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Metabolism of Polycyclic Aromatic Hydrocarbons: Etiologic Role in Carcinogenesis

OLAVI PELKONEN and DANIEL W. NEBERT

Department of Pharmacology, University of Oulu, Oulu, Finland, and the Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

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I. Introduction

THIRTY years ago, it was believed that practically all *xenobiotics** were pharmacologically active, toxic, carcinogenic, and/or mutagenic in their parent (nonmetabolized) form. The function of all drug-metabolizing enzymes was therefore regarded as *detoxication*. Recently it has become evident that although some chemicals are, indeed, active in their nonmetabolized parent form, others are inactive until metabolized (146, 206, 301); this process is called *toxification*. Detoxication and toxification enzymes coexist in the same cell, in some instances architecturally next to one another in the same membrane. Further, any given enzyme may be involved in detoxifying one chemical while toxifying a second chemical. Each enzyme is likely to differ among various tissues or organs, strains, and species; factors such as age (developmental enzymology), hormonal and nutritional variations, diurnal and seasonal rhythms, pH at the enzyme-active site, and saturating vs nonsaturating substrate concentrations all may contribute to the function of each of these enzymes. A delicate and very complicated balance exists, therefore, between detoxication and toxification.

One of the most useful theories to explain some mechanistic aspects of chemical carcinogenesis is the theory of toxification, i.e. the formation of reactive metabolites by enzymes and the covalent linkage of these activated intermediates with cellular macromolecules to initiate the carcinogenic process [for example, Miller and Miller (301)]. With this theory as a background, we examine the formation of reactive polycyclic hydrocarbon intermediates and factors affecting their interactions with DNA, RNA, and proteins. We have surveyed the literature concerning the effects of covalent binding of such reactive intermediates on the structure and function of biological macromolecules. We summarize some studies about possible correlations between the binding of these chemicals to DNA and their mutagenicity and carcinogenicity. Although these kinds of correlative studies cannot prove that DNA is the critical target for the carcinogenic action of chemicals, positive correlations at least do not refute this hypothesis.

During recent years, important experimental advances have revolutionized the study of the interactions of polycyclic hydrocarbons with macromolecules. First, the availability of compounds of high specific radioactivity has increased the sensitivity of detecting minute amounts of carcinogens bound to nucleic acids. Second, many different metabolites have been synthesized. Third, analytical instrumentation and methods for measuring specific nucleoside-hydrocarbon adducts and for measuring biochemical and biological activity of metabolites have been improved.

II. Overview of Xenobiotic Metabolism

A. How Many Different Drug-metabolizing Enzymes Exist?

Most xenobiotics are so fat-soluble that they would remain in the body indefinitely were it not for metabolism resulting in more water-soluble derivatives. These enzyme systems, located principally in the liver (but most likely present to some degree in virtually all tissues of the body), traditionally are divided into two groups: Phase I and phase II. During phase I metabolism, one or more water-soluble groups (such as hydroxyl) are introduced into the fat-soluble parent molecule, thus allowing a "handle," or a position, for the phase II conjugating enzymes to attack. Many phase I products, but especially the conjugated phase II products, are sufficiently water-soluble so that these chemicals are excreted readily from the body (146). Because there are several examples of phase II reactions preceding phase I reactions, Testa and Jenner (442) recommended use of the term "functionalization reactions" instead of "phase I reactions." "Conjugation reactions" might be a better term for "phase II reactions."

Listed in table 1 are a number of metabolic reactions that may be important in detoxication or toxification of

* Many different terms are being used to describe pharmacological and toxicological consequences of foreign chemical metabolism. In the absence of any consensus, we have chosen these italicized words for this review. "*Xenobiotic*" denotes all foreign chemicals not found normally in the body. "*Detoxication*," or detoxification, implies inactivation of an active chemical. "*Toxification*," or metabolic activation, denotes formation from the substrate of a more reactive intermediate or product.

TABLE 1

Metabolic reactions that may play a role in chemical mutagenesis or carcinogenesis

Reaction	Subcellular or Extracellular Location*	Examples of Toxification Reactions†
A. Oxidations (functionalization or phase I metabolism)		
1. Aliphatic or aromatic C-oxygenations (epoxidations, hydroxylations)	Ms	Acetanilide, bromobenzene
2. N-, O-, or S-dealkylations	Ms	Phenacetin
3. N-oxidations or N-hydrolations	Ms	Aniline
4. S-oxidations	Ms	Thioacetamide
5. Oxidative deaminations	Ms	
6. Dehalogenations	Ms	Methoxyflurane
7. Metallo-alkane dealkylations	Ms	Tetraethyl lead
8. Desulfurations	Ms	Parathion
9. Alcohol or aldehyde dehydrogenations	C	Xylyl and allyl alcohols
10. Purine oxidations	C	
11. Tyrosine hydroxylation	C	
12. Monoamine oxidations	Mt	
13. Diamine oxidations	C	
14. Aromatizations	Mt	
B. Reductions (functionalization or phase I metabolism)		
1. Azo reductions	Ms	Prontosil
2. Nitro reduction	Ms	Nitroquininoline N-oxide
3. Arene oxide reductions	Ms	
4. N-hydroxyl reductions	Ms	
5. Quinone reductions	Ms	
6. Carbonic anhydrase‡	C	Carbonyl sulfide
C. Hydrolyses (functionalization or phase I metabolism)		
1. Hydrolyses of esters	Ms, C, P	Cycasin
2. Hydrolyses of amides	Ms, C, P	
3. Hydrolyses of peptides	Ms, C, P	
4. Hydrolyses of epoxides	Ms, Mt, C	Polycyclic aromatics (table 4)
D. Conjugations (phase II metabolism)		
1. Glucuronidations	Ms	Morphine 6-glucuronide
2. Sulfate conjugations	C	Aromatic amines (table 4)
3. Glutathione conjugations	C	1,2-Dichloroethane
4. Acetylations	C	Procainamide, isoniazid
5. Glycine conjugations	C	
6. Serine conjugations	C	
7. N-, O-, or S-methylations	Ms, C	Isoprenaline
8. Ribonucleoside or ribonucleotide formation	C	
9. Glycoside conjugations	Ms	
E. Beyond conjugation or phase II metabolism		
1. C-oxygenations	Ms	Polycyclic aromatics (table 4)
2. Loss of glucuronides (β-glucuronidase)	C	Benzo[a]pyrene 3-glucuronide
3. Loss of glycosides	Feces (bacteria)	
4. Deacetylations	C	
F. Direct chemical reactions (oxidation/reduction)		

* The fundamental classification is according to Goldstein et al. (146) and Testa and Jenner (441). Ms, microsomal; Mt, mitochondrial; C, cytosolic; P, plasma.

† Toxification means that the metabolite has pharmacological activity and/or is toxic.

‡ Carbonic anhydrase, although a hydrase, is arbitrarily included in this section to simplify the table.

carcinogens or procarcinogens. Basically, any time a chemical bond is cleaved and/or electrons are passed one at a time, the possibility exists for unwanted reactions of such intermediates with nucleic acid or protein. The reactions can be complicated; factors include the degree of stability of short-lived chemical intermediates, the redox state, movement of unpaired electrons from one molecule to another (free radicals), and lipid peroxidation. As is evident from examples in table 1, many pathways have been shown to lead to toxification. Actually, at present there seems to be no a priori means of predicting the pharmacological and/or toxic activity of the metabolite. This last point may be of particular interest. Chemical carcinogens are not unique in their requirement of toxification. The number of xenobiotics is increasing that have been found to be metabolized to reactive intermediates capable of binding covalently to tissue macromolecules and producing harmful effects. It is not understood why some of these substances cause cancer and others do not. Generally, reactions involving DNA are believed to be most important for mutagenesis and carcinogenesis, but absolute experimental proof of

this hypothesis is lacking. Reactions involving proteins and cell membrane surfaces may be more important than those involving nucleic acids for toxicity and teratogenesis, but direct experimental proof of this hypothesis is also lacking.

1. Functionalization reactions: "Cytochrome P-450." The majority of phase I oxidations (146, 224, 441) are catalyzed by cytochromes P-450. "Cytochrome," derived from Greek, literally means "colored substance in the cell." The color is derived from the properties of the outer electrons of transition elements such as iron, and, indeed, cytochromes appear reddish in color when sufficient concentrations exist in a test tube.

"P-450" denotes a reddish pigment with the unusual property of having its major optical absorption peak (Soret maximum) at about 450 nm, when the material has been reduced and combined with carbon monoxide (339). Although the name P-450 was intended to be temporary until more knowledge about this substance was known, the terminology has persisted for 18 years because of the increasing complexity of this enzyme system perceived with each passing year and because of the lack of agreement on any better nomenclature.

P-450 clearly represents a family of hemoproteins (heme-containing proteins similar in some ways to hemoglobin) possessing catalytic activity toward thousands of substrates. This collection of enzymes is known to metabolize: almost all drugs and organic chemistry laboratory reagents; small chemicals such as benzene, thiocyanate, or ethanol; polycyclic aromatic hydrocarbons such as biphenyl and benzo[a]pyrene (ubiquitous in city smog, cigarette smoke, and charcoal-cooked foods); halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls, defoliants, insecticides, and ingredients in soaps and deodorants; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; strong mutagens such as N-methyl-N′-nitro-N-nitrosoguanidine and nitrosamines; aminoazo dyes and diazo compounds; various chemicals found in cosmetics and perfumes; numerous aromatic amines, such as those found in hair dyes, nitro aromatics, and heterocyclics; N-acetylarylamines and nitrofurans; wood terpenes; epoxides; carbamates; alkyl halides; safrole derivatives; antioxidants, other food additives, and many ingredients of foodstuffs, fermentative alcoholic beverages, and spices; both endogenous and synthetic steroids; prostaglandins; and other endogenous compounds such as biogenic amines, indoles, thyroxine, and fatty acids.

Until recently the general consensus among most laboratories (224, 399a) has been that three, or certainly less than two dozen, forms of P-450 exist and that overlapping substrate specificity accounts for all diversity seen when thousands of different chemicals are metabolized. At the other extreme has been the provocative hypothesis (314) that most organisms have the genetic *capacity* to produce as many distinct forms as there are inducers of P-450. Possessing the genetic capacity to synthesize hundreds or thousands of different forms of P-450 does not imply that all of them would exist at any one time. In this respect, the P-450 system might exist for the hundreds of thousands of environmental chemicals as the immune system exists for the approximately one million antigens on this planet; in other words, as a new chemical or antigen enters the body, a new drug-metabolizing enzyme or antibody might be mobilized in response to this stimulus. Much further work is needed to confirm or disprove this hypothesis about multiple forms of P-450.

2. Functionalization reactions: "Monooxygenase activity." Monooxygenases are enzymes that insert one atom of atmospheric oxygen into their substrates (161, 291). The various forms of P-450 represent a large subset of all monooxygenases. To perform this monooxygenation, the P-450 hemoprotein receives two electrons from the cofactors NADPH and/or NADH. These electrons are received one at a time, usually via reductases (flavoproteins). In certain bacteria such as *Pseudomonads*, the entire electron chain (NADH, reductase, an iron-sulfur protein, and P-450) is in the cytosol (soluble cytoplasm). In most organisms, however, the electron chain is deeply embedded principally in the endoplasmic reticulum (and to some degree the inner mitochondrial membrane and perhaps nuclear envelope). The endoplasmic reticulum centrifuged at 100,000 × *g* for an hour becomes the "microsomal pellet." The microsomal electron chain contains reductase and P-450, but the mitochondrial electron chain is more similar to bacteria containing reductase, iron-sulfur protein (called "adrenodoxin"), and P-450. In summary, P-450-mediated monooxygenase activities are ubiquitous in virtually all living things—certain kinds of bacteria, and presumably all plants and animals (224, 399a).

3. Functionalization reactions: Oxidations, reductions, and hydrolyses. P-450-mediated monooxygenase activities (table 1) include: aromatic and aliphatic hydroxylations of carbon atoms; N-, O-, and S-dealkylations; N-oxidations and N-hydroxylations; S-oxidations; deaminations; dehalogenations; metallo-alkane dealkylations; desulfurations; certain purine and monoamine oxidations; certain azo and nitro reductions; and certain arene oxide (436) and N-hydroxyl (514) reductions. Many of these reactions can result in the formation of reactive intermediates, e.g. dealkylations can lead to the methylation or ethylation (alkylation) of nucleic acid or protein, a potentially important mechanism for tumorigenesis or drug toxicity. Tetraethyl lead in gasoline, for example, is metabolized by P-450 in this manner to a reactive intermediate which is toxic to the central nervous system (146).

Human liver alcohol dehydrogenase (table 1) catalyzes the oxidation of the 3β-hydroxyl group of digitoxigenin and related derivatives; after oxidation of the 3β-hydroxyl group, cardiac activity of these digitalis-related genins is decreased by more than 90% (125). Genetic differences, or ethanol-induced differences, in alcohol dehydrogenase therefore may alter the required loading

and maintenance doses of digitalis due to this potential detoxication pathway. Liver alcohol dehydrogenase also may toxify chemicals, metabolizing several xylyl alcohols to aldehydes that are much more toxic to lung than liver tissue (355) and allyl alcohol to the neurotoxic acrolein (356). Alcohol dehydrogenase therefore is an excellent example of the dual nature of an enzyme designed to metabolize endogenous substrates: the enzyme is not only capable of detoxifying some foreign chemicals but is also capable of toxifying certain other drugs.

Quinone reduction by DT diaphorase (268) or by P-450 reductase (72) has been postulated to be an important step leading to glucuronide conjugation (table 1). Quinone-derived free radicals might also be generated by this catalytic activity or by some similar activity other than DT diaphorase. Carbonyl sulfide (78) is metabolized to hydrogen sulfide by carbonic anhydrase; hydrogen sulfide is responsibile for carbonyl sulfide toxicity. Carbonic anhydrase therefore represents an enzyme capable of toxification. Epoxide hydrolase adds water to arene oxides or epoxides to form dihydrodiols (335). This hydrolytic reaction occurs, for example, during phenytoin metabolism.

4. Conjugation reactions. Drugs and other foreign chemicals are most commonly conjugated with glucuronic acid (113), sulfate, or glutathione (table 1). Studies have just begun on characterization of the sulfotransferases (276). Glutathione transferases (196) act on a large number of chemicals—including arene oxides, epoxides, chlorodinitrobenzene, bromosulfophthalein (for testing liver function), and bilirubin; at least six glutathione transferases have been isolated and characterized so far.

5. Further metabolism of metabolites. Although dihydrodiols are generally, readily excreted, it is clear (301) that diol-epoxides are formed and that these highly reactive intermediates may be important in mutagenicity, tumorigenesis, toxicity (301, 335), and even birth defects (416). Diols therefore can undergo further C-oxygenations (table 1).

Once a conjugate has been formed, the general belief has been that the drug is excreted irreversibly. The glucuronide of 3-hydroxybenzo[a]pyrene treated with β-glucuronidase, however, forms reactive intermediates capable of binding covalently with nucleic acid and protein (235). By a similar mechanism, one might postulate that glycosides may react with various glycosidases, resulting in reactive intermediates capable of binding covalently with nucleic acid and protein. Evidence in favor of this mechanism enhancing mutagenesis in vitro has been reported (438). Following conjugation with acetic acid, many drugs can be deacetylated (table 1) to form reactive intermediates (301). Even conjugation with glutathione may yield a mutagenic product, as demonstrated with 1, 2-dichloroethane and 1,2-dibromoethane (384). There is a review emphasizing both detoxication and toxification aspects of conjugation reactions (68).

6. Direct chemical reactions (oxidation/reduction). Some chemicals by their inherent molecular properties possess a high redox potential. *o*-Aminophenol, for example, is capable of oxidizing ferro- to ferrihemoglobin. Nitrates also cause methemoglobinemia, and methylene blue is used to treat methemoglobinemia. Methylene blue (because of its redox potential) reduces the ferri- back to ferrohemoglobin. Such one-electron chemical reactions may play a role in mutagenesis.

In summary, a few enzymes may exist in the body only to take care of xenobiotics. Many enzymes designed for normal-body substrates, however, apparently also handle many carcinogens and procarcinogens. The result is a complicated and delicate balance of detoxication and toxification.

B. Extrahepatic Xenobiotic Metabolism

The route by which xenobiotics enter the body may determine their fate and organ specificity. For example, a compound entering the body via lungs will bypass the liver and may reach peripheral tissues in much higher concentrations than when the drug is absorbed from the gastrointestinal tract and subjected to the "first-pass" effect (396) in the gut and the liver. It is important to remember that, while the liver is the most extensively studied organ, extrahepatic tissues harbor their own xenobiotic-metabolizing enzymes which may differ radically from those of the liver in terms of activity and specificity. Extrahepatic metabolism might be of utmost importance in the availability of a chemical to different parts of the body and in the target tissue variability. This topic has been recently reviewed, for example, by Aitio (3), Bridges and Fry (56), and Vainio and Hietanen (461).

One of the difficulties in studying extrahepatic xenobiotic metabolism is that activities in extrahepatic tissues are usually much lower—on the order of one-tenth to one-thousandth—than those in liver. Enzyme profiles also differ from tissue to tissue. Consequently, with data from liver we usually cannot predict extrahepatic xenobiotic metabolism, except in special circumstances. These facts have important implications with respect to elimination and tissue-specific toxification and detoxication of carcinogens. The exposure of an individual to foreign chemicals may lead to induction that is both tissue- and inducer-specific. The "control state" is difficult to define for liver; but, as pointed out by Bridges and Fry (56), it is even more difficult to define for extrahepatic tissues. The last, but not the least, difficulty deals with the methodological problems: extrahepatic tissues may be very heterogenous with respect to cell population; the preparation of the microsomal fraction therefore may differ from tissue to tissue; and experimental conditions for enzyme assays may be different from those with liver.

C. Factors Affecting Xenobiotic Metabolism

1. Species and strain differences. Among the factors affecting xenobiotic metabolism (table 2), the most important may be genetic factors. The scientific literature is filled with reports describing species and strain differences in drug-metabolizing enzymes. The data are unpre-

TABLE 2
Some factors affecting xenobiotic metabolism

Factor		References
Genetic factors	Species	224, 336, 488
	Strain	224, 321
	Sex	222
	Interindividual	315, 316, 466
	Polygenic	
	Polymorphic	
Age	Specific ontogenetic (developmental) patterns, neonatal imprinting	325, 358
Immunological factors		465
Pregnancy		
Hormones	Overproduction	465
	Deficiency	465
Exogenous chemicals	Induction	88
	Enhancement	11, 82
	Inhibition	11
Temporal factors	Diurnal	376
	Seasonal	465
Nutrition	Overnutrition	69, 90, 91, 473
	Malnutrition	465
	Specific dietary constituents	255, 465
	Food contaminants	255, 465
Diseases	Concurrent illnesses	89, 223
	Specific organ pathology	465
Housing conditions		465
Stress		465

dictable. Whereas one enzyme may be undetectable in the hamster, high in the dog, and very high in the rat, a second drug-metabolizing enzyme activity may be just the reverse. In the sexually mature animal, the male rat tends to have many of these activities higher than the female; in the mouse, many activities are greater in the female than in the male. Thalidomide is highly teratogenic in the rabbit, monkey, and human, but not in the rat, mouse, or hamster. Recent evidence (147) suggests an involvement of P-450-mediated toxification of thalidomide in these sensitive species but not in thalidomide-resistant species.

Strain differences in drug-metabolizing enzymes are known to occur in the rat (321), mouse (241, 320, 321), and other laboratory animals (224, 321). Exploitation of such inbred mouse strain differences has led to a much better understanding of mammalian genetic systems—the glucuronidase locus (274), *Ah* locus (241), and *Coh* locus (273, 502) are three examples.

2. *Interindividual differences.* There are more than four dozen human pharmacogenetic disorders described in the literature [reviewed recently by Nebert (315, 319)]; these syndromes do not represent dissimilarities of 10% or 40% in some drug-metabolizing enzyme but rather reflect differences of 3-, 5-, or more than 20-fold between individuals in their response to some xenobiotic. Striking interindividual differences may occur even between two siblings. Hence, even if there are a dozen competing enzymic reactions for a xenobiotic, if one relatively important (e.g. rate-limiting step) enzyme activity is genetically 20-fold different between two individuals, minor (10% or 40%) differences in each of the other dozens of drug-metabolizing enzyme activities are relatively insignificant in the ultimate outcome of carcinogenesis or drug toxicity.

3. *Induction by exogenous chemicals.* At present more than 300 compounds are known to induce xenobiotic metabolism (88, 317). The specificity of induction depends on the inducer: there are at least six classes of inducers that cause distinctly differing responses: 1) drugs and halogenated insecticides (phenobarbital, DDT); 2) polycyclic aromatic hydrocarbons (3-methylcholanthrene); 3) steroids (pregnenolone-16α-carbonitrile) (115); 4) alcohol (456); 5) isosafrole (398); and 6) cholestyramine (157). Some inducers such as polychlorinated biphenyls exhibit properties of more than one class of inducers.

Other factors in table 2 tend to increase the variability of xenobiotic metabolism beyond the already large genetic variability. For example, when hospital patients have been studied (362, 431), one finds extreme variations in parameters of drug metabolism. Cytochrome P-450 levels vary from less than 1 to 37 nmol/g of liver, more than a 40-fold variation (431); the variability of aryl hydrocarbon hydroxylase activity is even larger, about 100-fold (362).

III. Methods to Detect the Formation of Reactive Intermediates

A. Nature of Reactive Intermediates

Chemical carcinogens, except direct alkylating agents (252), are often chemically unreactive. Miller and Miller (299, 300) were among the first to suggest that reactive intermediates of potential carcinogens arising through metabolism are positively charged electrophiles that react at negatively charged nucleophilic centers in proteins and nucleic acids. These electrophiles are very unstable and tend to bind covalently to any nucleophile present, including small nucleophiles such as water and glutathione. The covalent binding to critical macromolecules may be one of the necessary requirements for the initiation of chemical carcinogenesis. Toxification of chemical carcinogens occurs mainly by the functionalization reactions, but these reactions are by no means the only ones leading to reactive intermediates. In many instances a relatively stable *proximate* carcinogen is created by the functionalization reaction and the *ultimate* carcinogen is formed by the subsequent action of either conjugation enzymes or functionalization enzymes. Because proximate carcinogens are stable, their identification is usually not problematic.

B. Chemical and Biochemical Methods

Reactive metabolites can be detected by direct means such as chemical or biophysical assays or by indirect means such as studying their reactivity and its consequences (table 3). Although the structure of these inter-

TABLE 3
*Examples of methods to detect the formation of reactive intermediates from procarcinogens**

Nature of Method	Method	Examples	Remarks
Chemical	Derivatization	4-(*p*-Nitrobenzyl)pyridine derivatives of epoxides (525)	Biochemically impractical
Biophysical	Electron spin resonance	Benzo[a]pyrene 6-oxo radical (388)	Biological significance under debate (see section IV D)
Biochemical	Total covalent binding of radioactive carcinogens	Numerous studies in vitro (135, 153, 158, 449) and in vivo (275)	Sum of all intermediates; high blanks, ^{3}H-exchange might be problems
	Fluorescence assay	Benzo[a]pyrene binding to hepatocyte DNA (204) or cultured cell DNA (98, 192)	Sensitive; lacks specificity
	Chromatographic separation of nucleic acid adducts	Sephadex LH20 or HPLC chromatography of polycyclic aromatic hydrocarbons DNA adducts (22, 197)	Further identification by radioactivity or optical spectroscopy (360), fluorescence (98, 191, 457), linear or circular dichromism (132), or immunological techniques
Biological	Mutagenicity	*Salmonella*/liver test (9, 10); V79 cells with other cells or liver fractions for toxification (184, 185, 244)	Additive, synergistic or antagonistic biological activities of *all* intermediates formed from the parent substance
	Sister chromatid exchange	Human lymphocytes (364); Mouse egg cylinder (126); Chinese hamster cells (351, 352)	A rapid in vivo/in vitro assay
Immunologic	Radioimmunoassay for DNA adducts	Benzo[a]pyrene-DNA adducts (175, 374)	Possible to detect adducts produced in vivo (430)

* References are included in parentheses.

mediates can be inferred from the study of possible metabolites (and in some cases the reactive species can be isolated), in most cases direct chemical or biophysical determinations at present are impossible, because of the sheer reactivity and lability of metabolites. K-region epoxides were identified as metabolites of several polycyclic hydrocarbons (419). The first non-K-region epoxide polycyclic hydrocarbon to be characterized was the 10,11-epoxide (a diol-epoxide), detected as a metabolite of the 8,9-dihydrodiol of benz[a]anthracene (45). In most cases, however, it is necessary to use more indirect methods of detection, namely biochemical and biological methods. At the present time the most widely used biochemical method is to study the binding of metabolically formed reactive metabolites to suitable, or available, macromolecules. For years investigators have studied the total covalent binding of carcinogens to macromolecules, especially to DNA (57, 60, 148). The basic method was to expose tissue or cells in vivo to a carcinogen, isolate nucleic acids and proteins, wash them extensively with organic solvents and count the radioactivity, which was then expressed as "covalently bound substance." Another method was to incubate a carcinogen with an enzyme system, cofactors, and a suitable macromolecule, and then to repeat the process described above (129, 135, 153). Although a general correlation between the extent of "total covalent binding" and carcinogenic potency of a series of carcinogens is often observed (60, 148, 181, 186, 245), these methods measure all the possible reactive intermediates without regard to specific metabolite differences in reactivity, stability, and further metabolism. With regard to polycyclic hydrocarbons, the crucial improvement was the method refined by Baird and Brookes (22). The basic features of this method were the hydrolysis of the carcinogen-modified DNA and separation of unmodified nucleosides from modified ones by Sephadex LH20 column chromatography. This method allows the separation and subsequent identification of many metabolite-nucleoside complexes produced during the metabolism of any polycyclic hydrocarbon carcinogen.

C. Biological Methods

Another means of detecting the formation of reactive intermediates is to study their biological effects. Numerous target functions have been used, including mutagenesis in bacteria or mammalian cultured cells, toxicity in cultured or freshly prepared cells, effects on DNA repair and sister chromatid exchanges. With these methods one cannot identify different reactive metabolites produced from the parent compound, but one can develop an understanding of their biological effects which may be related to their effects on the whole organism [see articles in De Serres et al. (102) and Montesanto et al. (304)]. Although there were early bacterial mutagenesis tests to detect the formation of reactive intermediates from carcinogens (130, 131, 284), an important breakthrough in the area was the *Salmonella*/liver test (9, 10), which uses special strains of *Salmonella typhimurium*. This method has proved to be a rapid and very sensitive bioassay for reactive metabolites and has speeded up the progress enormously.

The bacteria or cells used in some test systems do not contain appreciable levels of carcinogen-metabolizing enzymes and, consequently, it is necessary to add a suitable enzyme preparation, for example, liver postmitochondrial

fraction from Aroclor 1254-pretreated rats as in the classical *Salmonella*/liver test. Purified enzymes have also been used in testing carcinogens for mutagenicity (142, 507).

D. Immunological Methods

Recently there has been the development of radioimmunoassays to measure carcinogen-DNA-adducts produced either in vitro or in vivo (table 3). Although practical applications still seem to be somewhat intangible at the present time, these methods may become useful in the intact animal in order for us to understand the sequence of events following exposure to specific carcinogens. Specific antiserum prepared against N-(guanosin-8-yl)-acetylaminofluorene has already been used to detect the formation and removal of the corresponding 2-acetylaminofluorene-DNA adducts from the DNA of mouse epidermal cells and human skin fibroblasts (373). This subject has been recently reviewed (372).

IV. Pathways of Toxification of Polycyclic Hydrocarbons

A. Reactive Metabolites of Polycyclic Aromatic Hydrocarbons

Many experiments leading to the concept of toxification of chemical carcinogens have been described by Miller (300), Heidelberger (164), Weinstein (475), and Boyland (50); in these reviews the interested reader can find an historical account. The evidence in favor of toxification being required for the initiation of chemically induced carcinogenesis is overwhelming. First of all, carcinogens, except direct alkylating agents, are quite inert and unreactive. Although it is theoretically possible that merely a physical disturbance (e.g. intercalation) in macromolecular structure can lead to the initiation of carcinogenesis, most investigators now do not believe this is possible with most polycyclic hydrocarbons.

Listed in table 4 and figure 1 are examples of polycyclic hydrocarbons, the toxification enzymes involved, and the postulated most reactive metabolites. With table 4 we do

TABLE 4

*Examples of toxification of polycyclic aromatic compounds by enzyme systems**

Compound	Toxification Enzymes	Probable/Suggested Intermediate in Carcinogenesis	References
Polycyclic aromatic hydrocarbons			
Benzo[a]pyrene	MO	4,5-Oxide	380
	MO, EH	7,8-Diol-9,10-epoxide	421
	MO	6-Oxo radical	257
7,12-Dimethylbenzo[a]anthracene	MO, EH	3,4-Diol-1,2-epoxide	95, 109, 111, 182, 281, 289, 290, 307, 426, 468, 469, 492
	HS	Hydroxymethyl derivatives	52, 99, 123
Benzo[a]anthracene	MO, EH	3,4-Diol-1,2-epoxide	260, 280, 282, 427, 445, 489, 494, 505
		8,9-Diol-10,11-epoxide	94, 168, 437, 470
Dibenzo[a,h]anthracene	MO, EH	3,4-Diol-1,2-epoxide	63, 334, 510
	MO, EH	10,11-Diol-12,13-epoxide	170
7-Methylbenzo[a]anthracene	MO, EH	3,4-Diol-1,2-epoxide	81, 283, 290, 454, 467
8-Methylbenzo[a]anthracene	MO, EH	3,4-Diol-1,2-epoxide	491
3-Methylcholanthrene	MO	1-Hydroxy-3-methylcholanthrene (proximate)	259, 443, 444, 500
	MO, EH	9,10-Diol-7,8-epoxide (ultimate)	96, 443, 444, 468, 500
Chrysene	MO, EH	1,2-Diol-3,4-epoxide	62, 263, 499, 508
5-Methylchrysene	MO, EH	1,2-Diol-3,4-epoxide	162, 163
Phenanthrene	MO, EH	1,2-Diol-3,4-epoxide	62, 499
1,4-Dimethylphenanthrene	MO, EH	1,2-Diol-3,4-epoxide	251, 261
Benzo[c]phenanthrene	MO, EH	3,4-Diol-1,2-epoxide	498
Cyclopenta[c,d]pyrene	MO	3,4-Oxide	144, 145, 504
Dibenzo[a,e]fluoranthene	MO, EH	3,4-Diol-1,2-epoxide	363
Benzo[e]pyrene	MO, EH	9,10-Diol-7,8-epoxide	277, 278, 497, 509
15,16-Dihydro-11-methylcyclopenta[a] phenanthrene-17-one	MO, EH	3,4-Diol-1,2-epoxide	2, 92, 485
Benzo[j]fluoranthene	MO, EH	9,10-Diol-11,12-epoxide	250
Benzo[k]fluoranthene	MO	8,9-Diol-10,11-epoxide	250
Dibenzo[a,h]pyrene	MO, EH	1,2-Diol-3,4-epoxide	77
Dibenzo[a,i]pyrene	MO, EH	3,4-Diol-1,2-epoxide	77

* Abbreviations used are: MO, monooxygenase; EH, epoxide hydrolase; HS, hydroxymethyl synthetase. For chemical structures, please refer to figure 1.

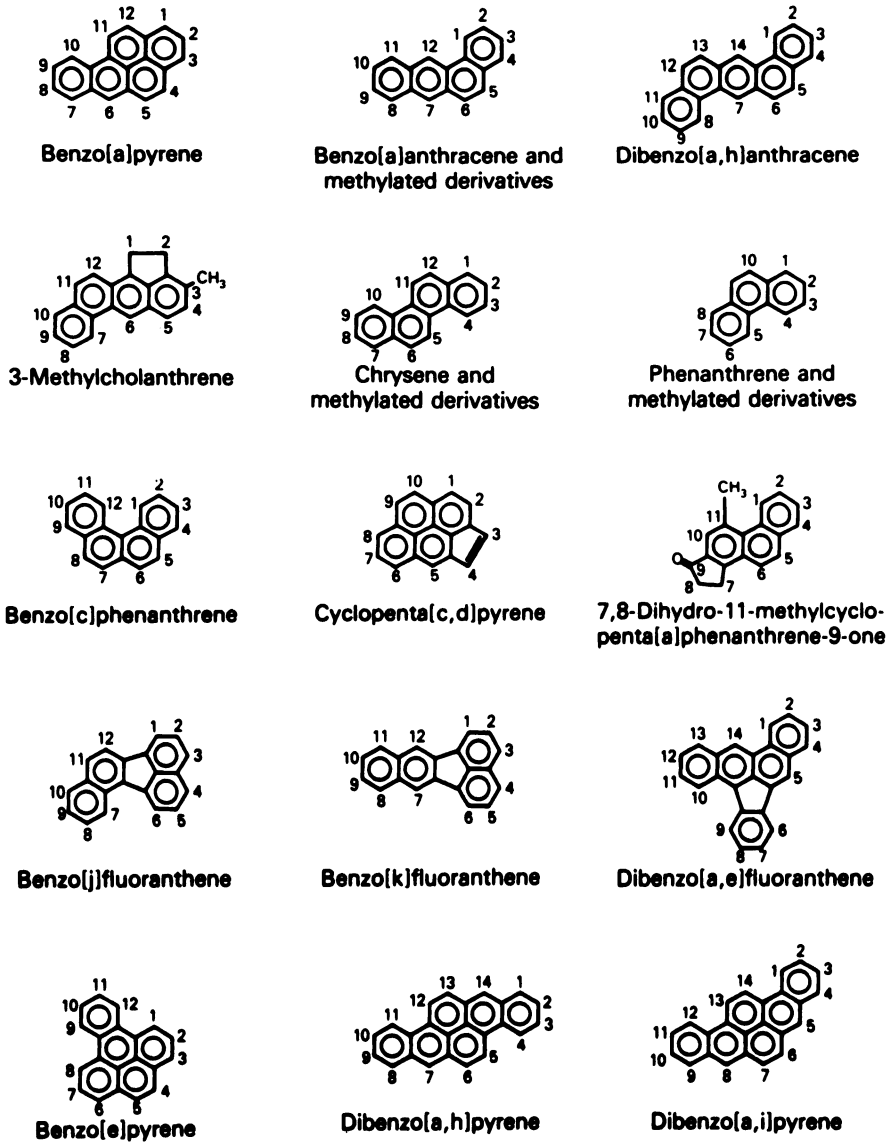

FIG. 1. Chemical structures of most of the polycyclic hydrocarbons described in table 4.

not intend to be comprehensive; nevertheless, we have tried to provide some original references in which detailed discussions of different compounds can be found. General approaches that have been adopted in the elucidation of metabolism and toxification of benzo[a]pyrene and other polycyclic aromatic hydrocarbons should apply as well to other chemical carcinogens.

There are certain carcinogens (e.g. asbestos, plastic film, certain heavy metals) that have not been shown to be mutagenic or to react with nucleic acids. Ashby et al. (14) suggested that substances found to be carcinogenic but not mutagenic are "epigenetic" carcinogens and that saccharin, phenobarbital, dieldrin, DDT, thioacetamide, thiourea, 3-aminotriazole, and asbestos belong to this class. Alternatively, it is also possible that the "correct" mutagenesis assay has not yet been developed for these compounds or that some of these chemicals belong more appropriately to the class of "tumor promoters" (105) instead of "tumor initiators."

Another class of potential carcinogens that may not act through the formation of reactive intermediates is the so-called "hypolipidemic hepatic peroxisome proliferators" (385). These substances include clofibrate.

B. Toxification of Benzo[a]pyrene

1. Historical aspects. On the basis of theoretical calculations Pullman and Pullman (381) postulated the importance of the K-region in carcinogenesis and for a long time investigations were designed along this theoretical line (108). In the early 1970s, research—especially from Brookes' and Sims' laboratories—began to cast doubt on the assumption that the K-region epoxide was most important in polycyclic aromatic hydrocarbon carcinogenesis. The best evidence was based on DNA-bind-

ing studies. By using the method for separation of metabolite-nucleoside adducts (22), Baird et al. (24, 25), and King et al. (234) demonstrated that nucleoside adducts of the K-region epoxides of 7-methylbenz[a]anthracene and benzo[a]pyrene differed from those produced in intact cells. Also, other studies could not confirm the theoretical prediction (298). Borgen et al. (47) demonstrated that benzo[a]pyrene-7,8-dihydrodiol was most efficiently converted to a DNA-bound derivative by hamster liver microsomes. Sims et al. (420) then suggested that benzo[a]pyrene is activated via a diol-epoxide pathway; this paper marked the beginning of tremendous scientific activity. In addition to what follows in this review, extensive data are also provided in several monographs and symposium proceedings (59, 124, 137, 151, 210, 211).

2. Metabolism and covalent binding of benzo[a]pyrene. The metabolism of benzo[a]pyrene (for reviews see refs. 58, 101, 117, 136, 405, 516) is extremely complicated, and only the most important pathways are presented in figure 2. A monooxygenase first introduces an oxygen atom into any of several positions of the molecule to produce primary, or "simple" epoxides (200, 419) (steps 1 in fig. 2). The formation of epoxides is catalyzed by different forms of cytochrome P-450 (101, 103, 117, 405, 484, 525). Step 2 is the spontaneous rearrangement reaction of epoxides to phenols [cf. Jerina and Daly 201]. For every system studied thus far, 3- and 9-phenols predominate but there also are significant amounts of 1- and 7-phenols (97). There had been some doubt as to the existence of an epoxide intermediate in the production of 1- and 3-phenols because of the lack of corresponding dihydrodiols, but Yang et al. (523) provided evidence that the 3-phenol is derived from 2,3-oxide. Another pathway for the epoxides is the reduction back to parent hydrocarbon (step 3) (44, 225, 436, 515). Step 4, the formation of quinones through the 6-phenol and 6-oxo radical (257), which can be detected by electron spin resonance (312, 388, 455), is less well characterized. It is not known whether the 6-phenol arises through an intermediary epoxide. Quinones also can be produced nonenzymically by lipid peroxidation (306) and aerobic oxidation. Epoxides can be further metabolized to dihydrodiols by the microsomal epoxide hydrolase (step 5) (172, 258, 335), or to glutathione conjugates catalyzed by cytosolic glutathione transferases (327) (step 6). Quinones also can be converted to glutathione conjugates (306). Glutathione conjugates are further metabolized to mercapturic acids (51) which can be detected in urine. Epoxides, dihydrodiols, phenols and quinones are traditionally regarded as "primary" metabolites of benzo[a]pyrene (407). All primary metabolites are further metabolized. Dihydrodiols can be dehydrogenated to catechols by a soluble dehydrogenase (step 7) (46, 447). Dihydrodiols, phenols, and quinones also are conjugated with glucuronic acid catalyzed by UDP glucuronosyltransferase (step 8) (326) or with sulfate catalyzed by sulfotransferase (step 9) (83, 86, 329). Phenols and dihydrodiols also can be substrates for the monooxygenase (step 10) (47, 71, 360, 420, 482). Hence, another position of the molecule can become epoxidated. This epoxide now can follow any of the various metabolic pathways already discussed. The final result is that a multitude of primary and secondary metabolites is produced.

In vitro DNA binding studies have demonstrated that several primary and secondary benzo[a]pyrene metabolites are able to bind covalently to DNA. These reactive intermediates are marked by an asterisk in figure 2. Simple epoxides are reactive enough to bind to exogenous DNA without further metabolism: there is evidence for the 4,5-oxide, 7,8-oxide, and 9,10-oxide (39, 360). Phenols can be metabolized to adducts that bind to DNA: for 1-

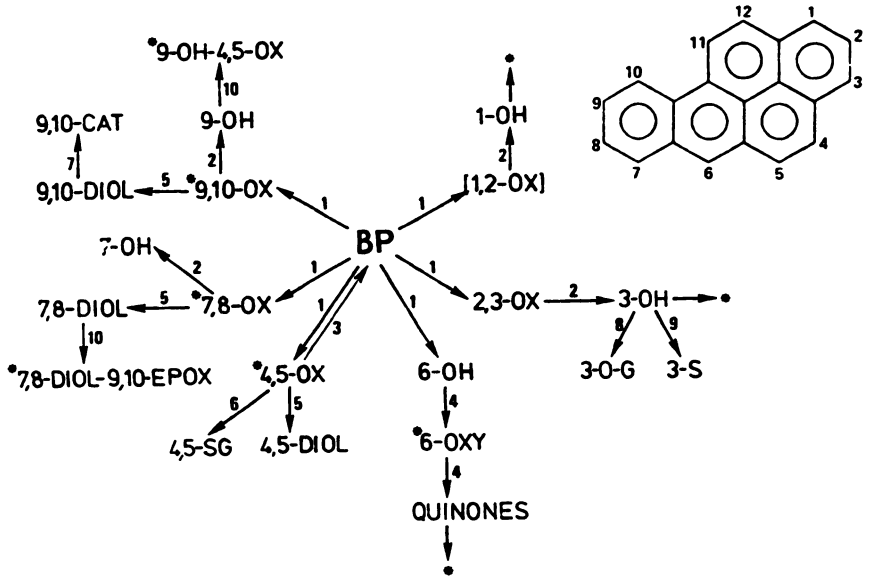

FIG. 2. Selected aspects of the metabolism of benzo[a]pyrene. Metabolites that have been shown to bind covalently to macromolecules are marked by an asterisk. OX, oxide; CAT, catechol; DIOL, *trans*-dihydrodiol; *OH*, phenol; EPOX, epoxide; SG, glutathione conjugate; O-G, glucuronide conjugate; S, sulfate conjugate; OXY, oxy free radical.

or 3-phenol (347, 348) the reactive intermediate has not been characterized, but the most probable derivative for 9-phenol is 9-hydroxy-4,5-oxide (230, 360, 451). Quinones can be converted by enzymatic catalysis to an unidentified species capable of binding to DNA (360, 451). Very little in vitro DNA binding is demonstrable with the 4,5-diol or 9,10-diol (451); the 7,8-diol is discussed in the following section.

3. The diol-epoxide pathway of benzo[a]pyrene metabolism. Since the pioneering findings of Borgen et al. (47) and Sims et al. (420), the pathway leading to benzo[a]pyrene 7,8-diol-9,-epoxide via the intermediary 7,8-oxide and 7,8-dihydrodiol has been under intensive investigation. As has been known for a long time, dihydrodiols of polycyclic aromatic hydrocarbons are in *trans* configuration (419). Furthermore, because of specific enzyme action, the dihydrodiols are also optically pure—with benzo[a]pyrene all the dihydrodiols are the (−)-enantiomers (518, 522). By further catalysis of the monooxygenase, two isomeric diol-epoxides from (−)-*trans*-7,8-dihydrodiol are formed, the relative amounts of which depend on the steroselectivity of the second oxygenation step (103, 104, 187, 521). The principal metabolite however, appears to be the *anti*-isomer (187, 198, 199, 232, 233, 446, 522). Nevertheless, both *anti*- and *syn*-isomers are very active in binding to DNA in vitro and to RNA and DNA of mammalian cells in culture (193, 240, 269, 305, 386, 433, 519). The diol-epoxides are further converted by solvolysis to tetraols (443, 520, 521) and by nonenzymic reduction to triols by NADPH (517). The structure of the principal benzo[a]pyrene diol epoxide-nucleoside adduct, the N^2-guanine derivative of the 7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene, has been resolved by using fluorometry, mass spectrometry, and circular dichroism (98, 198, 199, 294, 313, 342, 433, 476). Diol-epoxide adducts with other bases and positions also have been observed in different systems (17, 193, 295, 343, 344, 433).

4. Biological reactivity of benzo[a]pyrene metabolites. The importance of the diol-epoxide in the carcinogenic process of benzo[a]pyrene was suggested on the basis of findings that it was indeed the predominant DNA binding species in cells in culture (420) and that simple epoxides were not involved in the DNA binding in intact cells (25). However, at that time there was no convincing hypothesis to explain the exceptional reactivity of the diol-epoxide. Why was this metabolite more important than other potentially reactive metabolites of benzo[a]pyrene? In the ensuing attempts to corroborate the diol-epoxide hypothesis, numerous benzo[a]pyrene metabolites were synthesized and tested in different biological tests. Results of these studies are compiled in tables 5 and 6. Similar tables could be constructed for other polycyclic hydrocarbons, since the same experimental approaches are being used to elucidate their most reactive ultimate carcinogenic intermediates. For this the reader is referred to the references cited in table 4.

Mutagenicity studies in bacteria (the *Salmonella*/liver test) demonstrate that several phenols, the 7,8-diol, 4,5-oxide, and 7,8-oxide are reactive, the oxides being mutagenic without metabolic toxification. Among these primary metabolites, the 4,5-oxide is the most mutagenic compound. However, in malignant transformation and carcinogenicity test systems, the 7,8-diol and 7,8-oxide are the most reactive primary metabolites.

The data on biological reactivity of natural and synthetic benzo-ring derivatives of benzo[a]pyrene presented in table 6 provide convincing evidence that the diol-epoxides are indeed the most mutagenic, cell-transforming, and carcinogenic metabolites among various metabolites of benzo[a]pyrene. All other reactive metabolites can be excluded on different grounds: the 4,5-oxide, although a potent mutagen, is rapidly converted to a relatively inert diol by epoxide hydrolase (337) and in intact cells may not survive long enough to be effective (see section V.B.); benzo[a]pyrene 2-phenol, although a strong carcinogen, is not a major metabolite in vitro or in vivo (97). In summary, evidence from DNA binding, mutagenicity, malignant transformation, and carcinogenicity studies with various actual or potential benzo[a]pyrene metabolites strongly suggests that the benzo[a]pyrene-7,8-diol-9,10-epoxides are the ultimate carcinogenic forms of benzo[a]pyrene.

C. The "Bay-Region" Hypothesis

That benzo[a]pyrene-7,8-diol-9,10-epoxide is the most probable ultimate carcinogen gave rise to the bay-region hypothesis for the metabolic toxification of polycyclic aromatic hydrocarbons (for reviews see refs. 201, 203, 256). The unique structural feature of the diol-epoxide appears to be that the epoxide is on a saturated angular benzo-ring and that it forms part of a bay region of the polycyclic hydrocarbon. The key chemical feature of such epoxides is that they are highly susceptible to attack by nucleophiles.

The bay-region hypothesis states that the high chemical reactivity of such diol-epoxides can be attributed to their unique electronic properties. Perturbational molecular orbital calculations, which predict π-electron energy changes, indicate that epoxides on saturated benzo-rings (which form part of the bay region of a hydrocarbon) undergo ring opening to form a carbonium ion much more easily than do non-bay-region epoxides. Moreover, these calculations indicate that the more carcinogenic polycyclic hydrocarbons tend to form carbonium ions from their bay-region diol-epoxides more readily than weaker carcinogens. Studies on the quantitative relationship between the mutagenic activity and the calculated electronic properties of each of nine tetrahydroepoxides of polycyclic hydrocarbons (203) gave a high correlation coefficient of 0.74. Studies comparing rates of solvolysis (481) and rates of reaction with the nucleophile *p*-nitrothiophenolate (202) also satisfy the predictions of the quantum-mechanical calculations. Other studies (377) have shown that the correlation between ease of carbonium ion formation and carcinogenicity among 45 deriv-

TABLE 5

*Biological reactivity of different primary metabolites and/or derivatives of benzo[a]pyrene**

	Mutagenicity		Malignant Transformation	Carcinogenicity
	Bacteria	Animal cells		
Phenols				
1	++ (140, 507)	± (187)		− (423, 490)
2	++ (507)			++ (423, 490)
3	+ (140, 507)	± (187)		− (423, 490)
4	± (140, 507)			− (218, 423)
5	− (507)			− (218, 423)
6	+ (140, 507)	± (187)	− (290)	− (218, 423)
7	± (140, 507)	− (187)		− (218, 423)
8	− (507)	− (187)		− (218, 423)
9	± (140, 507)	− (187)		− (218, 423)
10	− (507)			− (218, 423)
11	− (507)			+ (423, 490)
12	+ (507)			− (423, 490)
Quinones				
1,6	− (140, 495)	− (495)		− (425)
3,6	− (140, 495)	− (495)		− (425)
6,12	− (140, 495)	− (495)		− (425)
Dihydrodiols				
4,5	− (280, 507)	− (187)	− (279, 290)	− (80, 425)
7,8	+ (280, 507)	++ (187)	+ (180, 183, 217, 279, 290)	++ (80, 262, 264, 266, 428)
9,10	± (280, 507)	− (187)	− (279, 290)	− (80, 425)
11,12	− (507)			− (425)
Oxides				
4,5	++ (280, 500, 503)	+ (187)	± (279)	(±) (242, 265, 425, 428, 493)
7,8	+ (140, 503)			+ (242, 265, 266, 425, 428, 493)
9,10	+ (495, 503)			− (242, 265, 425, 428, 493)
11,12	+ (495)			(±) (242, 425, 493)
Others				
6-CH_3		± (187)		+ (425)
6-CH_2OH		± (187)		+ (425)
Tetraols		− (180)		− (219, 425)
Triols		− (180)		

* References are included in parentheses.

TABLE 6

*Biological reactivity of different benzo-ring metabolites of benzo[a]pyrene**

Benzo-ring Derivative	Mutagenicity		Malignant Transformation	Carcinogenicity
	Bacteria	Animal cells		
(+)-Trans-7,8-dihydrodiol		+ (180)		+ (220, 242, 262)
(−)-Trans-7,8-dihydrodiol		++ (180)		+++ (220, 262)
7,8-Diol-9,10-epoxide (*cis*,II,1,*syn*)	+++ (282, 496, 511)	+ (282, 331, 511)	+ (279, 288)	− (217, 219, 264, 428)
7,8-Diol-9,10-epoxide (*trans*,I,2,*anti*)	++ (282, 496, 511)	++ (282, 331, 511)	++ (279, 288)	+++ (217, 219, 428) ± (264)
(+)-Anti-isomer (7β,8α-dihydroxy)	+ (501)	+++ (501)		+++ (64, 424)
(−)-Syn-isomer (7β,8α-dihydroxy)	++ (501)	+ (501)		+ (64, 424)
(−)-Anti-isomer (7α,8β-dihydroxy)	+ (501)	+ (501)		± (64, 424)
(+)-Syn-isomer (7α,8β-dihydroxy)	+ (501)	+ (501)		± (64, 424)
9,10-Diol-7,8-epoxide (*syn*)	+ (282)	− (282, 288)	− (288)	
9,10-Diol-7,8-epoxide (*anti*)	+ (282)	− (282, 288)	− (288)	
7,8-Diacetyl				++ (242)
7,8-Catechol				− (242)
7,8-Dihydro	++ (506)		+ (290)	++ (242)
9,10-Dihydro	+ (506)		− (290)	− (242)
7,8-Quinone	− (506)			
7,8,9,10-Tetrahydro	− (506)			
7,8-Dihydroxy-7,8,9,10-tetrahydro	− (506)			

* References are included in parentheses.

atives of polycyclic aromatic hydrocarbons is quite good.

Many other studies with different polycyclic aromatic hydrocarbons have attempted to corroborate the bay-region hypothesis. DNA binding, metabolic, carcinogenic, and mutagenic studies on a variety of polycyclic hydrocarbons (table 4 and fig. 1) support the view that

the "bay-region" metabolite is probably the ultimate carcinogenic form. Exceptions to this rule exist: benzo[a] anthracene appears to be toxified to at least one potent intermediate *not* associated with its bay region (94, 470); cyclopenta[c,d]pyrene is potentiated through the K-region metabolism (144, 145, 500); dibenzo[a,c]anthracene has at least one major non-bay-region diol-epoxide (170).

The bay-region hypothesis predicts that the diol-epoxides of some weakly carcinogenic polycyclic hydrocarbons such as benzo[e]pyrene should be strong carcinogens (202). This discrepancy seems to be now more or less resolved. Although the bay-region epoxide of 9,10-dihydro-benzo[e]pyrene is a strong mutagen and carcinogen (497), the corresponding diol-epoxide is not appreciably produced in in vitro or in vivo situations (277, 278, 509). Further, synthetic benzo[e]pyrene 9,10-diol-11,12-epoxides have very low mutagenic activity, probably because of their structural rigidity (497).

D. Other Toxification Pathways

Although the diol-epoxide pathway of toxification for benzo[a]pyrene and other polycyclic aromatic hydrocarbons and the bay-region hypothesis for its explanation have received wide acceptance, many other pathways and mechanisms have been suggested (e.g. 160). Some of these are shown in table 4 as alternative toxification pathways for benzo[a]pyrene, benzo[a]anthracene, and 7,12-dimethylbenzo[a]anthracene.

As shown in table 5, several primary metabolites of benzo[a]pyrene clearly are mutagenic with or without toxification, although only a few of them are carcinogenic in the experimental systems employed. However, it is possible that some of these reactive intermediates could be ultimate carcinogens in other experimental situations. One should not abandon this possibility, because evidence for the bay-region hypothesis, although impressive, is not without flaws. Furthermore, rather unexpected findings have emerged. Nagao et al. (311) demonstrated that the (−)-*trans*-7,8-diol is a direct mutagen to *Salmonella typhimurium* TA 100, but not to TA 98. Hsu et al. (177, 178) showed that this diol, without toxification, became bound noncovalently to single-stranded DNA and caused the loss of ØX174 DNA infectivity. These studies suggest possible biological activity for the 7,8-diol that is independent of the diol-epoxide pathway.

Metabolites could disturb DNA structure without binding covalently. In vitro incubation studies indicate that the benzyl-ring tetraols of benzo[a]pyrene bind noncovalently to DNA by an intercalation mechanism (133).

Benzo[a]pyrene toxification via the free-radical pathway at carbon-6 position has received much attention during recent years. The 6-hydroxybenzo[a]pyrene is unstable in solution and rapidly undergoes radical formation to the 6-oxobenzo[a]pyrene (257, 270). This radical may interact with macromolecules or be converted to quinones (237, 270). The binding of benzo[a]pyrene to rat skin has been claimed to occur at positions 1, 3, and 6, supporting the free-radical scheme for toxification (392), whereas other studies have claimed the absence of significant binding involving the 6-position (231, 345). However, the biological damage need not necessarily be in direct relation with covalent binding of the parent compound with macromolecules. In this regard, studies from the laboratory of Ts'o are interesting; they propose that quinones of benzo[a]pyrene and other biologically active quinones owe their activity to oxidation-reduction cycles involving quinone, hydroquinone, and molecular oxygen (271). The reactive reduced oxygen radicals and semiquinone radicals formed during these cycles may be responsible for the cellular injury and inhibition of cellular processes in cell culture studies (272) and for single-strand DNA breaks observed in in vitro experiments (271).

Hydroxylation of methyl side chains of certain alkylated hydrocarbons has been suggested as a toxification pathway (see table 4 and ref. 106). Although alkyl side chain hydroxylation could be significant in some cases, e.g. in the toxification of 7,12-dimethylbenz[a]anthracene with respect to adrenal gland necrosis (53), recent studies (table 4) also support the important role of the diol-epoxide pathway in the toxification of alkylated aromatic hydrocarbons (6).

The toxification of benzo[a]pyrene by hydroxymethylation catalyzed by the "hydroxymethyl synthetase" has been suggested (395, 429). The presence of this pathway has not, however, been confirmed.

Toxification mechanisms not related to drug-metabolizing enzymes have been little studied. Marnett et al. (286, 287) have demonstrated that, in the presence of prostaglandin synthetase and arachidonic acid, benzo[a] pyrene becomes covalently bound to DNA and is mutagenic. Under in vitro conditions, both γ-radiation (138) and UV-light (434) convert benzo[a]pyrene to reactive form(s) that bind covalently to macromolecules and are mutagenic to bacteria. The significance of these toxification mechanisms is not known.

If the polycyclic hydrocarbon nucleus bears substituents containing atoms other than carbon, the toxification pathway may be totally different. For example, the strong mutagenicity of nitropyrenes is probably associated with the formation of hydroxylamino derivatives by bacterial nitroreductases (70, 297). Because polycyclic hydrocarbons undergo substitution reactions with SO_2 and NO_2 relatively easily, it is therefore possible that these substituted polycyclic hydrocarbons play a role in environmentally induced tumorigenesis.

V. Factors Determining the Covalent Binding of Reactive Intermediates to Cellular Macromolecules

A. Modifying Factors in in Vitro Experiments

Xenobiotic metabolism is extremely sensitive to numerous endogenous and exogenous factors (table 2). Dif-

ferent aspects of these modifying factors have been recently summarized (422). In principle, two groups of factors affect the outcome of in vitro experiments: those related to the source of enzyme preparation and those related to incubation conditions. Numerous in vitro and in vivo studies with benzo[a]pyrene and other carcinogens clearly demonstrate that metabolite profiles vary enormously depending on the pretreatment, diet, and genetic background (453). Clearly the "primary" in vitro production of reactive intermediates can be modified (42, 323). For the detection of reactive metabolites, DNA usually has been used as the target and the Sephadex LH20 column chromatography or high-performance liquid chromatography has been used for separation and quantitation of metabolite-nucleoside adducts. Species differences are observed both in absolute and relative amounts of reactive intermediates formed (360, 459). Sometimes these dissimilarities can be traced to different catalytic properties of purified enzymes, as illustrated by studies with cytochrome "P-448" in rat and rabbit liver (226). A well-known strain difference in mice with regard to the responsiveness of aryl hydrocarbon hydroxylase induction by polycyclic aromatic hydrocarbons is also reflected in the metabolite-nucleoside adduct patterns produced in vitro (42, 43, 323, 360). Other inducers such as phenobarbital, pregnenolone-16α-carbonitrile, or alcohol have rather similar effects in both responsive and nonresponsive strains (43). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, a potent inducer, increases the different benzo[a]pyrene metabolite-nucleoside complexes in nonresponsive mice to the same degree as that found in responsive mice (43). Dietary manipulations such as sucrose feeding (43, 255), starvation (255), or specific purified diets (255) have profound effects on the production of reactive intermediates, although the measurable levels of xenobiotic-metabolizing enzymes do not change appreciably.

The selective induction of epoxide hydrolase, UDP glucuronosyl-transferase, and glutathione transferase by dietary antioxidants (40, 213, 308, 401)—such as butylated hydroxyanisole or ethoxyquin—have pronounced effects on the binding of benzo[a]pyrene to DNA. Ethoxyquin feeding increases liver-microsome-catalyzed formation of the benzo[a]pyrene diol-epoxide-DNA adduct (214). How these findings can be reconciled with the known protective effect of ethoxyquin against benzo[a]pyrene-induced tumor formation (474) is not clear.

Other relatively selective inducers and activators of drug-metabolizing enzymes are being found. For example, *trans*-stilbene oxide (308) and benzil (404) are quite specific inducers and activators, respectively, of epoxide hydrolase.

In addition to species, strain, interindividual, and dietary differences in the metabolism of carcinogens, one must also consider differences on the basis of experimental techniques employed. The substrate, as well as enzyme, concentrations in the incubation mixture are of critical importance in terms of specific adducts produced (33, 359). Adduct patterns are dependent on the source of microsomes—whether placenta, lungs, bowel, or liver are used (255, 361). Changes in concentrations of cofactors and other constituents can affect markedly adduct patterns (15, 127). The addition of epoxide hydrolase to the in vitro microsomal incubation mixture strikingly decreases the mutagenicity of benzo[a]pyrene and decreases the mutagenicity and covalent binding of benzo[a]pyrene-4,5-oxide (337, 507), but has little effect on the activity of the bay-region diol-epoxide (31, 448). The addition of glucuronic acid or active sulfate decreases the binding to DNA and mutagenicity of benzo[a]pyrene and several of its metabolites, presumably by changing the metabolite profile and by trapping potential promutagens (347, 348). However, there are also studies demonstrating the enhanced in vitro metabolism and binding to DNA of benzo[a]pyrene intermediates, as well as enhanced mutagenicity, in the presence of UDP glucuronic acid (120, 328, 330). The reason for these discrepancies is unknown.

Other carcinogens and noncarcinogenic compounds modulate the metabolism, covalent binding, and biological effects of carcinogens under in vitro and in vivo conditions. These synergistic and antagonistic modifications have been summarized by Ashby and Styles (13) and Wiebel et al. (483).

B. Role of Cellular Integrity

It long has been recognized that certain enzyme activities in the intact cell differ from those observed when subcellular fractions have been isolated. The importance of the balance of different enzymes and the intactness of cells is emphasized by studies demonstrating differing DNA binding patterns among microsomal in vitro systems, isolated organs, cell cultures, and intact animals (table 7). For example, when benzo[a]pyrene is incubated with DNA and mouse or rat liver microsomes, at least nine distinct metabolite-nucleoside complexes can be separated after the appropriate hydrolysis of DNA (360, 451, 459). The most prominent peaks can be attributed to nucleoside adducts of 7,8-diol-9,10-epoxide, the K-region oxide, and 9-hydroxy-4,5-oxide (360); the phenol-oxide-nucleoside adduct is usually the largest. When benzo[a]pyrene is allowed to react with DNA in isolated perfused liver, four peaks can be detected, the most abundant being the nucleoside adduct of the diol-epoxide (212). In isolated perfused lung, even fewer metabolite-nucleoside adducts are observed and again the diol-epoxide-nucleoside adduct predominates (100, 459). DNA isolated from skin epidermis, trachea, bronchi, isolated hepatocytes, or cells in culture exposed to benzo[a]pyrene almost always contains only one prominent peak, namely that associated with the diol-epoxide (16, 18, 23, 25, 152, 199, 233, 269, 346, 476, 487). In cultured human lymphocytes, the prominent adduct bound to DNA is the 4,5-oxide (41). Most likely the further detoxication of primary benzo[a]pyrene metabolites resulting in metabolites not available for covalent binding (e.g. conjugation or diol formation) is responsible for the differences in

TABLE 7

*The formation of certain benzo[a]pyrene metabolite-nucleoside adducts in different experimental systems**

Adducts of	In Vitro Liver or Lung Microsomes with Added DNA (360, 459)	Isolated Perfused Liver (212)	Isolated Perfused Lung (100, 459)	In Vivo Intact Cells and Tissues and Section (359)	Disappearance from DNA In Vivo	Further Metabolism of Active Intermediate in Intact Cells
7,8-Diol-9,10-epoxide	++	+++	+++	+++	Slow (333, 359)	Slow (448)
4,5-Oxide	++	+	−	±	Rapid (122, 359)	Rapid (448)
9-Hydroxy-4,5-oxide	+++	++	±	±	Rapid (333)	Probably rapid
Activated quinones†	++	+	±	−		Probably rapid (72, 73, 268)

* References are included in parentheses.

† According to the model proposed by Lorentzen and Ts'o (271), quinones can produce superoxide anions and other oxygen radicals as a consequence of oxidation/reduction cycles, which then damage DNA without the covalent binding of the parent quinone.

metabolite-nucleoside adducts observed in vivo, as compared with microsomal metabolism in vitro.

Analogous dissimilarities between in vitro and in vivo experimental systems have been shown with other polycyclic aromatic hydrocarbons. Bigger et al. (33–36) have demonstrated that in four intact cellular systems—mouse skin in vivo, mouse embryo cells in culture, rat liver cells in culture, and human skin cells in culture—binding of 7,12-dimenthylbenz[a]anthracene to DNA occurs via the diol-epoxide, whereas under in vitro conditions with rat liver microsomes or 9,000 × *g* supernatant fraction, a wider variety of toxification routes is operative and diol-epoxide adducts represent only a small contribution to the total binding.

Jaggi et al. (195) compared the DNA binding of benzo[a]pyrene in rat liver after in vivo administration, in liver perfusion in situ, isolated liver cells, liver homogenate, and liver microsomes incubated with DNA. The amount of benzo[a]pyrene bound per mg of DNA varied by more than three orders of magnitude between the two extreme systems—in vivo vs the incubation of benzo[a]pyrene with DNA in the presence of microsomes. Consequently, they felt that the biological relevance of in vitro systems is very poor and argue very strongly in favor of in vivo approaches in binding studies (275).

In another in vivo study (48), benzo[a]pyrene was shown to be bound to rat liver and lung DNA; the major adduct in both liver and lung DNA appeared to result from the further metabolism of benzo[a]pyrene phenols. A very small amount of the diol-epoxide adduct was detected in rat lung only. These adduct patterns are in contrast with most cell culture and perfusion studies in which the diol-epoxide adduct is the most prominent. It is possible that the discrepancy can be explained on the basis of pretreatment or diet of the animals.

C. Metabolic Differences among Cell Cultures, Intact Cells, the Perfused Organ, and the Intact Animal

1. Primary cells in culture and continuous cell lines. It has been long realized that the metabolism in fetal rat primary hepatocytes (349) or continuous hepatoma-derived cell lines (350) differs from metabolism in intact liver. The benzo[a]pyrene metabolite profile changes (84, 119, 408) as a function of time in culture, and the induction of drug-metabolizing enzymes by phenobarbital in culture clearly is not the same as that in the intact animal (84, 349, 350). In addition, subcellular fractions differ from intact cells in the profile of metabolites (99, 405) and DNA adducts (34, 323, 332) formed after metabolism of various potent carcinogens such as aflatoxin B_1, benzo[a]pyrene, and 7,12-dimethylbenz[a]anthracene. These data reflect the rapid loss of many forms of P-450 and perhaps other drug-metabolizing enzymes just as soon as cells are treated with trypsin and first put into culture (38, 317). The reason for such a loss is not understood but must be appreciated in any study involving the activation of procarcinogens to carcinogens.

The toxification of benzo[a]pyrene and most other polycyclic aromatic hydrocarbons to mutagenic intermediates (185) by continuous cell lines has been reported dozens of times, whereas toxification of aflatoxin B_1, for example, to mutagenic intermediates often does not occur (247). These data are explainable by the fact that the form(s) of P-450 necessary for polycyclic hydrocarbon oxygenation remain in cultured primary and continuous cell lines, whereas the form(s) of P-450 responsible for the 2,3-oxide formation of aflatoxin B_1 can disappear rapidly in culture for unknown reasons (317, 406). All of the extensive studies involving human cultured tissues (19, 20, 174, 176) may have this same major liability, although aflatoxin B_1 2,3-oxide was identified as a metabolite in human cultured bronchus and colon (19).

2. Freshly prepared intact cells. After perfusion of liver (or other organs) with collagenase, hyaluronidase, and other degradative enzymes, it is possible to isolate freshly prepared intact cells (32). These liver cells appear to maintain most of their drug-metabolizing capacity for at least the first 2 to 4 hours after isolation. Orrenius and co-workers (66, 341, 452, 458) have applied this system to drug toxicity in many excellent studies. These studies have been extended to isolated cells from rat small intestine (149) and kidney (340). The advantages of this system are that all drug-metabolizing enzymes appear to be active and able to excrete mutagenic, carcinogenic, and/or toxic intermediates into the medium, as well as into any other neighboring cells. The multiplicity of P-450 forms has not yet been thoroughly characterized with this system, however, so it is quite conceivable that

metabolite profiles of certain procarcinogens would differ between these intact cells and the cells in their intact organ. The major disadvantage of this system is that these cells are no longer in the same architectural arrangement as was the case in the intact organ. These cells are principally parenchymal cells with the mesenchymal stroma removed. If the proximity of one "high metabolizing" cell to another "target" cell is important within the same organ—and such hypotheses abound, especially among the various cell types in the lung (37, 85)—the data with freshly prepared cells in vitro may differ from data with the intact organ.

3. The perfused organ. Studies involving perfused liver (212), lung (100, 459, 460), testis (253), and other perfused, superfused and explanted organs (158) have become increasingly popular. In terms of metabolism of procarcinogens and carcinogens, perfusion systems obviously will approximate what occurs in the intact animal. With respect to long-term superfusion and explant systems, it is not known how faithfully they represent the intact organ or organism. However, because of data with long-term cultured cells, one should be cautious about accepting too easily the results obtained with explant systems as representing what is expected in the intact organ or organism.

Perfusion experiments may give useful information regarding the balance of toxification and detoxication enzymes in the perfused organ and the probability of the interaction of reactive intermediates with macromolecules (see e.g. 114, 212, 459). By examination of the perfusate, the presence or absence of relatively stable procarcinogens or carcinogens might be established.

An excellent demonstration of the usefulness of a perfused organ was recently provided by Meerman et al. (296). The authors showed that, by omission of sulfate from the perfusion medium or by the inhibition of sulfation by pentachlorophenol, N-O-sulfation of N-hydroxy-2-acetylaminofluorene is responsible for most of the reactive metabolite(s) which react with protein and RNA in isolated perfused liver.

4. The intact animal. Many important aspects of chemical carcinogenesis can be studied only in intact animals, including organotropism and pharmacokinetics. Whole-animal experiments obviously have advantages over any perfused organ or in vitro assay, because the animal is a combination of all organs and tissues interconnected by blood and lymphatic vessels, with the architectural arrangement of each cell intact in each tissue, and with the interrelationship of all subcellular organelles intact in each cell. Nevertheless, this complexity makes it difficult to study the importance of, for example, organ-specific metabolic factors in the carcinogenicity of chemicals. There is some evidence that several indirect-acting carcinogens, including many hepatocarcinogens, bind more extensively to DNA of their target tissues, probably because of the toxification-detoxication balance is favorable for formation and binding of reactive intermediates [see Lutz 275]. However, evidence is still very fragmentary.

In intact animals there is one distinct factor that has been not usually taken into account and which may be important in the whole-body metabolic fate of carcinogens—namely, microbial metabolism in the gut. Bacteria possess certain drug-metabolizing capacities (171, 438), and laboratory animals as well as the human possess numerous types of bacteria principally in the colon. Bacterial cells, in fact, comprise approximately half of all human fecal solids (432). "Fecalase," a preparation of human feces, recently has been proposed as a model for studying the toxification of dietary glycosides to mutagens by intestinal microflora (438). The formation of glucuronides and glycosides (conjugation reactions of table 1) does not ensure detoxication and excretion of these innocuous products. Glucuronidases (especially high in concentration in the kidney and urinary bladder) and glycosidases (especially from bacteria in the colon) therefore provide mechanisms for producing reactive intermediates from many conjugated products. If these intermediates are extremely short-lived, mutagenesis, carcinogenesis, or toxicity would occur in the kidney, bladder, or colon epithelium; if these intermediates are less short-lived (having half-lives on the order of seconds), it is possible that even distant organs would be targets for such carcinogens. Such a complicated arrangement certainly cannot be taken into account if one studies the perfused organ, cells in culture, or any in vitro experimental system.

D. Intracellular Localization of Benzo[a]pyrene Toxification

The enzymes listed in table 1 predominate (i.e. more than 95%) in the cytoplasm—endoplasmic reticulum (microsomes) and soluble (cytosolic) fractions. Mitochondrial enzymes of these types generally appear to be much less than 5%, and any enzymes of the nuclear envelope appear to contribute less than 1% of the total cellular metabolic activity of any one of these enzymes. Recent excitement has occurred with regard to "nuclear membrane cytochrome P-450" and the possibility of other similar enzymes capable of forming reactive mutagens and carcinogens. The attractiveness of this hypothesis is obvious: if a short-lived intermediate is formed in the proximity of nuclear DNA, would this not be far more important than reactive intermediate formation occurring a much greater distance away from DNA on the other side of the nuclear membrane in the cytoplasm? On the other hand, recent studies (7, 21) have raised the possibility that mitochondrial DNA might be the target for reactive metabolites of polycyclic hydrocarbons.

Evidence has been presented that the monooxygenase activities capable of toxification of benzo[a]pyrene are located in the nuclear envelope and nuclei (118, 221, 228, 391, 417) and that these activities are inducible by polycyclic aromatic hydrocarbon inducers (5, 54, 205, 228,

365–367, 391, 393, 394, 472). Other polycyclic hydrocarbons such as 7,12-dimethylbenzo[a]anthracene and its derivatives are also apparently metabolized by the nuclear envelope monooxygenase system (79). The presence of cytochrome P-448 in the nuclei of rat liver cells has been suggested by immunochemical (450) and spectrophotometric (390) studies. It has been demonstrated that incubation of isolated nuclei with benzo[a]pyrene results in covalent binding of benzo[a]pyrene metabolites to DNA, RNA, histones, and nonhistone proteins (4, 6, 55, 205, 365–367, 393, 394, 463). The formation of benzo[a] pyrene 7,8-diol-9,10-epoxides by isolated nuclei has been demonstrated (54, 368). Experiments devised to elucidate the relative contributions of nuclear and microsomal enzyme activities to DNA binding of benzo[a]pyrene, however, have yielded equivocal results. Both increases (4, 6, 205, 365, 366) and decreases (394, 463, 471) in benzo[a]pyrene binding to DNA have been reported when microsomes are added to incubation systems containing nuclei or when nuclear and microsomal enzyme activities have been induced differentially. Although the nuclear toxification hypothesis remains attractive, almost all studies have relied upon light microscopy to determine the "degree of purity" of nuclear membrane. Miniscule contamination of nuclear fraction with microsomal fragments (more than 100-fold more metabolically active) would cause artifactual data; a recently published study (216) supports this possibility that most "nuclear membrane drug-metabolizing enzyme activity," in fact, reflects microsomal contamination of the isolated nuclear membrane fraction. Yet, most authors contend that the nuclear membranes have been clearly shown to possess drug-metabolizing activities.

Another question of potential importance is the generally held belief that metabolites more polar than the parent compounds do not so easily enter cells or pass through biological membranes. On the contrary, oxygenated benzo[a]pyrene intermediates have been shown to pass readily through membranes. Radiolabeled benzo[a] pyrene 7,8-dihydrodiol is taken up by cells in culture more rapidly than benzo[a]pyrene and also is metabolized more rapidly to more polar, excretable products (215). The 7,8-diol-9,10-epoxide injected intraperitoneally into newborn Swiss-Webster mice is at least 150 times more effective than benzo[a]pyrene in causing pulmonary and lymphatic tumors (64).

Lastly, there is the question as to the significance of the half-life of reactive intermediates in terms of intracellular location of toxification. Benzo[a]pyrene 7,8-diol-9,10-epoxide, for example, is considered to be highly reactive and extremely short-lived. Yet, this chemical given intraperitoneally to newborn mice causes tumors in distant tissues and organs (64). Short-lived intermediates apparently can be stabilized by proteins and lipids in solution and need not be as "short-lived" as anticipated. The half-life of the benzo[a]pyrene *trans*-diol-epoxide is 3 minutes (256) which, in terms of animal studies, is sufficient to reach any cell in the body. It remains to be proven, therefore, whether nuclear P-450 is any more important than cytoplasmic P-450 in forming this highly reactive potent carcinogen. Still, it needs to be emphasized that the possible role of nuclear drug-metabolizing capability in carcinogenesis should be further examined.

E. Cellular Defense Mechanisms

Excess amounts of numerous cellular nucleophiles may be important in "scavenging" reactive intermediates before the intermediates reach critical targets. Liver injury by bromobenzene or acetaminophen, for example, does not become manifest until the liver concentration of reduced glutathione has decreased to less than 20% of its original concentration; only after this degree of glutathione depletion will massive covalent binding of the agent occur (207, 208). Similar nucleophiles also might be of importance for carcinogenic intermediates (155, 156).

The covalent binding of reactive intermediates to macromolecules need not necessarily lead to harmful effects, because cells have means to dispose of, or to repair, damaged macromolecules (159, 389). Among the strongest evidence in favor of the somatic mutation hypothesis in chemical carcinogenesis is the increased risk for neoplasms in patients having hereditary diseases characterized by defects in DNA repair mechanisms. Cell cultures from patients with xeroderma pigmentosum or ataxia telangiectasia have greatly decreased capacity to repair DNA damage caused by certain agents (409). Repair of DNA damage induced by the reactive metabolites of polycyclic aromatic hydrocarbons can occur in both human (75, 121, 333, 414) and rodent cells (61, 75, 110, 413). Further, some studies have shown that cellular DNA repair mechanisms may remove certain metabolite adducts very effectively but others very poorly. For example, the benzo[a]pyrene 4,5-oxide adduct is removed from mouse skin DNA more rapidly than the diol-epoxide adduct (121, 122, 359). In cultured human fibroblasts, DNA strand breaks induced by 9-hydroxybenzo[a]pyrene are repaired rapidly, whereas those induced by the 7,8-dihydrodiol are repaired at a much slower rate (333). Dipple and Hayes (107) demonstrated that mouse embryo cells efficiently excise DNA damage introduced by 7-bromomethylbenzo[a]anthracene and 3-methylcholanthrene, but are inefficent in excising damage introduced by 7,12-dimethylbenzo[a]anthracene.

F. Pharmacokinetics of Procarcinogens and Carcinogens

Circumstantial evidence exists about the importance of pharmacokinetics in the action of some carcinogens. For example, it is believed that the carcinogen, 3,3-dimethyl-1-phenyltriazene is N-demethylated in the liver and the carcinogenic metabolite, 3-methyl-1-phenyltriazene is transported to target tissues, principally the brain (30). Perhaps the most convincing evidence, how-

ever, comes from studies of carcinogenic polycyclic aromatic compounds in inbred strains of mice.

The murine *Ah* locus controls the induction of many drug-metabolizing enzyme activities by numerous polycyclic aromatic compounds (241, 324). A cytosolic receptor highly specific for these inducers has been shown to be essential for the induction process (74, 338, 375). "*Ah*-responsive" mice (Ah^b/Ah^b) possess high levels of this receptor and therefore have drug-metabolizing enzymes easily inducible by these polycyclic aromatic compounds. "*Ah*-nonresponsive" mice (Ah^d/Ah^d), on the other hand, lack detectable levels of the cytosolic *Ah* receptor and thus have drug-metabolizing enzymes that are much more difficult to induce with these same polycyclic compounds. The heterozygote (Ah^b/Ah^d) is *Ah*-responsive, indicating a Mendelian dominant type of inheritance.

In more than a dozen studies of various types of tumorigenesis and toxicity, a pattern has emerged with the use of these mice [reviewed by Nebert (318)]. If the chemical administered to intact mice is an inducer of P_1-450 (a form of P-450 controlled by the *Ah* receptor), not only the dose and timing of the dose are important, but the route of administration and the site at which the tumor or toxicity occurs—relative to the route of administration—are critically important. Hence, polycyclic aromatic compounds applied topically, subcutaneously, or intratracheally cause more tumors or toxicity in *Ah*-responsive than in *Ah*-nonresponsive mice in tissues *at the site of exposure*: epidermal carcinoma or ulceration, subcutaneous fibrosarcomas, and various types of pulmonary tumors, respectively. These compounds administered intraperitoneally cause more hepatic necrosis and ovarian toxicity in *Ah*-responsive than in *Ah*-nonresponsive siblings; again, liver and ovary are viewed as intraperitoneal organs close to the site of the administered drug.

On the other hand, polycyclic aromatic compounds given orally or subcutaneously cause more aplastic anemia, leukemia, and lymphatic tumors in *Ah*-nonresponsive than *Ah*-responsive mice. These malignancies and toxicity are manifest in tissues *distant* from the site of the administered drug. In the example of oral benzo[a]pyrene, pharmacokinetic studies have shown (322) a 10- or 20-fold higher uptake of benzo[a]pyrene in the marrow and spleen of *Ah*-nonresponsive than *Ah*-responsive mice, thus confirming the phenomenon called "first-pass elimination" kinetics (396).

In the case of malignancy or toxicity *at* the site of exposure, therefore, the compound induces its own metabolism (particular forms of P-450)—much more in *Ah*-responsive than *Ah*-nonresponsive mice—leading to high levels of reactive intermediates that cause the local cancers or toxicity. In the example of malignancy or toxicity in tissues *distal* from the site of administration, on the other hand, the compound in cells at the site of application induces its own metabolism more in *Ah*-responsive than in *Ah*-nonresponsive mice, but the detoxication pathways also appear to be important. The concentration of nonmetabolized parent drug that reaches distal tissues, such as bone marrow and lymph nodes, is therefore much greater in nonresponsive than in responsive mice; this higher level of chemical thus causes more toxicity or tumorigenesis in *Ah*-nonresponsive than in *Ah*-responsive mice.

Recent studies (318) confirm and extend this hypothesis: marrow cells in culture from *Ah*-responsive mice are more easily killed by benzo[a]pyrene in the growth medium than cells from *Ah*-nonresponsive mice; *Ah*-nonresponsive mice that receive transplanted *Ah*-responsive marrow develop aplastic anemia when given daily doses of benzo[a]pyrene in their diet. The intestinal epithelium and perhaps the liver of the *Ah*-responsive mouse thus appear to be the ultimate barrier in preventing aplastic anemia induced by oral benzo[a]pyrene.

VI. Effects of Covalent Binding of Carcinogens on the Properties and Function of Biologically Important Macromolecules

A. Effects of General Properties of DNA

Although the molecular mechanisms of chemical carcinogenesis are presently not understood, it is generally agreed that "initiation" registers a *permanent* change in molecules and/or functions in the affected cell. This phenomenon must represent some change in the properties or functions of a biologically important macromolecule and must be causally linked with the initiation of cancer. There certainly are similarities between carcinogenesis and mutagenesis. Damage of the DNA strands leading to mutation is a permanent change and is propagated to daughter cells, thus fulfilling two important requirements of the carcinogenic process. Nevertheless, the concept of mutation as an initiating event is still an hypothesis and other possibilities continue to be examined. Histones (150), nonhistone proteins (150), RNA (143), and other cellular proteins (227) have been postulated as important targets for chemical carcinogens, and actual binding of polycyclic hydrocarbon reactive intermediates to these macromolecules has been demonstrated (400, 526, 527). Despite such findings, however, DNA has remained the center of most investigations.

Potentially useful information about changes in DNA structure and function is listed in table 8. For example, Weinstein and coworkers (254, 380) have demonstrated that modification of native thymus DNA by the benzo[a]pyrene diol-epoxide leads to a decrease in the denaturation temperature and hyperchromicity, suggesting local points of disturbances in DNA structure. They also demonstrated that the modification leads to decreased template efficiency in vitro and to shortening of RNA transcripts. It is not clear, however, whether transcription of mammalian genes with the use of *Escherichia coli* RNA polymerase is a valid assay (353). Mizusawa and Kakefuda (302) and Pulkrabek et al. (379) have demonstrated

TABLE 8

Effects of benzo[a]pyrene-7,8-diol-9,10-epoxide modifications on the structure and function of DNA and/or RNA*

Property	Effect	References
Double helix structure	Decrease in Tm	380
	Decrease in hyperchromicity	380
	Increased susceptibility to endonuclease	380
	Increased lability to strand scission	127, 128
Transcription†	Decreased template efficiency	254
	Shortening of RNA transcripts	254
	Inhibition of plasmid transcription	302, 379
Translation	Inhibition of amino acid incorporation	154
Nucleosome structures	Undisturbed reconstitution	512
Viral infectivity	Loss of infectivity	176, 379, 421
Virus production (SV40)	Inhibition	76
Reconstitution of viral particles	Partial inhibition	421

* (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene.

† It is possible that transcription with the use of *E. coli* RNA polymerase to transcribe mammalian genes is an artifact (353).

that treatment of plasmid DNA with the diol-epoxide leads to inhibition of transcription, probably because of damaged DNA. These examples demonstrate that DNA modification by the postulated ultimate carcinogenic form of benzo[a]pyrene can adversely affect the structure and function of native DNA. The extent of modification in these studies usually was relatively high, several percent; hence, it is not known how significant these in vitro findings are, when extrapolated to the in vivo situation.

Other studies (238, 239, 512) demonstrate that the benzo[a]pyrene diol-epoxide-modified DNA has the capacity to associate with histones to form nucleosome structures that are comparable to unmodified DNA. All regions of chromatin-associated DNA seem to be susceptible (at least to a small extent) to attack by activated derivatives of benzo[a]pyrene, although the diol-expoxide reacts preferentially with the DNA present in the "open" (internucleosomal or linker) regions of the chromatin (238, 239). In vivo and cell culture studies have demonstrated that the binding of polycyclic aromatic hydrocarbons occurs preferentially to either reiterated DNA sequences (415) or nuclear matrix (169); the functional significance of this binding has not yet been studied.

In the context of chromosomal modifications, the effect of polycyclic hydrocarbons on sister chromatid exchange should be mentioned. Although the molecular basis of the phenomenon itself is not understood, sister chromatid exchange and polycyclic hydrocarbon-induced initiation of tumorigenesis might be caused by a common event. It is well known that polycyclic aromatic compounds increase the frequency of sister chromatid exchange in exposed cells under various experimental conditions (134, 249, 364). The significance of this increase is unclear, because it does not seem to be directly related to chromosomal aberrations or gene mutation produced in cultured cells (87, 134) or to reactive intermediates binding to DNA (402). Whatever its biological role, the induction of sister chromatid exchange appears to be a sensitive indicator of exposure to reactive intermediates (cf. table 3).

The best we can say at this moment, therefore, is that these various perturbations—modification of DNA, inhibition of transcription, and sister chromatid exchange—observed in vitro may be directly related to in vivo carcinogenic effects initiation. It also could be that we are not investigating the right parameter or function. On the other hand, carcinogenic substances may affect many functions simultaneously in a synergistic manner, so that low concentrations and minute interactions could give rise to attenuated biological responses.

B. "Specific" Interactions of Reactive Electrophiles with DNA

Benzo[a]pyrene *trans*-7,8-diol-9,10-epoxide is believed to bind covalently predominantly to the N^2 of guanine (477) and to lie in the minor groove of DNA, though considerable amounts of covalent binding between this diol-epoxide and both adenine and cytosine also have been characterized (477). Binding to other positions of guanine also has been reported (344). This (thermodynamically) "very good" fit in the minor groove of DNA (476) has been shown to lead to RNA polymerase problems in transcription plus relatively slow recognition by the DNA repair system (254); these aberrations may be important in making this particular diol-epoxide such a potent carcinogen. Benzo[a]pyrene *cis*-7,8-diol-epoxide and benzo[a]pyrene 4,5-oxide appear *not* to fit very well in the minor DNA groove. The DNA adducts of the *cis*-7,8-diol-epoxide and 4,5-oxide are cleaned up more quickly by the DNA repair system than the DNA adduct of the *trans*-7,8-diol-9,10-epoxide (121, 359). Differences in the efficiency of DNA repair of 7,12-dimethylbenzo[a]anthracene-nucleic acid adducts also have been described (439). Other potentially important well characterized electrophiles include N-hydroxy-2-acetylaminofluorene binding to the C^8 of guanine (190), aflatoxin B_1 2,3-oxide binding to the N^7 of guanine (116), and alkylation of O^6 of guanine by reactive metabolites of nitrosamines (252). Whether these "specific binding sites" for metabolites in the DNA are related to mutagenesis or cancer, however, remains to be determined.

Carcinogenesis by certain electrophiles thus may reflect the combination of the "most detrimental" metabolic reactions and the "most effective" fit between the reactive intermediate and the DNA helix. The most effective fit denotes the best position whereby a DNA adduct most efficiently would elude the DNA-damage-detection-and-repair systems yet cause problems with DNA polymerase and/or transcription into a "normal"

mRNA precursor. (It should be noted that the possibility has *not* been ruled out that interactions between an electrophile and RNA or protein also may lead to effective carcinogenesis). Hence, it is predicted that an increasing number of chemicals will be found and characterized in these classes: those that are metabolized by one or more drug-metabolizing enzymes and fit effectively into a DNA pocket; those that are metabolized by drug-metabolizing enzymes but do not interact effectively with DNA; those that are not metabolized by drug-metabolizing enzymes yet, when produced synthetically in vitro by a chemical reaction and added to DNA, will fit effectively into a DNA pocket; and those large cumbersome molecules that fit neither into the drug-metabolizing enzyme active-site nor any DNA pocket. These types of interactions obviously will not apply to carcinogens that act via free-radical, methyl carbonium anions, and other similar types of mechanisms.

VII. Experimental Methods Used to Study Chemical Carcinogenesis

Although a vast multitude of factors always must be taken into consideration, the basic protocol of the classical carcinogenesis assay is simple. The test substance is applied once, or any number of times according to a schedule, with or without the subsequent application of a promoter onto the skin, or elsewhere, of an animal (usually mouse or rat); the appearance of tumors during a certain period of time is then noted. There essentially are three shortcomings in this assay: it takes time, it is expensive, and weak carcinogens are not very easily detected. This assay also does not imply anything as to the mechanism of chemical carcinogenesis, yet such an assay is usually the final conclusive test about the possible carcinogenicity of the compound.

With classical carcinogenicity testing, a long time is required to arrive at an answer, and, more often than not, the answer is equivocal. Quicker methods therefore have been developed. The only test that observes the production of malignant cells is the so-called malignant transformation assay (see section VIII C): cells are exposed to a carcinogen and, during subculturing, the appearance of transformed colonies of cells is quantified. Although this test is quicker than classical carcinogenicity testing, there are difficulties: the criteria—about which cells are "malignantly transformed" and which are not—are not uniformly accepted, and it is difficult to find suitable cell lines. Nevertheless, the malignant transformation assay may be helpful in the experimental study of mechanisms of initiation and promotion of chemical carcinogenesis.

Carcinogenesis is thought to be a multistep process. This fact precludes any direct linkage between the formation of reactive intermediates, the covalent binding to macromolecules, and the final outcome—uncontrolled growth. All other tests that have been suggested for the detection of carcinogenic substances, and those that usually offer the more direct link between covalent binding and the biological consequence, suffer from the same shortcoming: the tests imply a certain specific mechanism of chemical carcinogenesis, most often a somatic mutation. These short-term tests include the *Salmonella*/liver mutagenicity assay and a large number of other tests that employ bacteria, yeast, *Neurospora*, *Drosophila*, plants, or mammalian cells as targets with mutagenicity, toxicity, or chromosomal damage as end-points. Actually, these tests are not *carcinogenicity* tests at all. Many of these tests measure the possibility of the formation of biologically active products from substances to be studied. This fact should be kept in mind, because much unnecessary confusion exists regarding the difference between real carcinogenicity tests and indirect tests based on the correlative implications.

VIII. Relationships Amongst DNA-Binding, Mutagenesis, Malignant Transformation, and Carcinogenesis

A. Theoretical Considerations

In the foregoing discussion, we have listed the overwhelming evidence indicating that polycyclic hydrocarbons must usually be metabolically activated before they exert their carcinogenic actions. The next step—interacting with a critical subcellular target—is less clear, and what happens beyond that is unknown. Several competing hypotheses—genetic and epigenetic—have been proposed. Different hypotheses implicate dissimilar target molecules for reactive, labile carcinogens. The difficulty in some epigenetic hypotheses, such as that of Pitot (370) postulating a heritable change in cytoplasmic membranes, is that target molecules for carcinogens are completely hypothetical and the postulated mechanism has no experimental evidence thus far. Also, mechanisms by which carcinogens might activate latent oncogenic viral genomes (440) or might select for preneoplastic cells (378) are hypothetical and lack direct evidence.

The somatic mutation theory implicates DNA damage as an important step in neoplastic transformation, and several lines of indirect evidence are in favor of somatic mutation as a mechanism of initiation of carcinogenesis (480). There is also, however, a considerably body of evidence *not* favoring the somatic mutation hypothesis [see McCann et al 293]. The proposed correlations between the covalent binding of reactive intermediates to DNA with mutagenesis, malignant transformation, and carcinogenesis are evaluated below.

B. Reactive Intermediates and Mutagenesis

It is generally believed that, without repair, DNA lesions lead to mutagenesis. Consequently, covalent interactions of reactive intermediates with DNA may be expected to have a direct correlation with mutagenesis, except when reactive intermediates generate free radicals that cause DNA damage without covalent binding of the metabolite (see section IV D). Most compounds studied

are completely inactive without the cell's own (or exogenous) activating enzyme systems [e.g. Hollstein et al. (173)]. Studies with direct-acting chemical mutagens such as ethyl methanesulfonate and other chemical treatments have demonstrated that several types of base damage can be linked with base-pair transformations and frame-shift mutagenesis (112, 229, 383). With other compounds, the relationship between covalent binding and mutagenesis is not so clear. The major benzo[a]pyrene adduct bound to *S. typhimurium* DNA has been characterized as the bay-region diol-epoxide bound to the exocyclic amino group of guanine (399). This lesion can be effectively removed from *E. coli* DNA by an excision repair mechanism similar to that which excises pyrimidine dimers (194). However, in bacteria the diol-epoxide seems to be an indirect mutagen that acts through host-mediated functions, i.e. the SOS (error-prone) repair pathway, in the process of mutation fixation (194). Consequently, in bacteria defective in this SOS function, benzo[a]pyrene diol-epoxide is not mutagenic at all.

In eukaryotic test systems, there exists a considerable lack of information on the mutagenic effectiveness of carcinogens bound to DNA and on the mutagenicity of a specific type on DNA damage. Some data are available for mispairing with O^6-methylguanine produced by alkylating agents, but otherwise there are only a few studies correlating the extent of DNA binding with mutagenesis in the same test system (1, 285, 332, 403). Generally, the correlation between the extent of DNA binding and mutagenicity (measured by a change at a particular locus) is good. The use of benzo[a]pyrene diol-epoxide-modified templates for prokaryotic polymerases (513) suggests the possibility that benzo[a]pyrene adducts on DNA bases may have no mutagenic effect. These data point to other mechanisms such as the formation of benzo[a]pyrene phosphoester adducts (128), breaks (127), or transpositions (67, 478). In summary, although the binding of reactive intermediates to DNA can generally be associated with mutagenesis, exact mechanisms are far from being clearly understood.

C. Reactive Intermediates and Malignant Transformation

Toxification is also needed for malignant transformation (165–167, 479). Cells not possessing active toxification systems cannot be transformed, unless the active enzymes are supplied either by feeder cells or by the addition of suitable tissue fractions (173). With respect to the target macromolecule of activated intermediates, Barrett et al. (28) suggest that DNA damage might be causally linked with transformation. They demonstrated that malignant transformation can be experimentally induced in Syrian hamster cells by means of direct perturbation of DNA by bromodeoxyuridine and near-ultraviolet light, treatments known to cause DNA damage. But whether a somatic mutation is sufficient to cause malignant transformation is doubtful at this moment, because several studies (26, 27, 183, 354) have demonstrated that the frequency of malignant transformation is much higher than the frequency of mutagenesis. Data especially from the laboratory of Ts'o (26, 27) have illustrated the multistep nature of malignant transformation and the dependence of calculated frequency on the specific stage chosen. In some experimental systems the frequency of morphological transformation actually approaches 100%, a value never achieved when other criteria for transformation or mutagenesis are used as end-points (cf. 354).

D. Correlations among Mutagenesis, Malignant Transformation, and Carcinogenesis

In 1955 Burdette (65) concluded on the basis of literature survey that "a general correlation between mutagenicity and carcinogenicity cannot be proposed from present evidence." "Somatic chromogene mutation has not been excluded as a mode of origin of cancer, but the possibility that this is more than an unusual occurrence or that it adequately explains more than specific cases of carcinogenesis remains in doubt." Boyland (49) stated that "there are no short-term tests that could give a valid indication as to whether a substance is carcinogenic or not." At present, the correlation seems quite good and short-term tests usually appear valid, at least within most classes of organic chemical carcinogens. Venitt (464) concluded that bacterial short-term tests "will continue to prove their worth in a variety of investigations requiring . . . undoubted relevance to carcinogenesis." Ames (8) stated flatly that "a very high percentage of carcinogens tested are mutagens, and most mutagens appear to be carcinogens," and consequently "bacterial mutagenicity tests can be used as specific and sensitive prescreening methods to detect potential carcinogens."

A reversal of this opinion has been based on the awareness of the necessity of toxification of procarcinogens and promutagens. The most widely used short-term test, the *Salmonella*/liver mutagenicity test, has demonstrated that a high percentage of organic chemical carcinogens tested thus far are mutagens and that alleged noncarcinogens are usually without mutagenic potency (292, 293, 309, 310, 382, 418, 435; see articles in De Serres et al. (102) and Montesano (304). Also, the correlation among malignant transformation tests and cytogenetic tests and carcinogenicity of chemicals is relatively good (173). These excellent correlations sometimes have been taken as conclusive proof for the somatic mutation hypothesis of chemical carcinogenesis, although direct evidence in favor of this hypothesis is still absent [see e.g. Rubin (397)].

Some recent surveys have yielded somewhat weaker correlations between mutagenicity and carcinogenicity. The thorough review of Rinkus and Legator (387) pointed out that certain classes of carcinogens (such as polycyclic aromatic hydrocarbons) can be detected in the *Salmonella*/liver system with high reliability, whereas

many other classes are poorly detected. Upon closer investigation of the failures, Rinkus and Legator concluded that false negatives appear to arise for two reasons: an inability to devise an in vitro toxification system that can be reliably used in a standard way; and an inability to detect the entire spectrum of mutational events. It is interesting that there are almost no false-negatives among polycyclic aromatic hydrocarbons.

The problem of false-positives is already apparent from a closer look at table 5. Many benzo[a]pyrene metabolites are clearly mutagenic, but only few appear to be carcinogenic. Enzymatic differences, as well as other reasons for this discrepancy, have been discussed extensively in previous sections of this review. In this context, it is interesting to note the results of three recent studies on the mutagenicity of carcinogenic and noncarcinogenic polycyclic aromatic and heteropolycyclic hydrocarbons (12, 93, 141). Although over 90% of carcinogens were found to be mutagenic, over 50% of alleged noncarcinogens were equally mutagenic, and in one of the studies (93) there was no quantitative relationship between mutagenic and carcinogenic potencies. The same difficulty in quantitative comparisons is apparent from data in table 9. The extent of reaction with DNA in vitro and mutation frequency in bacteria do not show any correlation with carcinogenicity, whereas DNA binding in mouse skin and mutagenicity in living cells *do* show a reasonably good correlation. It is interesting that Bartsch et al. (29) in the *Salmonella*/liver test *did* find close positive associations between the mutagenicities of the dihydrodiols that could yield bay-region diol-epoxides and the extent of reaction with mouse skin DNA and the carcinogenic potencies of the parent hydrocarbons. This study clearly points out the importance of trying to elucidate the proximate carcinogen, because with in vitro systems the many other reactive intermediates also being produced may disturb in vitro-in vivo correlations.

It is generally thought that most problems in short-term tests—especially when using prokaryotes—arise from the artificiality of the toxification. Various laboratories recently have attempted to improve upon this shortcoming by using more "natural" toxification systems such as living cells (139, 184, 185, 209, 246–248, 303, 371). Whether these modifications will help in improving the correlation between mutagenesis and carcinogenesis remains to be seen.

E. DNA Binding and Biological Responses: Specific Examples

1. DNA binding and skin carcinogenesis. Phillips et al. (369) recently studied the extent of reaction with DNA of seven tritium-labeled polycyclic hydrocarbons topically applied on mouse skin. The level of binding of hydrocarbon to DNA was determined from the amounts of radioactivity eluted from Sephadex LH20 columns. The seven hydrocarbons possessed activities as tumor initiators or complete carcinogens in mouse skin. The correlation of binding with either carcinogenicity or tumor-initiating activity was not perfect: similar binding of 7-methylbenzo[a]anthracene, 3-methylcholanthrene, and benzo[a]pyrene to DNA was detected, despite their different biological activities. However, a very interesting finding was that only one hydrocarbon-nucleoside product peak was obtained in the Sephadex LH20 elution profile of benzo[a]pyrene-modified DNA hydrolysates, whereas three peaks were obtained with hydrolysates of either 3-methylcholanthrene- or 7-methylbenzo[a]anthracene-modified DNA. It is possible that these individual adducts play unequal roles in expressing the biological effects that follow treatment with the parent compound.

2. DNA binding and mutagenesis. Newbold et al. (332) and Wigley et al. (486) studied five polycyclic hydrocarbons with respect to cell-mediated mutagenesis and DNA binding in cultured Chinese hamster cells. The DNA binding was analyzed by Sephadex LH20 column chromatography in order to determine the nature and true extent of reaction of the metabolites with DNA. Values for true binding reflected differences in carcinogenic potency between the compounds. Induced mutation frequencies were also related to the extent of true DNA

TABLE 9

DNA binding and biological activities of certain selected polycyclic aromatic hydrocarbons

Hydrocarbon	Extent of Reaction with DNA		Mutation Frequency		Tumor initiation on Mouse Skin ‖	Carcinogenicity¶ on Mouse Skin
	In Vitro*	In Mouse Skin†	Bacteria‡	Cells§		
7,2-Dimethylbenzo[a]anthracene	23	69	820	281	819	151
3-Methylcholanthrene	10.7	21	180	366	102	80
Benzo[a]pyrene	12	16	540	425	25	75
Dibenzo[a,h]anthracene	6.7	13	1240	17	22	26
Dibenzo[a,c]anthracene	21	2.5	1250	22	0.8	0
Benzo[a]anthracene	10.8		830	9	0.9	0

* Levitt et al. (267); the activating system was fresh microsomes from 3-methylcholanthrene-pretreated C57BL/6N mice; pmol hydrocarbon bound/mg DNA.

† Brookes and Lawley (60); μmol of hydrocarbon bound/mol of DNA phosphorus.

‡ Levitt et al. (267); revertants/plate with the best strain and concentration.

§ Huberman and Sachs (185); mutation frequency in V79 cells to 8-azaguanine resistance/10^5 survivors, concentration of hydrocarbons 1.0 μg/ml, except with DMBA, 0.1 μg/ml.

‖ Scribner, as quoted by Phillips et al. (369); tumors/μmol.

¶ Iball (189); the percent of tumor incidence divided by the average latency, × 100.

binding for each compound. At equivalent extents of DNA reaction with hydrocarbon products, levels of induced mutation were not significantly different. This finding is very interesting, because it suggests that biological consequences depend on the extent of true binding, not on the nature of the specific metabolite-nucleoside adduct. However, it may be that the diol-epoxide-nucleoside adducts of all hydrocarbons investigated are so similar in structure that their effects on DNA structure, function and repair also are very similar.

In the studies described above, several important points should be emphasized. It is clear that the measurement of "total" covalent binding of hydrocarbons to DNA is not enough and may lead to erroneous conclusions. The ratio of specific, "true" binding (represented by metabolite-nucleoside adducts) to apparent total binding—which usually includes contaminants and tritium incorporation into unmodified nucleosides—is not necessarily constant, either with a single compound in different experimental situations or with several hydrocarbons in the same experimental setting. It is worth noting that use of "true" binding instead of "total" binding yields better correlations among DNA binding, mutagenesis and carcinogenesis, and gives further credence to claims that DNA is the critical target for carcinogens.

3. Future trends. It is interesting that so many studies correlating biological response with macromolecular binding have involved polycyclic aromatic hydrocarbons—one small class relative to all carcinogens! One reason could be that historically, since Percival Pott's observation of scrotal cancer among chimney sweeps in 1775, polycyclic aromatic compounds have been popularized by several key investigators in the whole field of chemical carcinogen research. Another reason might be that the best correlative data have been obtained with polycyclic aromatic compounds; this has been true since the classical study of Brookes and Lawley (60). This success no doubt reflects the predominance and/or persistence of polycyclic hydrocarbon-induced form(s) of P-450 among extrahepatic tissues and cells in culture (317). We suspect that correlations between biological response and macromolecular binding within other classes of carcinogens are not as good as those with polycyclic aromatic hydrocarbons.

Recent studies demonstrate that we should analyze specific carcinogen-nucleic acid interactions in order to get more meaningful information. However, is the binding of carcinogens to DNA causally linked with tumorigenesis? After all, correlations do not constitute a direct proof. Insertion of naked DNA without any contaminating RNA or proteins, or a cloned gene for "transformation," into a normal cell, and then observing this cell become transformed in culture and tumorigenic in animals, would constitute *direct* proof that DNA alone is causally linked with cancer. Several laboratories presently are, in fact, very near this goal.

Although it is not yet clear what "signal" leading to carcinogenesis is evoked by the ultimate carcinogen metabolite, transmission of the signal clearly appears to involve DNA rather than RNA or protein (412). Transfection of DNA from certain chemically transformed cell lines into NIH 3T3 mouse cells can induce the appearance of transformation foci (411). NIH 3T3 cells that have been transformed by transfected DNA from human, rabbit, and mouse tumors and tumor cell lines have been shown in certain cases to be tumorigenic in mice and to contain human DNA sequences when the original transfected DNA had been human in origin (243, 410).

If such studies as these prove that DNA, and DNA alone, is the critical target for initiation of carcinogenesis, what is the next step? We feel that the next step in this field will be the union of pharmacologists, geneticists, virologists, and immunologists. There must be some commonality among polycyclic hydrocarbon adducts which damage DNA, regulatory genes that control growth factors, viral oncogenes, and the immune system. The recent findings of tumor viruses regulating eukaryotic gene expression (179), eukaryotic genetic material controlling viral expression (188, 357, 462), and similar DNA rearrangements implicated in the etiology of both murine leukemia and Burkitt's lymphoma (236) we believe are examples of the types of exciting research that will bring all these research fields together.

IX. Conclusions

Polycyclic aromatic hydrocarbons are metabolized by way of numerous pathways catalyzed by enzymes principally in the endoplasmic reticulum, but also in other parts of the cell. Functionalization reactions are especially important in the toxification of polycyclic hydrocarbon carcinogens into ultimate carcinogenic forms, although conjugative enzymes sometimes participate in certain toxification reactions. Numerous factors, genetic as well as environmental, affect the activity and the balance of different enzymes participating in the toxification and detoxication of carcinogens.

Methods have been developed in which biological consequences of DNA damage caused by reactive metabolites can be detected (for example, mutagenesis in prokaryocytes or eukaryocytes) or in which specific metabolite-DNA adducts can be identified and quantified. These methods have made it possible to elucidate probable ultimate carcinogenic forms and to study cellular factors affecting carcinogen-DNA interactions. Numerous reactive metabolites of, for example, benzo[a]pyrene generated by microsomes are capable of binding to exogenous DNA in vitro, but with intact cells, isolated perfused organs, or in the intact animal, most of these reactive forms are detoxified or otherwise dissipated before reaching and interacting with nuclear DNA. The principal binding species in vivo appears to be the bay-region diol-epoxide for most polycyclic aromatic hydrocarbons.

Binding of carcinogens to DNA affects the structure

and function of DNA causing local aberrations and impaired transcription. The in vivo significance of these findings, however, is unclear.

Reasons why carcinogens act on specific target tissues characteristic to each carcinogen are incompletely understood. Differences in enzyme profiles among tissues no doubt contribute to the target tissue variability. The actual intracellular location where toxification occurs is not known. Conjugated metabolites of carcinogens may be reactivated spontaneously or by enzymic hydrolysis; this fact raises the possibility of transport of metabolites to distant target tissues.

Although the necessity of toxification of polycyclic hydrocarbon carcinogens is well established, the critical target(s) for their action is(are) not known. A large proportion of recent investigations have been based on the assumption that carcinogens act through somatic mutation, and consequently carcinogen-nucleic acid interactions have geen the principal focus. Because chemical carcinogenesis is a multistep process, the direct correlation between DNA binding and the endstage—uncontrolled growth of a cell type—is difficult to study. Excellent correlations among mutagenesis, malignant transformation, and carcinogenesis may reflect the fact that the production of reactive intermediates is required for all these phenomena. Recent studies measuring "true" instead of "total" binding of polycyclic hydrocarbon metabolites to DNA have yielded better correlations between mutagenesis and carcinogenesis. Nevertheless, much more must be known about the oncogenic process before one is able to decide whether DNA alone is the critical target for carcinogens and whether somatic mutation is always the initial step in the oncogenic process.

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