrodiol metabolites can be bioactivated by hepatic enzymes, perhaps by way of catechols, quinones, or oxides.

The binding of K-region arene oxides, dihydrodiols, and phenols to DNA, RNA, and protein present in cultured cell lines has been examined (*104, 105*). In one of these studies (*104*), both arene oxides and phenols bound to a great extent to macromolecules. In the other study with a different cell line, distinctly greater binding occurred with the oxides. Neither study revealed any correlation between the extent of binding and the carcinogenicity of the parent hydrocarbon, thus providing evidence that both the extent and the nature of binding are important. The K-region oxide of dibenzo[*a,h*]anthracene binds more extensively than the parent hydrocarbon to an intracellular protein in mouse embryonic cells (*106*).

Attempts to establish correlations between metabolism, covalent binding, and carcinogenicity are even more difficult in the case of alkyl-substituted polycyclic aromatic hydrocarbons than they are with the unsubstituted polycyclic hydrocarbons. With the alkyl-substituted compounds, benzylic hydroxylation followed by conjugation with a group such as sulfate could, as would the formation of an arene oxide, generate a potent alkylating agent. 7-Hydroxymethyl-12-methylbenzo[*a*]anthracene and 7-methyl-12-hydroxymethylbenzo[*a*]anthracene are, however, far less potent carcinogens than the parent hydrocarbon (*107*). It is interesting that the 7-hydroxymethyl compound is extremely active in producing adrenal necrosis (*108*). Study of covalent binding of bromomethyl derivatives of benzo[*a*]anthracene as model alkylating agents of DNA did not reveal any correlation between the carcinogenicity of such derivatives and the total extent of reaction with DNA or the extent of reaction with particular nucleoside residues in the DNA (*109*). The binding of the 7-methyl, the 7-bromomethyl, and the 7-methyl-K-region oxide derivatives of benzo[*a*]anthracene to the DNA in cultured cells affords a different spectrum of products in each case (*101*). The mechanism involved in the carcinogenicity of alkyl-substituted hydrocarbons thus remains even less resolved than that of the unsubstituted polycyclic hydrocarbons. The ambiguous results obtained with both classes of polycyclic hydrocarbons attest to the difficulties inherent in attempts to correlate binding phenomena with biological effects.

Summary

Arene oxides have been identified as intermediates in the metabolic formation of phenols, *trans*-dihydrodiols, and premercapturic acids in mammals. The steady-state concentrations of intermediate arene oxides are related to the rates at which they are formed, their ability to isomerize to phenols, to react with nucleophiles such as glutathione, and to undergo enzymatic hydration. The covalent binding of these bioactivated intermediates to intracellular macromolecules provides a molecular basis for the cytotoxicity of aromatic hydrocarbons and the carcinogenicity of polycyclic hydrocarbons. The nature of the sites of binding to biopolymers, and the complex set of kinetic and structural parameters that influence both the reactivity of arene oxides and their ability to bind to critical target molecules are under investigation, but such factors have not been completely defined for any aromatic hydrocarbon. That bioactivated intermediates other than arene oxides also have a role in the carcinogenicity of polycyclic hydrocarbons remains a possibility.

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Immunologic Modification: A Basic Survival Mechanism

Cellular adaptation contributes to tumor growth, allograft survival, and mammalian embryogenesis.

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Changes in phenotypic expression of immunogenicity often contribute to survival of cells or tissues that otherwise would be eliminated by an immune reaction. Such cellular adaptation has been demonstrated extensively in a variety of experimental systems, the diversity of which suggests that this is a basic biological mechanism. Examples of these adaptations induced in vitro and in vivo are (i) altered potential for immune activity in response to undefined physiologic changes resulting from tissue passage in vitro, (ii) modulation of antigen expression induced by exposure to specific antibodies, (iii) alteration of the antigen recognition mechanism by exposure to specific antigens, and (iv) changes induced by exposure of allografts to immunologically unresponsive recipients. Although in the latter case it is initially the response that is modified, there is ample evidence that the tissue against which the response is directed also is affected. Cellular antigens and the responding organism interact in a dynamic way, and, although it is sometimes difficult to define the primary change, both participate in affecting the end result, be it survival or death of grafted cells. Examples with implications for evolutionary significance are drawn from various species, and we did not attempt an exhaustive review of the literature. In particular, we have called attention to changes in immunologic function occurring in cell and tissue culture after exposure to antibodies in vitro and in vivo, after exposure to antigenic material and ribonucleic acid (RNA) preparations, and in the fetal-maternal interrelationship. Such changes in expression of immunogenicity and responsiveness are recorded for hematopoietic and fixed tissues, both normal and neoplastic.

Phenotypic Alteration of Cell Surface Antigens

Since Schlesinger (*1*) has reviewed the work on phenotypic expression of cells in vitro, we present here only a few examples of reversible change in antigenic specificity in order to complete the correlation with other systems.

After an initial adaptation to the environment in vitro, tissue culture cell lines may maintain their differentiated phenotypes for many years (*2*). Although the expression of some traits such as pigment and cartilage formation (*3*) and antigen expression (*4*) may appear lost, they may be recovered either spontaneously or by changing the culture conditions, indicating modification of phenotype rather than selection of genetic variants (*5*).

A greater reduction in immunogenicity induced by organ culture explantation as compared to that induced by cell culture may be associated with a more favorable physiological environment usually obtainable when disassociated cells are used. However, since specificity of cultured cells has not been tested routinely by allotransplantation [terminology defined in (*6*)], modified immunogenicity may have been overlooked when unaltered antigenicity was present. While cellular antigens may be demonstrable by in vitro reactivity with serum or lymphoid cells and by their ability to respond to cellular or humoral reactions in sensitized recipients, these same antigens do not invariably evoke an effective primary immune response (*7*).

The expression of cellular antigens and serum proteins is influenced by the stage of differentiation of the organism and the presence or absence of neoplasia. The time of expression and the quantity of alloantigens on cells of different tissues also varies (*8*). A "thymus specific" antigen detectable only on brain and thymic cells has been identified in several species (*9*). Likewise, antigens present during fetal life may be greatly reduced or absent in adults only to reappear with the development of neoplasia; for example, the carcinoembryonic antigen de-

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