THE *Ah* LOCUS: GENETIC REGULATION OF THE METABOLISM OF CARCINOGENS, DRUGS, AND OTHER ENVIRONMENTAL CHEMICALS BY CYTOCHROME P-450-MEDIATED MONOOXYGENASES

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THE CYTOCHROME P-450-MEDIATED MONOOXYGENASE SYSTEMS

Probable Mechanism of Action and Reactions Catalyzed by Monooxygenases

Numerous xenobiotics, including many chemical carcinogens, are so hydrophobic that they would remain in the body indefinitely were it not for Phase I and Phase II drug-metabolizing enzymes.[1] During Phase I metabolism, one or more polar groups (such as hydroxyl) are introduced into the parent molecule, thereby presenting the Phase II conjugating enzymes (e.g., UDP glucuronosyltransferase) with a substrate. The conjugates are sufficiently polar to be excreted from the cell and from the body.

The cytochrome P-450-mediated monooxygenases are a collective example of Phase I enzymes. These membrane-bound multicomponent enzyme systems, requiring molecular oxygen and NADPH,[2] are known to metabolize: polycyclic hydrocarbons, such as BP* (ubiquitous in city smog, cigarette smoke, and charcoal-cooked foods), MC, and biphenyl; halogenated hydrocarbons, such as polychlorinated and polybrominated biphenyls, insecticides, and ingredients in soaps and deodorants; strong mutagens, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and nitrosamines; aminoazo dyes and diazo compounds; *N*-acetylarylamines and nitrofurans; numerous aromatic amines, such as those found in hair dyes, nitro aromatics, and heterocyclics; wood terpenes; epoxides; carbamates; alkyl halides; safrole derivatives; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; most drugs; ethanol; both naturally occurring and synthetic steroids; and other endogenous substrates, such as biogenic amines, indoles, thyroxine, and fatty acids.[3-6]

The isolation of highly purified liver microsomal P-450 from phenobarbital- and β-

* Abbreviations used: BP, benzo[*a*]pyrene; MC, 3-methylcholanthrene; AHH, aryl hydrocarbon (BP) hydroxylase (EC 1.14.14.2); B6, the inbred C57BL/6 mouse strain; D2, the inbred DBA/2 mouse strain; C3, the inbred C3H/fCum mouse strain; GSH, reduced glutathione; 2-AAF, 2-acetylaminofluorene.

naphthoflavone-treated rabbits,[7,8] from MC-treated rats and rabbits and phenobarbital-treated rats,[9] from phenobarbital-treated rabbits,[10,11] and from phenobarbital-treated and control rabbits and control, phenobarbital-, and MC-treated rats[12] has been reported by numerous laboratories. These very highly purified samples have led to recent studies which greatly enhance our knowledge about the chemical, catalytic, and electrophoretic properties of this group of enzymes.

A scheme to account for the mechanism of substrate oxygenation catalyzed by P-450 is probably best illustrated in Figure 1. The binding of substrate to the oxidized cytochrome is necessary before rapid electron transfer can occur. The uptake of two electrons is indicated, with formation of the ferrous hemoprotein and a second electron bound elsewhere or with formation of a hemoprotein having both electrons accommodated within the P-450 iron's *d* orbitals. Oxygen then combines with the protein and undergoes a two-electron reduction, perhaps to yield O_2^{2-} and perhaps with the intermediate formation of $\cdot O_2^-$ superoxide.[13] The fully reduced oxygen is then pictured as undergoing protonation to form HO_2^-, followed by elimination of water and formation of an Fe-O complex having an overall charge of 3+ and a formal oxidation state of 5+. Insertion of the oxygen atom into a favorably positioned C-H bond of the bound substrate, R-H, then yields the hydroxylated product R-OH, with regeneration of oxidized P-450. The activated oxygen may be an oxenoid species or other resonance structures involving higher oxidation states of the iron, or the porphyrin may contribute to stabilize the activated oxygen.[8,14]

This scheme (Figure 1) also illustrates the manner by which hydrogen peroxide may support substrate hydroxylation in the absence of O_2 and an external donor. Postulating HO_2^- as an intermediate explains the observation that the hydrogen peroxide-de-

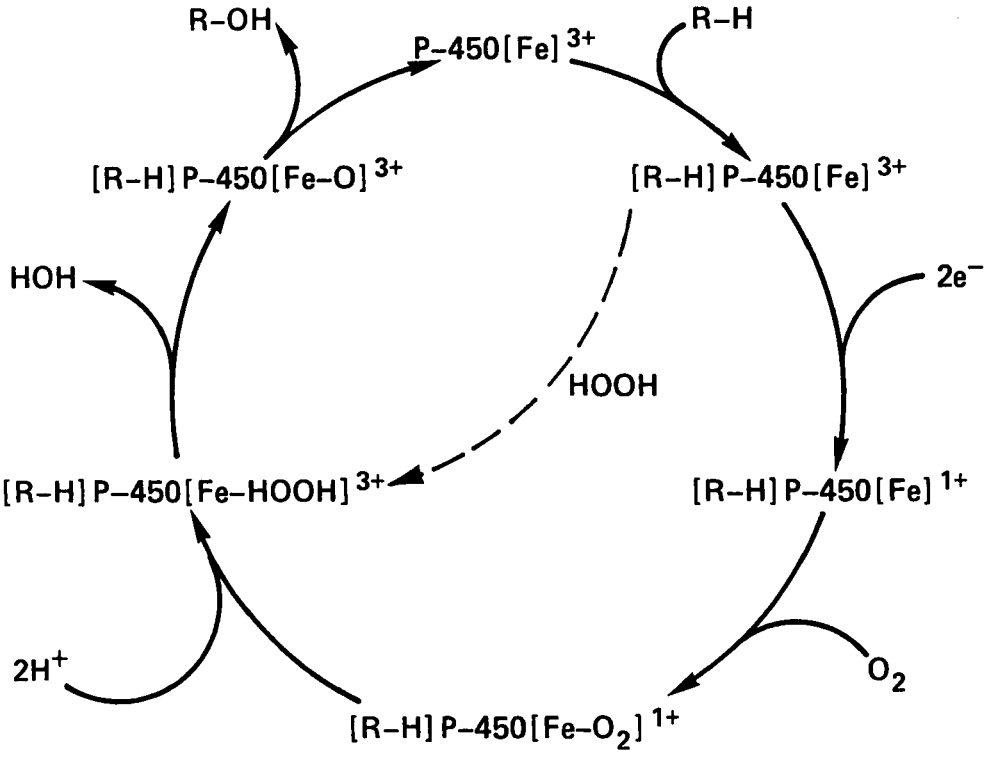


FIGURE 1. Proposed scheme for the mechanism of catalysis by various forms of cytochrome P-450. R-H denotes the substrate; R-OH denotes the oxygenated product.

pendent hydroxylation increases in rate with increasing pH. This proposed scheme shares common features with the mechanism of action of peroxidases.[15] Peroxides may react with P-450 to produce the same species of activated oxygen as that generated by the aerobic pathway; this hypothesis could be tested by seeing if the same substrate specificity for a form of P-450 exists in the presence of molecular oxygen and NADPH as exists in the absence of O_2 and an external electron donor. To our knowledge these experiments, which should be carried out separately with several forms of highly purified P-450, have not been yet performed.

P-450 can catalyze at least nine distinct chemical reactions involving the insertion of activated oxygen into a favorably positioned C-H, N-H, S-H, or C-X (X = halogen) bond (Figure 2). Growing evidence[12] indicates that the numerous forms of P-450 are able to perform these various oxygenations with greatly varying degrees of efficiency, presumably correlated with the specificity of the enzyme active-site of each form of P-450. Cytochrome P-450 is also known to catalyze the reduction of several substrates, e.g., tertiary amine *N*-oxides[16] and BP 4,5-oxide.[17,18]

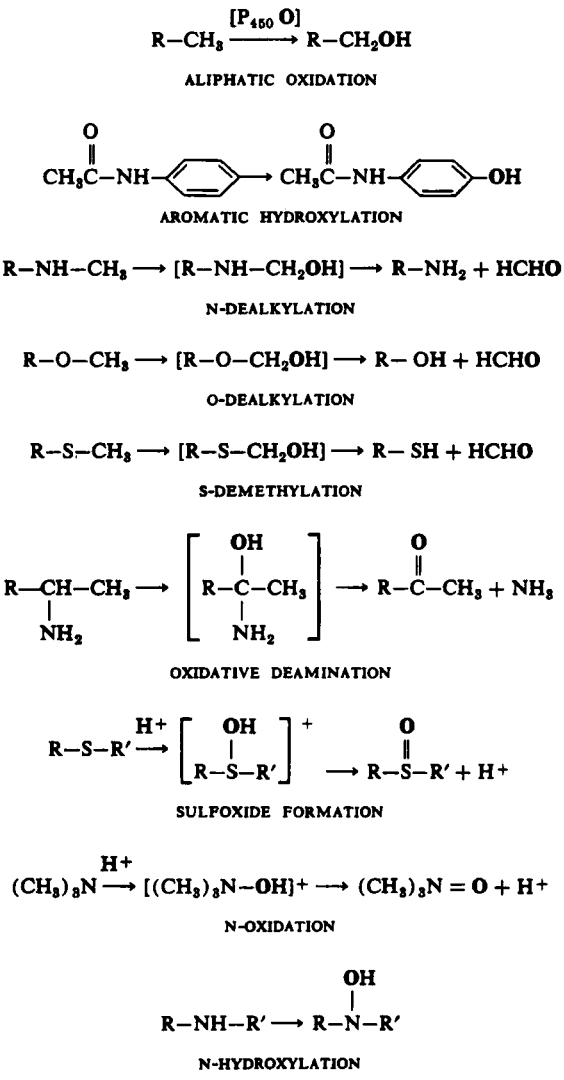


FIGURE 2. Examples of typical types of reactions catalyzed by cytochrome P-450-mediated monooxygenases.

Location of Monooxygenases in the Cell

It has been suggested that monooxygenase activity and therefore presumably P-450 exists in the nuclear membrane[19] in addition to the endoplasmic reticulum. Several studies[20] show no important difference between microsomal and nuclear monooxygenase activity with respect to metabolism, induction, or inhibition of metabolism by specific chemicals. The possibility remains that nuclear membranes are contaminated with minute amounts of the microsomal electron transport chain during the separation and purification procedures. Microscopic analysis is not good enough to detect these levels of contamination. An exhaustive study with several biochemical markers specific for one or another membrane of the cell is indicated to prove beyond the shadow of a doubt that more monooxygenase activity exists in nuclear membranes than can be accounted for by any microsomal membrane contamination. The subcellular location of the drug-metabolizing enzyme systems may be important in the etiology of tumorigenesis or drug toxicity if reactive intermediates are extremely short-lived and if, for example, DNA in the nucleus is the critical subcellular target for initiation of cancer or toxicity.

Evidence for Multiple Forms of P-450

Various substrates thus combine with molecular oxygen and the enzyme active-site of one of the various forms of cytochrome P-450 to yield a trimolecular complex (Figure 3); one atom of oxygen is incorporated into the substrate, the other into cellular water. The reactive intermediate is called an epoxide if the bond involves an alkyl portion of the substrate and an arene oxide if the bond involves an aryl portion of the substrate. Whereas many phenolic products had been believed to form via direct oxygen insertion, many have been recently demonstrated (by means of newer more sophisticated chemical techniques) to occur by way of arene oxide formation.[21] Nevertheless, the possibility certainly remains[22] that a direct oxygen insertion occurs in some circumstances. The amount of epoxide that is not converted to the various oxygenated and

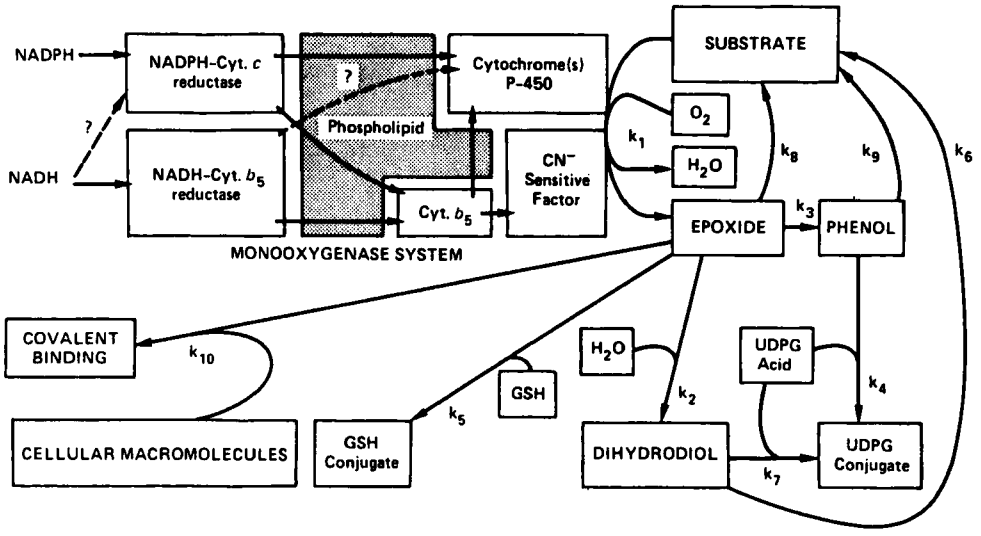


FIGURE 3. Scheme for the membrane-bound multicomponent monooxygenase system(s) and the various possibly important pathways for hydrophobic substrates.[3-5,23] For any given substrate, the relative rates of k_1 through k_{10} are currently not known and most likely differ among different tissues, strains, and species; age, nutritional, hormonal, diurnal variations, pH, and saturating vs. nonsaturing conditions all possibly may be important factors in affecting these various rates. (Modified from Nebert, D. W., Robinson, J. R., Niwa, A., Kumaki, K., and Poland, A. P., *J. Cell Physiol.*, 83, 393, 1975. With permission.)

conjugated innocuous products is free to bind covalently with cellular nucleic acids and proteins (presumably in a random fashion), and this reaction with cellular macromolecules may be important in the mechanism of genetic differences in chemical carcinogenesis,[6] drug toxicity,[6,24,25] and mutagenesis.[26,27] The reactive intermediate may bind in a manner more "specific" than random covalent binding: for example, the binding of BP 7,8-diol-9,10-epoxide to the ^2N-amino of guanine.[28]

It has become increasingly apparent that different forms of P-450 may generate different ratios of metabolites from the same substrate. Comparing MC vs. phenobarbital as the inducer in rat liver, for example (Figure 4), various groups have shown that hydroxylations may occur predominantly in different chemical positions on the molecule for such substrates as biphenyl,[34] testosterone,[35] 2-acetylaminofluorene,[36,37] bromobenzene,[38] *n*-hexane,[39] and BP.[40-42] Such differences in the metabolite profile of a polycyclic hydrocarbon or other foreign chemical reflect presumed differences in the enzyme active-sites of the various forms of P-450 and in the nature of the intermediates formed; differences in the reactivity of these intermediates might result in marked dissimilarities in the toxicity or carcinogenicity of a given compound.

THE *Ah* LOCUS IN THE MOUSE

The AHH Assay

The assay for AHH activity[43-45] — with the use of BP as the substrate and "3-hydroxybenzo[*a*]pyrene" as the product formed — has remained for years a reliable index of BP metabolism, although it had been known[46] that numerous oxygen-containing metabolites other than the 3-hydroxy derivative are formed. It was believed until very recently that the formation of 3-hydroxybenzo[*a*]pyrene may occur either by a direct hydroxylation or in a two-step process (reactions k_1 and k_3 of Figure 3).[3] It now appears that 3-hydroxybenzo[*a*]pyrene arises from a very reactive 2,3-oxide.[21] An additional point of interest is that an impure preparation of "recrystallized 3-hydroxybenzo[*a*]pyrene" may actually contain several different phenolic metabolites of BP, all of which are activated and exhibit fluorescence at similar wavelengths. It was recently reported[48] that 9-hydroxybenzo[*a*]pyrene is similar to 3-hydroxybenzo[*a*]pyrene in its degree of fluorescence at similar wavelengths of activation and emission.

Therefore, the previously described AHH assay[43-45] probably takes into account the formation of several phenolic products (the 1-, 3-, 6-, 7-, and 9-hydroxy derivatives[47]). Diols and quinones do not contribute significantly to fluorescence in this assay.[48] What other reactions might accurately reflect the metabolism of BP? A recently described[49] radiometric assay involves the quantitation of tritium removed from generally labeled [^{3}H]BP to water.

When we compared quantitatively the fluorescent AHH assay[44] with the radiometric assay,[49] however, more "BP metabolized" was found in the former than in the latter.[23] An apparent discrepancy exists. The "total" tritium exchange should include all quinone and other nonphenolic derivatives and therefore should theoretically reach a higher value than the method in which only the phenolic products are measured. This apparent discrepancy can be explained by the fact that generally labeled [^{3}H]BP is used. As shown in Figure 5, a BP molecule not having tritium on both positions involved in the epoxidative process will not be included in the assay[49] for "total" tritium exchange. When the oxygen becomes more strongly associated with carbon 7 than carbon 8 of BP, the hydrogen atom at position 7 by means of the "NIH shift"[50] will migrate to position 8. Because the carbon-tritium bond is thermodynamically stronger than the carbon-hydrogen bond, the hydrogen atom rather than the tritium atom will leave to become incorporated into water. Thus, the only metabolites measured by the

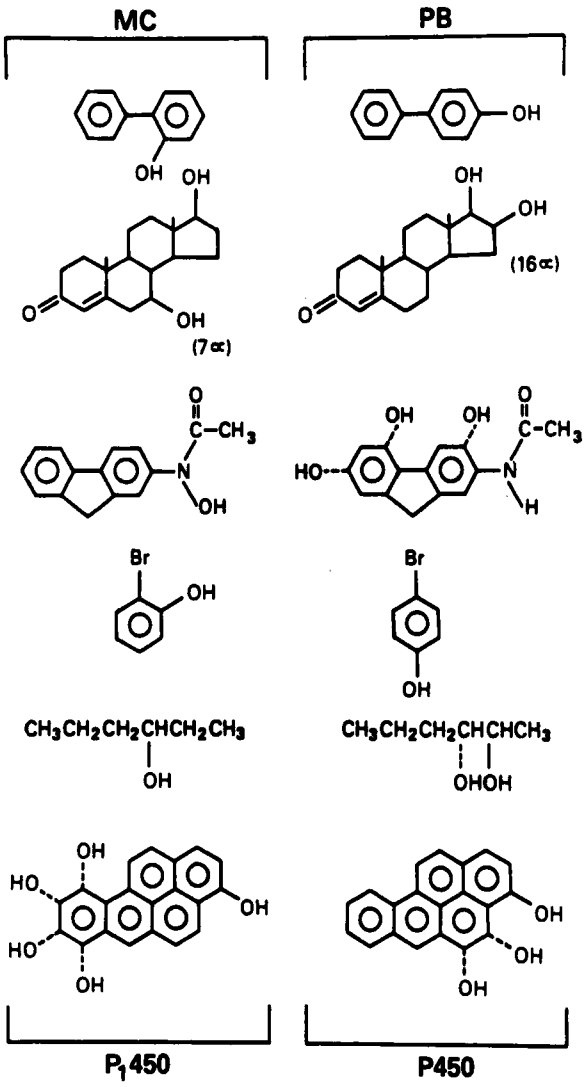


FIGURE 4. Chemical structures of known differences in metabolite formation when each of these six substrates is oxygeneated in vitro with liver microsomes from MC- or phenobarbital (PB)-treated rats.[6] These products are not formed exclusively by one or another form of cytochrome, but rather there is overlapping of substrate specificity, i.e., an increased appearance of these products when MC treatment is compared with phenobarbital treatment or when microsomes from these rats are compared with microsomes from control rats. Similar differences in metabolite profile exist in mice for biphenyl,[29] 2-AAF,[30] and BP,[31] but not fr testosterone[32] or bromobenzene.[33] To our knowledge, *n*-hexane metabolites have not been examined in MC- and PB-treated mice. (From Thorgeirsson, S. S. and Nebert, D. W., *Adv. Cancer Res.*, 25, 149, 1977. With permission.)

"total" tritium exchange assay[49] are those BP molecules having tritium on the two adjacent carbons at which the oxygen attack occurs or those phenols which are formed directly at a particular carbon-tritium bond by an oxygen "insertion" reaction, if this ever happens.[3] In fact, the rate of tritium exchange relative to that of phenolic product

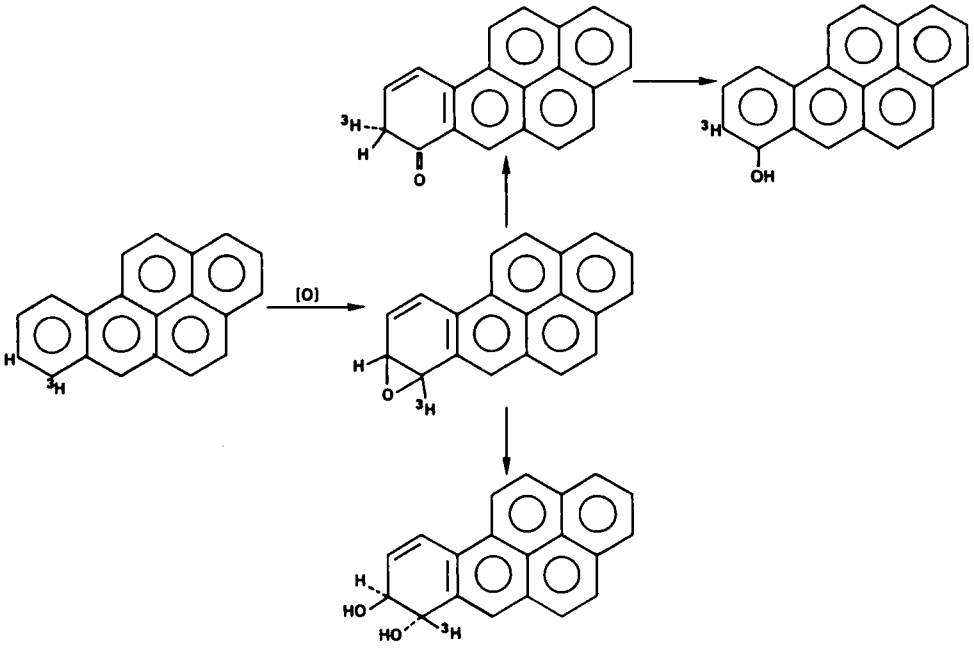


FIGURE 5. Hypothetical example of tritiated BP, illustrating why the AHH assay using generally tritiated BP is less sensitive than the fluorescent AHH assay due to tritium retention.

formation could be used as an index for measuring the uniformity of tritium labeling for any radioactive preparation of [^{3}H]BP.

Genetic Expression of AHH and Cytochrome P_1-450 Induction

There is now immunological evidence in rat liver[51] for at least six different forms of P-450. One of these forms, cytochrome P_1-450, is induced by polycyclic aromatic compounds in the B6 and in other responsive mouse strains, but the induction of this form of cytochrome by polycyclic aromatic compounds* is absent in liver and markedly decreased in lung, bowel, kidney, lymph nodes, skin, bone marrow, pigmented epithelium of the eye, and ovary in the D2 and other nonresponsive mouse strains.[6,23,52] This "responsiveness" to aromatic hydrocarbons was designated[53,54] the *Ah* locus: the allele Ah^b denotes the B6 and Ah^d the D2 inbred strain.

Numerous studies[23] indicate that an important product of the *Ah* (regulatory) locus in mice is a cytosolic receptor[55] capable of binding to certain polycyclic aromatic inducers (Figure 6). Such a complex in some manner activates structural gene(s), thereby leading to increases in enzymes which metabolize these inducers (and other polycyclic aromatic noninducing compounds). In addition to innocuous products, reactive metabolites may also be generated.

The AHH assay is a reliable, simple, and very sensitive assessment of aromatic hydrocarbon responsiveness following treatment of animals with polycyclic hydrocarbon inducers. Using AHH induction as an indicator of phenotype at the *Ah* locus, several laboratories have found that about half or slightly more than half of all inbred mouse strains examined are responsive (as are wild mice, randombred mice, and about 20 inbred strains of rats tested [unpublished data]) and the remaining mouse strains are

* In the general sense, Cytochrome "P-450" denotes all forms of CO-binding hemoproteins associated with membrane-bound NADPH-dependent monooxygenase activities. "P_1-450" is defined as that form(s) of cytochrome which increases during polycyclic aromatic inducer treatment of the laboratory animal or cells in culture. Two forms of P_1-450 have been characterized electrophoretically and catalytically, and even more than two are believed to exist.[4]

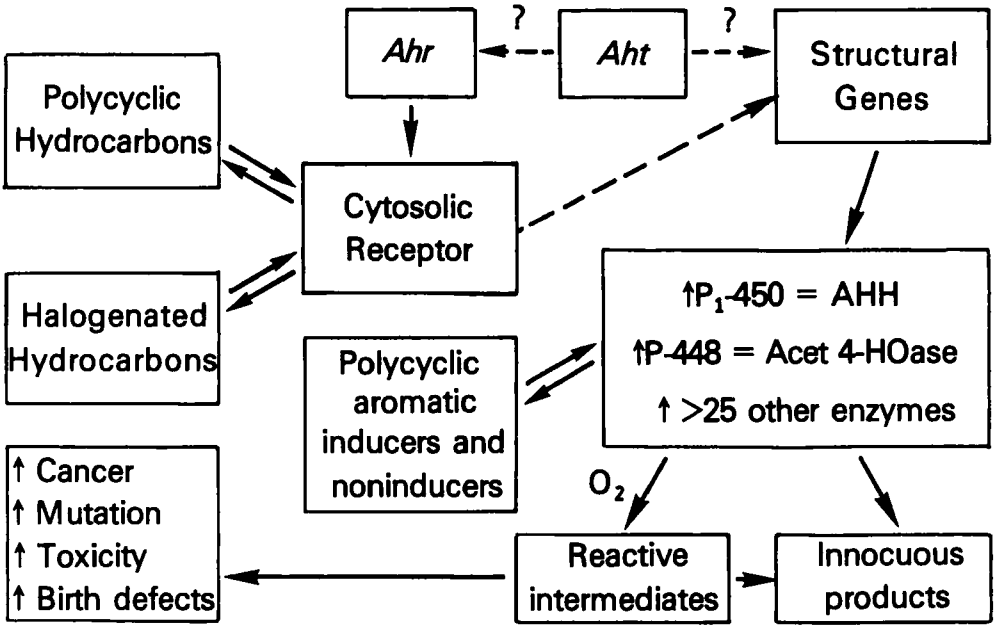


FIGURE 6. Simplified scheme demonstrating the relationship of the *Ah* locus in the mouse with cancer, mutagenesis, toxicity, and birth defects. *Ahr*, regulatory, and *Aht*, temporal, gene(s) associated with this genetic system. Acetanilide 4-hydroxylase (Acet 4-HOase) is associated with a form of P-450 which, when reduced and bound with C), gives a Soret peak at about 448 nm (i.e., P-448).

nonresponsive. There has evolved during the past 60 or 70 years of developing these inbred mouse strains, therefore, a stable mutation whereby certain strains lack (either quantitatively or qualitatively) the gene product of the *Ah* locus, the cystolic receptor molecule.[55]

Induction of AHH activity and cytochrome P_1-450 by MC is expressed almost exclusively as an autosomal dominant trait among offspring of the appropriate crosses between B6 and D2 inbred strains[45,56-58] and as an additive trait among offspring of the appropriate crosses between the C3 and D2 inbred strains.[59,60] On the other hand, the lack of induction of AHH activity and P_1-450 by MC is expressed as an autosomal dominant trait among offspring of the appropriate crosses between C57BL/6N and AKR/N parent strains.[60] The simplest genetic model to explain most (but still not all) of the data includes a minimum of six alleles and two loci[60] (reviewed in Reference 23). However, for all intents and purposes, we may regard genetic expression at the *Ah* locus in offspring from the appropriate crosses between B6 and D2 strains and between C3 and D2 strains, respectively, as dominant and additive. Because of the distinct phenotypes generated in progeny from the $F_1 \times D2$ backcross and the F_2 generation, therefore, we can evaluate the possible importance of steady-state levels of reactive intermediates in the mechanism of chemically induced carcinogenesis, mutagenesis, or toxicity among siblings in the same litter or among individuals sharing the same uterus.

The genetic regulation of an induced enzyme in one tissue need not be the same in other tissues. However, the induction of AHH and several other monooxygenase activities as well[23,61] appears to have similar genetic expression in all tissues examined[58] (reviewed in Reference 6). There are difficulties in a very careful genetic analysis of this point because most "control" responsive mice have, in fact, slightly induced AHH activity in many of their tissues which can be lowered by changing the diet. Hence, the slightly higher hepatic and pulmonary AHH activities seen in the responsive "con-

trol" B6 and C3 mice (Figure 7) may reflect the enzyme activities slightly induced by environmental factors.

One can also see in Figure 7 that induced AHH in $(B6D2)F_1$ liver or lung is slightly less than that in B6 liver or lung. The approximately 50:50 bimodal distribution among $(B6D2)F_1 \times D2$ offspring and approximately 25:75 nonresponsive to responsive bimodality among the $(B6D2)F_2$ generation can be seen clearly in both liver and lung of MC-treated animals. The additive expression of AHH induction is found in the liver (and lung; data not illustrated) of $(C3D2)F_1$ individuals; however, the approximately 50:50 intermediate to high bimodality among the $(C3D2)F_1 \times C3$ offspring, the approximately 50:50 low to intermediate bimodality among the $(C3D2)F_1 \times D2$ offspring, and the approximately 25:50:25 low to intermediate to high trimodal distribution among the $(C3D2)F_2$ generation that can be seen easily in the liver is not easily demonstrable in the lung because of relatively small differences between these groups and relatively large variation among pulmonary AHH in these MC-treated animals.

The differences in hepatic and nonhepatic induction of AHH activity (and presumably cytochrome(s) P_1-450) are apparent among B6, $(B6D2)F_1$, and D2 mice as a function of both time and dose of inducer. A responsive polycyclic hydrocarbon-treated mouse is therefore subject to both quantitative and qualitative increases in the steady-state levels of certain reactive intermediates because of both an increase in cytochrome(s) P_1-450 content and an increased P_1-450/P-450 ratio in numerous tissues. The relative content of P_1-450 compared with other forms of P-450 may be especially large (e.g., ratios of 10:1 or 50:1) in tissues such as skin and lung, but never reaches even a 1:1 ratio in liver.[63] This relatively large change in the profile of cytochrome(s) P-450 compared with smaller increments of change in epoxide hydrase,[64] UDP glucuronosyltransferase,[65] and GSH S-transferase[66] activities with BP as substrate might be a factor in explaining why polycyclic hydrocarbons cause tumors in skin and lung, but rarely in liver.

In studies involving the association of the *Ah* locus with cancer, mutation, or toxicity, the routine use of offspring from appropriate crosses between B6 and D2 parent strains (Figure 8) is ideal because expression of AHH induction by MC is most closely approximated a single-gene difference: Ah^b is the dominant allele for responsiveness; Ah^d is the recessive allele, the Ah^d/Ah^d animal being genetically nonresponsive. One therefore can determine whether this single allelic difference is advantageous or disadvantageous with respect to risk for cancer or toxicity when all individuals receive the same dose of the same drug. We can thus evaluate the possible importance of steady-state levels of reactive intermediates in the mechanism of chemically induced carcinogenesis, mutagenesis, or toxicity among individuals in the same family or among siblings sharing the same uterus. This genetic probe is a particularly powerful experimental model system in the research areas of pharmacology, toxicology, teratology, and chemical carcinogenesis, because the test compounds studied often cause undesirable side effects (e.g., sedation, diarrhea, malnutrition, hormonal imbalance, etc.) that are hard to distinguish from specific pharmacologic, toxicologic, or carcinogenic effects of the compounds.

Inducible Monooxygenase "Activities" Associated with the Ah^b Allele

Recent studies have shown (Figure 9) that the induction of at least 20 monooxygenase activities is closely associated with the Ah^b allele. Cytochrome P-450-mediated monooxygenases not associated with the *Ah* locus include the induction of: aminopyrine *N*-demethylase, *d*-benzphetamine *N*-demethylase, diphenylhydantoin hydroxylase, hexobarbital monooxygenase, aniline hydroxylase, benzenesulfonanilide hydroxylase, chlorcyclizine *N*-demethylase, ethylmorphine *N*-demethylase, pentobarbital hydroxylase, and testosterone 7α-, 16α-, and 6β-hydroxylases. Also not associated with the *Ah*

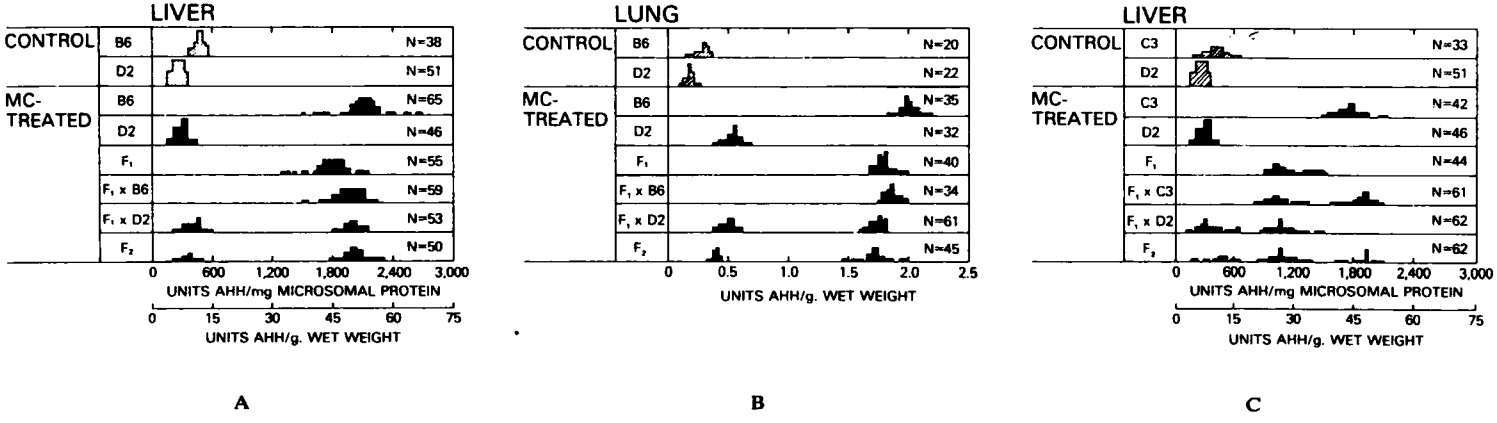


FIGURE 7. Genetic variance in hepatic (A) and pulmonary (B) AHH activity in control and MC-treated offspring from appropriate crosses between B6 and D2 inbred strains and hepatic AHH activity (C) in control and MC-treated offspring from appropriate crosses between C3 and D2 inbred strains.[52] Histograms for liver samples represent specific AHH activity in control mice and in mice treated i.p. 24 hr beforehand with MC (100 mg/kg body weight); controls received i.p. corn oil. For lung samples, the mice received intratracheally 24 hr beforehand MC, 500 μg in 20 μℓ of 0.2% gelatin-0.85% NaCl; controls received the sterile vehicle alone. One unit is defined[45] as that amount of enzyme catalyzing per minute at 37° the formation of the hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[*a*]pyrene. Specific AHH activity denotes units per milogram of microsomal protein. The mice weighed between 15 and 20 g. The number of mice examined individually is given at the right for each group. (From Kouri, R. E. and Nebert, D. W., *Origins of Human Cancer,* Hiatt, H. H., Watson, J. D., and Winsten, J. A., Eds., Cold Spring Harbor Laboratory, New York, 1977, 811. With permission.)

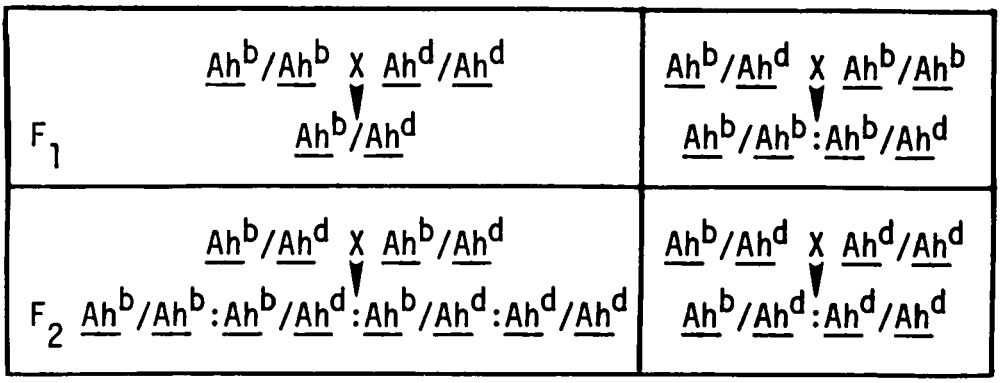


FIGURE 8. Simplified genetic scheme for aromatic hydrocarbon "responsiveness" in the mouse.[26] (From Nebert, D. W. and Felton, J. S., *Cytochromes P-450 and b5*, Cooper, D. Y., Rosenthal, O., Snyder, R., and Witmer, C., Eds., Plenum Press, New York, 1975, 127. With permission.)

locus is the induction of NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase, epoxide hydrase, and GSH S-transferase.[6,52,73] Other inducible enzymes that are not monooxygenases, but that appear to be associated with the Ah^b allele (and therefore may require the cytosolic receptor protein), include microsomal UDP glucuronosyltransferase,[67] cytosolic reduced NAD(P):menadione oxidoreductase,[74] and cytosolic ornithine decarboxylase.[75] This concept of a class of inducers binding to a cytosolic receptor with very great affinity, thereby evoking a "pleiotypic response," has been discussed elsewhere in greater detail.[73,74]

How so many substrates with very different chemical structures can be oxygenated by a single enzyme active-site (Figure 9) is not understood. One likely possibility is that there are several (or many) forms of cytochrome P_1-450. There is recent evidence in the mouse,[76] rabbit,[77] and rat[78] for at least two electrophoretically and catalytically distinct forms of P_1-450. Very recent studies[167] indicate there may exist between three and five such forms of P_1-450, which all appear to be structural gene products controlled by the regulatory *Ah* locus.

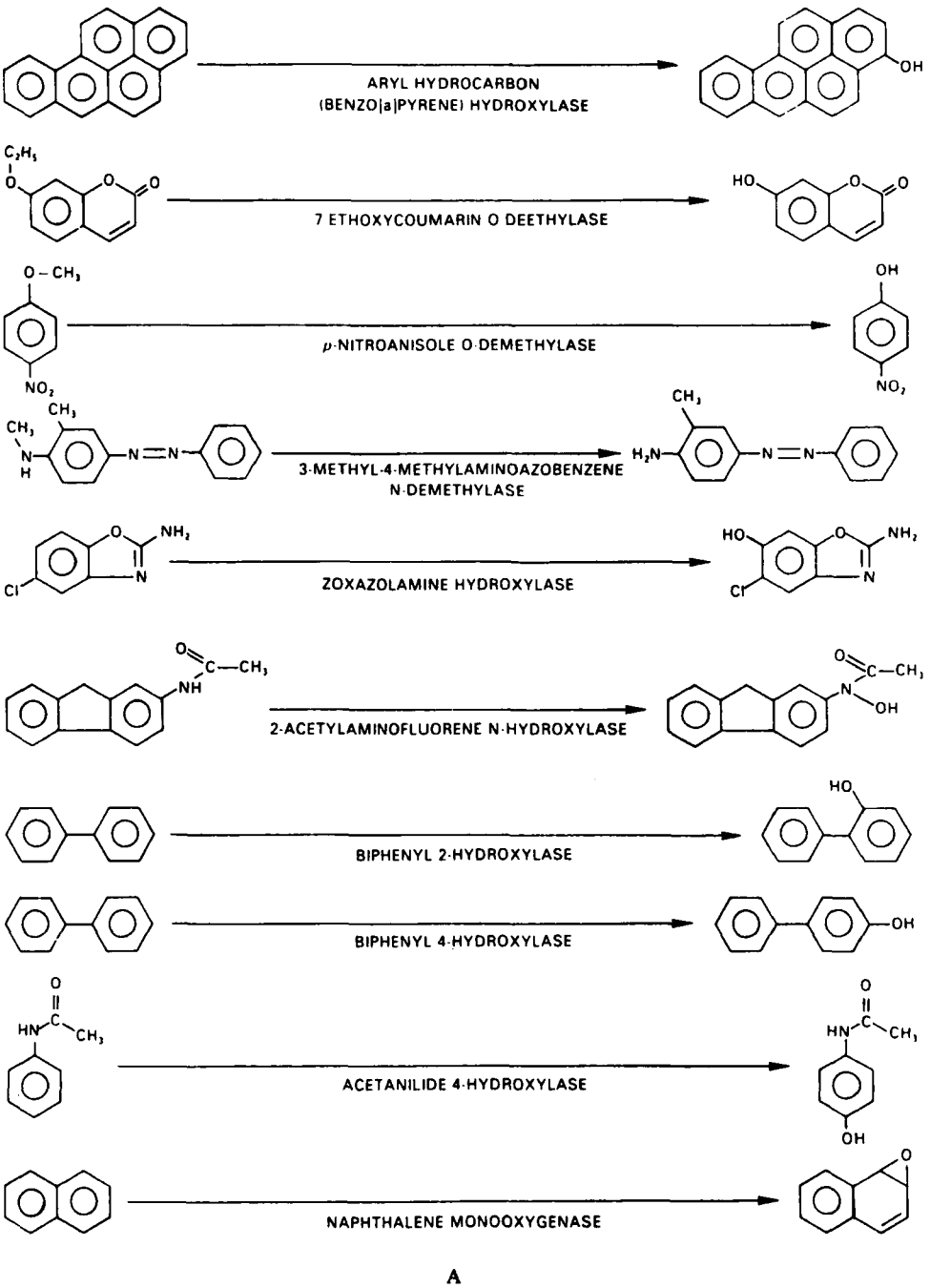
IMPLICATIONS OF THE *Ah* LOCUS IN CANCER AND TOXICOLOGY RESEARCH

Polycyclic Hydrocarbons

Tumorigenesis

Figure 10 illustrates pathways of major importance in the metabolism of BP. Increased K-region oxygenation (at lower left) occurs under conditions of high P-450/P_1-450 ratios; increased oxygenation of the non-K-region (pathway up the center) appears to be very important for covalent binding of metabolites to DNA[4] (reviewed in References 6, 52, and 73), and this pathway is favored under conditions of a high P_1-450/P-450 ratio in the tissue.[31,40,42,88-91] The 7,8-diol-9,10-epoxide of BP is felt to be an ultimate carcinogen: its extremely short half-life and its inability to be a substrate for UDP glucuronosyltransferase or epoxide-GSH S-transferase apparently account for its marked potential for carcinogenicity[92,93] and for mutagenicity and toxicity in vitro.[94,95,100] Differences in BP-initiated s.c. fibrosarcomas between B6 and D2 mice[52] appear to be associated, at least in part, with the *Ah* locus,

It should be emphasized that induction or suppresion by any chemical of any of the enzymes involved in the metabolic pathways illustrated in Figures 3 and 10 also may affect the incidence of tumorigenesis. Depending upon the experimental conditions and the tissue studied, for example, induced monooxygenase activity may also lead to



A

FIGURE 9A and 9B. Chemical reactions representing induced monooxygenase "activities" associated with cytochrome(s) P_1-450 induction and the *Ah*^{b} allele in the mouse. Not shown is UDP glucuronosyltransferase activity with 4-methylumbelliferone as substrate,[67] a membrane-bound metabolically coordinated enzyme whose induction appears to be closely correlated with the *Ah*^{b} allele. Only the substrate and the major product are shown. The activities are listed in chronological order;[29,30,48,68-72] and unpublished data.

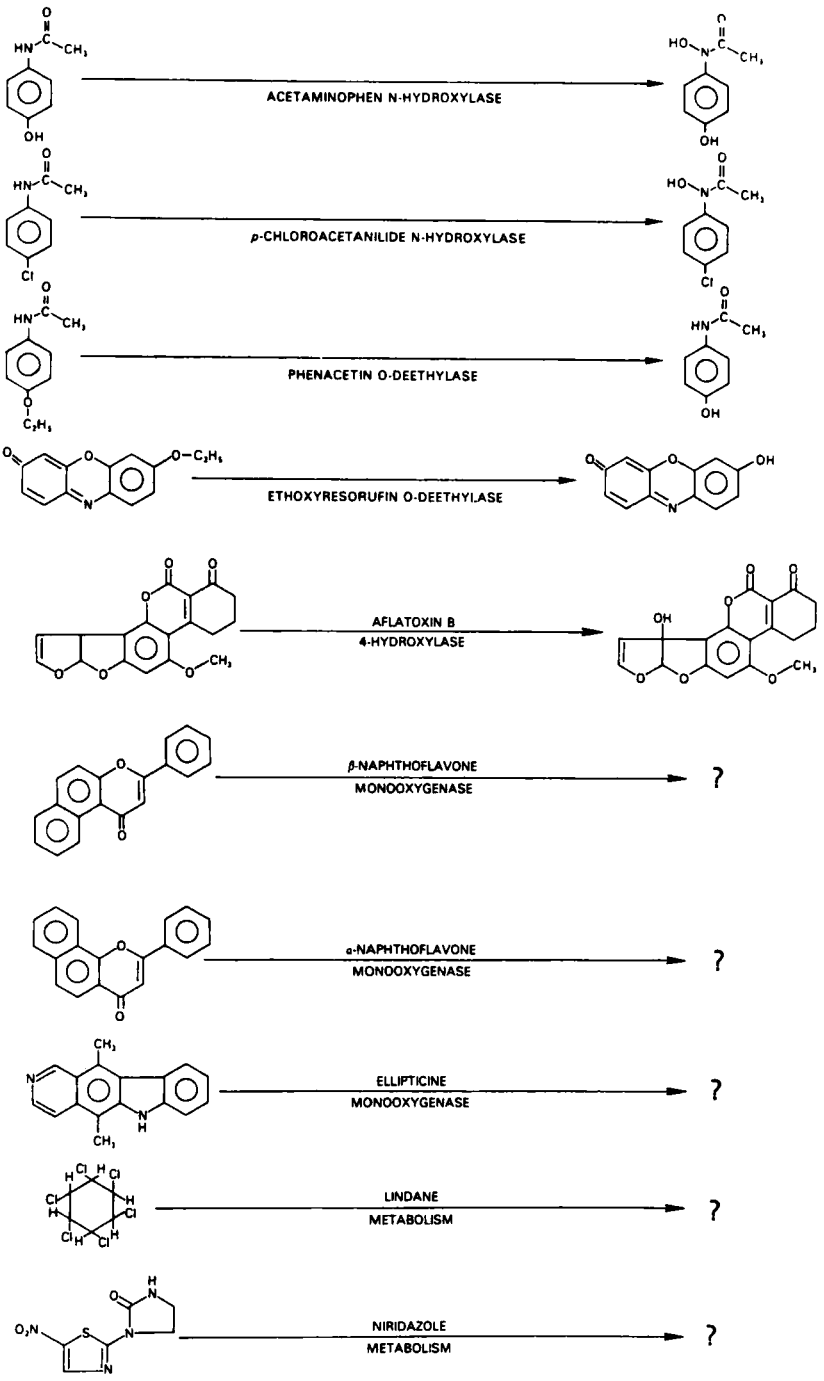


FIGURE 9B

decreases, rather than increases, in polycyclic hydrocarbon-initiated neoplasia,[96] bone marrow toxicity,[97,98] and leukemia.[99]

Fibrosarcomas initiated by s.c. administered MC are associated with genetically mediated aromatic hydrocarbon responsiveness among 14 inbred strains of mice.[52] Table 1 demonstrates that the carcinogenic index for s.c. MC in offspring from crosses involving the B6 and D2 inbred parental strains is, in fact, associated with the *Ah*^{b} allele:

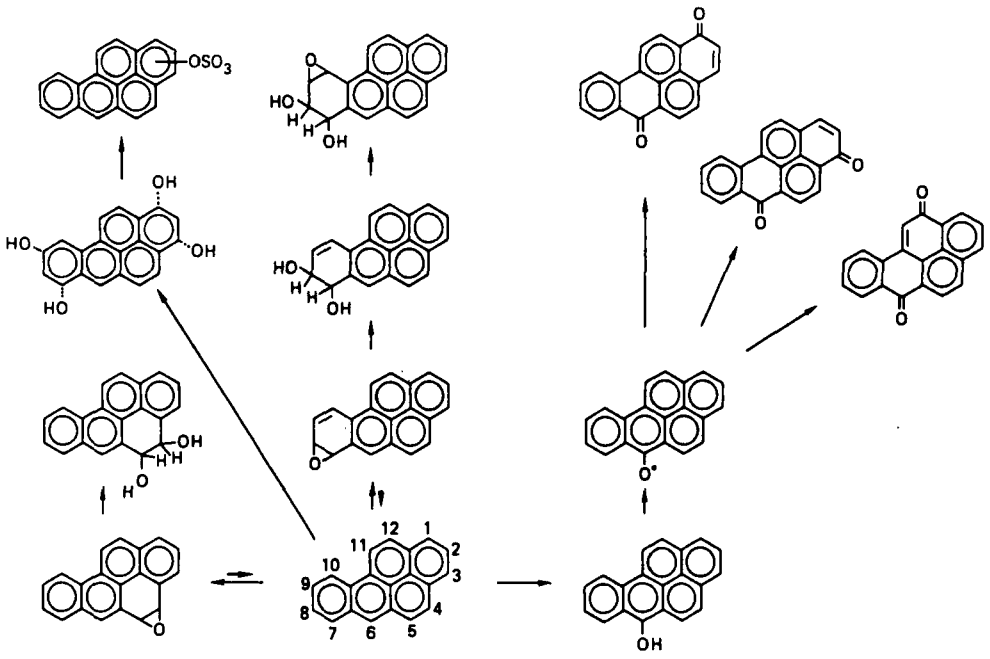


FIGURE 10. Chemical structures of known metabolites of BP (bottom center with carbon atoms numbered from 1 to 12).[79] The in vivo formation of BP phenols in the 1, 3, 7, and 9 positions[47] and subsequent sulfate conjugation of these phenols[80] are shown at upper left. The K-region arene oxide (bottom left) is formed predominantly by a form(s) of P-450 other than P_1-450 and is subsequently converted to the diol by epoxide hydrase. The 7,8-oxide is formed predominantly by P_1-450; following diol formation via epoxide hydrase, the 7,8-diol-9,10-epoxide is formed predominantly by P_1-450. The 6-phenol can rearrange to the free radical 6-oxybenzo[*a*]pyrene, which subsequently is converted to the three quinones.[81] The relatively easy abstraction of a proton from 6-hydroxybenzo[*a*]pyrene by molecular oxygen in solvents used during ESR analysis[82] accounts for the paramagnetic ESR signal and probably has little relationship to carcinogenicity per se.[83,84] The 6-hydroxybenzo[*a*]pyrene is quite inactive as an agent causing transformation in M2 mouse fibroblasts[85] or as a carcinogen applied topically to mouse skin,[86] although it is weakly mutagenic.[87] (Fom Pelkonen, O., Boobis, A. R., and Nebert, D. W., *Carcinogenesis: A Comprehensive Survey, Vol. 3,* Jones, P. W. and Freudenthal, R. I., Eds., Raven Press, New York, 1978, 383. With permission.)

the carcinogenic index is greater than 42 in all responsive phenotype groups and less than 12 in all nonresponsive phenotype groups.

With respect to the carcinogenic index for s.c. MC in offspring from crosses involving the C3 and D2 lines, however, unexpected values in Table 1 can be seen. Although the intermediate phenotype has intermediate carcinogenic index values among $(C3D2)F_1$ individuals and among offspring from the $(C3D2)F_1 \times D2$ backcross (37 and 46, respectively), the values among progeny of the $(C3D2)F_1 \times C3$ backcross and the $(C3D2)F_2$ generation are more susceptible to MC-initiated tumors than can be accounted for by their inducible AHH activity alone (carcinogenic indices of 60 and 61, respectively). There was also nearly a doubling (carcinogenic index of 17) in nonresponsive F_2 individuals. We conclude that there probably exist other genes carried by the C3 mouse that make this strain particularly sensitive to MC tumorigenesis.

The carcinogenic index for s.c. BP (far right of Table 1) is disproportionately low among $(C3D2)F_1$ progeny (value of 19) and among all offspring from both backcrosses and the $F_1 \times F_1$ intercross. The intermediate phenotype of the $(C3D2)F_1 \times D2$ backcross is particularly resistant to BP tumorigenesis, having a carcinogenic index (value of 1) lower than that for the D2 parent. It seems likely that the D2 strain carries other genes that confer even a higher resistance to BP-induced tumors than would be expected from their AHH content alone. Nonetheless, the *Ah* locus still plays a major role in

TABLE 1

Relationship Between Aromatic Hydrocarbon Responsiveness and Susceptibility to S.C. MC- and BP-Initiated Tumors Among Offspring from Appropriate Crosses Involving the B6, C3, and D2 Strains of Mice

Strain or offspring	Expression at *Ah* locus*	Carcinogenic index for MC	Strain or offspring	Expression at *Ah* locus*	Carcinogenic index for MC	Carcinogenic index for BP
B6	+ +	61	C3	+ +	73	56
D2	0	11	D2	0	10	4
B6D2F_1	+ +	43	C3D2F_1	+	37	19
F_1 × B6	+ +	58	F_1 × C3	+ +	74	27
				+	60	24
F_1 × D2	+ +	54	F_1 × D2	+	46	1
	0	8		0	9	1
F_2	+ +	63				
	0	6	F_2	+ +	69	31
				+	61	7
				0	17	2

Note: Animals received as weanlings 150 μg of MC or BP in trioctanoin s.c., and the carcinogenic index was determined over an 8-month period.[52] The carcinogenic index is defined as the percent incidence of s.c. fibrosarcomas, divided by the average latency in days, times 100.

* The phenotypic expression at the *Ah* locus is ranked as: + +, fully responsive; 0, nonresponsive; +, intermediate responsive; as judged by the data illustrated in Figure 2.

From Kouri, R. E. and Nebert, D. W., *Origins of Human Cancer*, Hiatt, H. H., Watson, J. D., and Winsten, J. A., Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1977, 811. With permission.

the susceptibility of these animals to BP tumorigenesis because both the (C3D2)F_1 × C3 progeny and the (C3D2)F_2 generation demonstrate a close association between tumor susceptibility caused by BP and inducible AHH activity. Thus, though some other genes may also influence susceptibility to BP- and/or MC-initiated tumors, the primary determinant for cancer susceptibility is the allele(s) regulating inducible AHH activity in numerous tissues of the mouse. Among recombinant inbred sublines having C57BL/6N and AKR/N as the progenitor strains in which the lack of induction is expressed as an autosomal dominant trait, susceptibility to MC-initiated tumors remains linked with inducible AHH activity.[73]

The model system of tumorigenesis initiated by s.c. MC or BP suffers from the shortcoming that AHH activity is determined in liver, whereas tumor formation occurs in s.c. connective tissue. The lung offers an alternate model system in which pulmonary AHH can be specifically and preferentially induced by intratracheal MC.[102,103] AHH induction in the lung appears to be under similar genetic control as that in the liver.[6,23,52,73] Mouse lung is known to be susceptible to MC tumorigenesis; moreover, MC-caused bronchogenic squamous cell carcinomas in mice have been described,[104] and it is well known that carcinomas—not sarcomas—are the most frequently observed type of tumor in man. A statistically significant ($p < 0.01$) correlation between lung tumors produced by intratracheal MC and the Ah^b allele has been found.[52] This correlation is most clearly seen in offspring from the (B6D2)F_1 × D2 backcross in which the responsive individuals were observed to be more than three times more susceptible to lung cancer than the nonresponsive individuals. Again, some contribution of genes other than the *Ah* locus seems to be responsible for the increased susceptibility to MC-initiated pulmonary tumors found in the F_1 and F_2 offspring and the progeny from

both backcrosses. Most likely, genes controlling DNA repair, susceptibility to oncogenic virus infection, or immunological surveillance (e.g., differences in the *H-2* locus) may be important in the overall susceptibility of certain tissues of an individual to chemically-induced cancer. Although these other genes may also influence susceptibility to BP- and/or MC-initiated tumors, however, one primary determinant for cancer susceptibility is the Ah^b allele regulating inducible AHH activity in numerous tissues of the mouse.

Hepatic and nonhepatic AHH activity and its associated cytochrome P_1-450 can be stimulated in nonresponsive inbred strains by the potent inducer TCDD to levels just as high as those in responsive strains; however, the ED_{50} is approximately 15 times higher in nonresponsive strains than in responsive strains.[73] The carcinogenic index for s.c. MC is increased in TCDD-treated nonresponsive D2 mice to about 60% of that of B6 mice in the presence or absence of TCDD.[105] We believe the most likely explanation for this effect is that TCDD acts as a cocarcinogen by inducing P_1-450 in the nonresponsive mouse. The newly induced cytochrome is now capable of metabolizing MC to the ultimate carcinogen more readily.[105]

In summary, MC and BP are either metabolized to higher steady-state levels of a proximal or ultimate carcinogenic intermediate(s) in the s.c. connective tissue or lung of responsive mice because of increased P_1-450 content in responsive mice, compared with nonresponsive mice, or are predominantly metabolized to a particular proximal or ultimate carcinogen(s) because of a marked change in the P_1-450/P-450 ratio.

Binding of Metabolites to DNA In Vitro

To understand further the interaction between covalently bound carcinogens and nucleic acid, Baird and Brookes developed a method[101] for the enzymic degradation of nucleic acid containing bound carcinogens and the fractionation of the resulting mixture by Sephadex® LH20 column chromatography. This method has shown great promise in that distinct peaks eluted from the column can be demonstrated to change in elution profile, depending on the carcinogen incubated with microsomes and cofactors, whether rat liver microsomes or cells in culture are used,[52,73] and on the use of microsomal inhibitors in vitro.[88] The nature of carcinogenic metabolites (from, e.g., BP, DMBA, and 7-methylbenzo[*a*]anthracene) bound to DNA nucleosides has been studied not only by column chromatography,[88,89,101,106] but also recently by high-pressure liquid chromatography.[107,108]

Figure 11 illustrates the results obtained with hepatic microsomes from the control or MC-treated responsive B6 mouse and the control or MC-treated nonresponsive D2 mouse. Instead of five peaks designated A through E by Brookes and co-workers,[109] nine peaks were reproducibly found in this laboratory. Peaks E and H (which correspond to peaks A and D named by Brookes and co-workers[109]) were particularly large with microsomes from the responsive B6 mouse. Peaks E, G, and H (which correspond to peaks A, C, and D, respectively, named by Brookes and co-workers[109]) were the largest with microsomes from the nonresponsive MC-treated D2 mouse. Whereas peaks E and H were much larger with the B6 than with the D2 microsomes, peak G was in fact larger with D2 than with B6 microsomes. Peaks A, B, C, D, F, and I were also larger with microsomes from the responsive strain than with microsomes from the nonresponsive strain.

Because inbred mouse strains differ at thousands of genetic loci, the definitive experiment is to study responsive (Ah^b/Ah^d) and nonresponsive (Ah^d/Ah^d) progeny from the $B6D2F_1$ × D2 backcross.[6] In doing this experiment, we found that the result was very similar to that found with the inbred B6 and D2 strains. All peaks, with the exception of peak G, appear to be principally associated with BP metabolism mediated by P_1-450 and therefore to be controlled by the Ah^b allele.

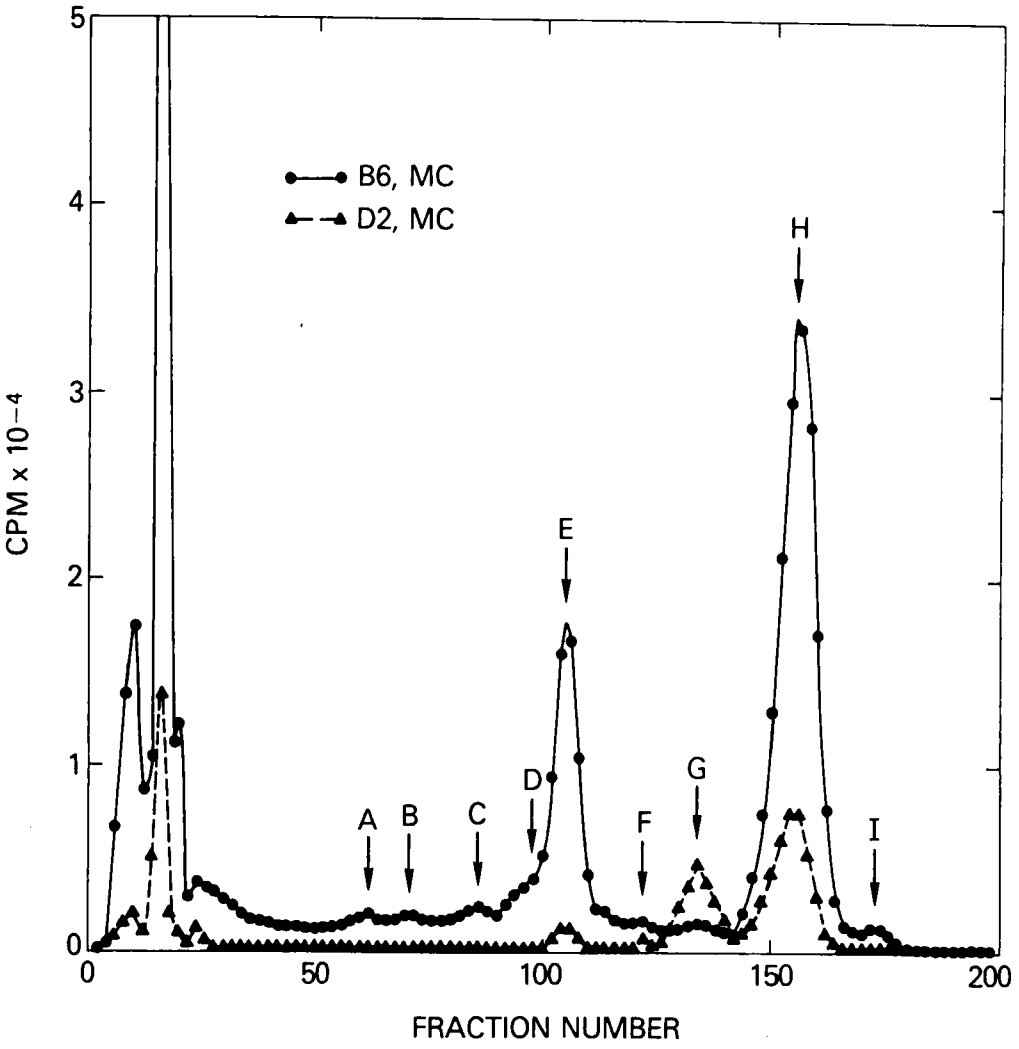


FIGURE 11A and 11B. Sephadex® LH20 column chromatography of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an in vitro incubation with hepatic microsomes from MC-treated B6 or D2 mice.[99] In this figure, the treatment of the animals, the preparation of the hepatic microsomes, and the separation of the metabolite-nucleosides after the incubation are previously described.[79,101] Deproteinized salmon sperm DNA (20 mg) was incubated with 4 mg of microsomal protein, 25 μmol of $MgCl_2$, 1 μmol of EDTA, 7 μmol of NADPH, 100 μmol of glucose-6-phosphate, 1.4 units of glucose-6-phosphate dehydrogenase, 1 mmol of potassium phosphate buffer, pH 7.5, and 60 nmol of [^{3}H]BP (1.19 mCi, specific activity 20 Ci/mmol) added in 200 μl of acetone. The 10-ml reaction mixture was incubated at 37° for 30 min. The DNA was reisolated, purified, digested with enzymes, then chromatographed on an 80-cm Sephadex® LH20 column eluted with a 30 to 100% methanol gradient in water at a flow rate of approximately 1 ml/min. Two hundred fractions of 5.1 ml each were collected. Radioactivity (in cpm) was determined for 1-ml portions of alternate fractions. The ordinate in B is a tenfold expansion of the ordinate (from the same experiment) in A. (From Nebert, D. W., Boobis, A. R., Yagi, H., Jerina, D. M., and Kouri, R. E., *Biological Reactive Intermediates*, Jallow, D. J., Kocsis, J. J., Snyder, R., and Vainio, H., Eds., Plenum Press, New York, 1977, 125. With permission.)

With the use of synthetic and biologically produced metabolites[113] and on the basis of our studies on the effects of microsomal enzyme inducers and inhibitors,[88] all peaks have been tentatively assigned to one or more metabolites of BP. Of the nine peaks, it is of interest that eight (all except perhaps peak G) involve more than a single monooxygenation by forms of cytochrome P-450.[88,113]

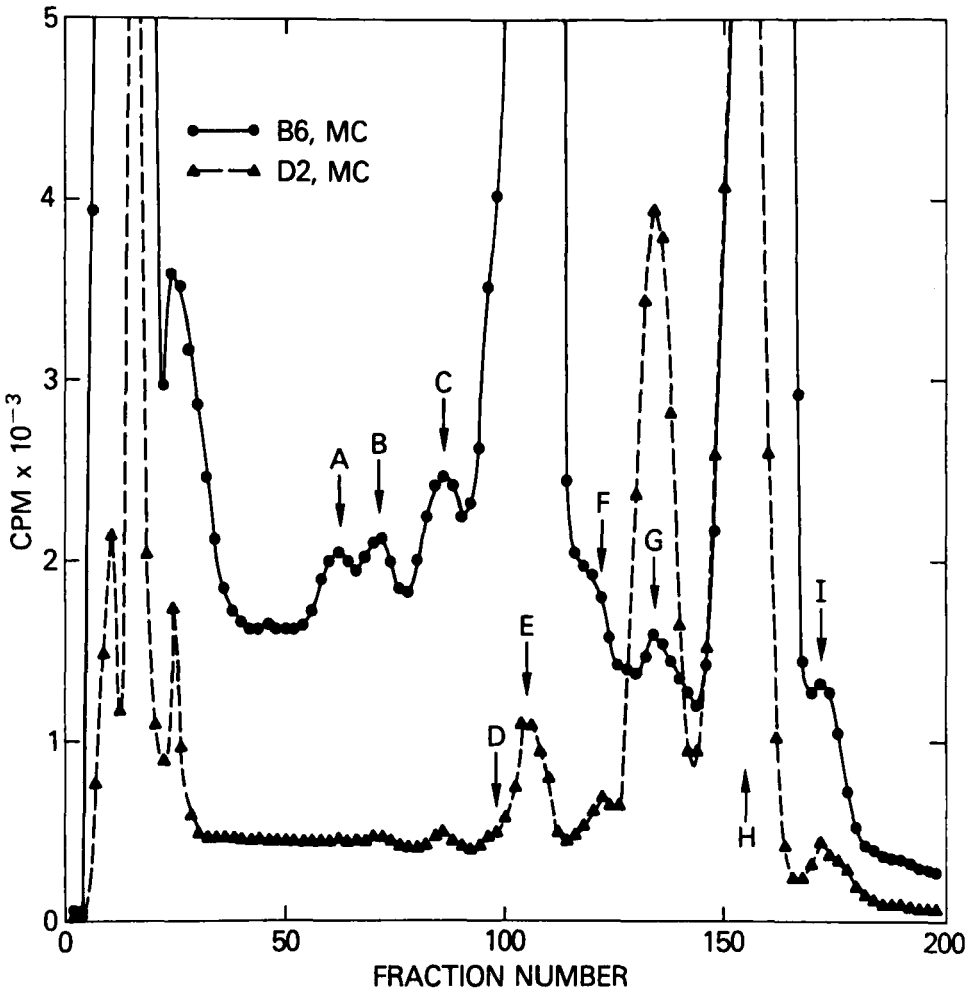


FIGURE 11B

BP thus may be metabolized to four different "types" of reactive intermediates capable of binding to DNA: (1) primary arene oxides; (2) diol-epoxides; (3) phenols oxygenated further; and (4) quinones oxygenated further (or quinone-derived free radicals). These last three types of microsomally activated intermediates are therefore the result of two- or three-step enzymic processes in which P-450-mediated monooxygenations occur at least twice.

The previous concept of BP glucuronidation being a "pathway to safety" might have to be revised in light of recent findings. BP glucuronide conjugates can be metabolized in vitro by β-glucuronidase presumably to BP-oxy free radicals capable of binding covalently to DNA and proteins.[110] Increases in glucuronide conjugation of BP phenols have also been shown to enhance BP 7,8-diol-9,10-epoxide covalent binding to DNA in vitro, presumably by removing phenols from the membrane and allowing further oxygenation of BP diols.[111] Studies in the intact animal — for example, among inbred strains of mice known[112] to vary widely for β-glucuronidase induction — are now certainly indicated in order to see if BP tumorigenesis can be modified via the BP glucuronide pathway.

Genetic differences in the binding of reactive metabolites to DNA catalyzed by liver microsomes from MC-treated B6 and D2 mice were studied with numerous substrates

(Table 2). The total covalent binding of reactive intermediates to DNA displayed a strain different with BP, MC, BA, DMBA, dibenzo[*a,h*]anthracene, 2-acetylaminofluorene, and dopamine. No strain differences were found with benzidine. The ratio between the responsive B6 and the nonresponsive D2 ranged from 2- to more than 12-fold with the various substrates.

Mutagenicity in Salmonella typhimurium In Vitro

A sensitive and simple bacterial test for the detection of chemical carcinogens has been developed.[114] About 300 carcinogens and noncarcinogens of widely varying chemical structures have been tested, and there exists a high correlation between carcinogenicity and mutagenicity: about 90% (157 out of 175 compounds) of carcinogens were mutagenic, and few chemicals believed to be "noncarcinogens" showed any degree of mutagenicity.[114-116]

Also shown in Table 1 are genetic differences in mutagenicity with the bacterial test system catalyzed by liver S-9 from MC-treated B6 and D2 mice with ten nonradioactive substrates. The mutagenicity displayed distinct strain differences with BP, MC, benzo[*a*]anthracene, dibenzo[*a,h*]anthracene, dibenzo[*a,c*]anthracene, 2-acetylaminofluorene, 6-aminochyrysene, and β-naphthylamine. The "background" revertant rates per plate are approximately 30, 35, and 250 for TA1538, TA98, and TA100, respectively, but are somewhat variable between experiments; therefore, it is difficult to describe "fold increases" in B6 samples compared with D2 samples. 7,12-Dimethylbenzo[*a*]anthracene and α-naphthylamine, however, showed only very small differences between B6 and D2.

Hypothesis That Oxygenation of Polycyclic Hydrocarbons by P_1-450 is Particularly Pronounced in the "Bay Region"

It is very tempting to speculate that the enzyme active-site(s) of cytochrome(s) P_1-450 preferentially attacks the benzylic ring at the bond farther from the "bay region". Following hydration by the closely coupled[117] epoxide hydrase, the *trans*-dihydrodiol is formed. The benzylic ring now contains only a nonaromatic bond adjacent to the bay region, and P_1-450 preferentially oxygenates the *trans*-dihydrodiol to the diol-epoxide.[79,88] Figure 12 shows such postulated diol-epoxides for seven polycyclic aromatic compounds of interest to this review. All these different chemicals have been shown to be at least weakly carcinogenic in one or another experimental model system. Marked genetic differences in MC and BP tumorigenesis[52] and in MC, BP, and 6-aminochrysene mutagenesis[6] are associated with the *Ah*^{b} allele. During the next few years, it will be interesting to find out if P_1-450 catalyzes preferentially each of these carcinogens to the diol-epoxide of the benzylic ring adjacent to the bay region, providing a common molecular basis for the initiation of polycyclic aromatic hydrocarbon-induced tumorigenesis — a hypothesis recently advanced by Jerina and Daly.[118] Moreover, because there is evidence for differences at the human *Ah* locus[52,119] (reviewed later in this chapter), elucidation of P_1-450-catalyzed metabolites of numerous environmental carcinogenic pollutants becomes extremely important.

N-acetylarylamines

Tumorigenesis

Much of our knowledge of the metabolic activation, binding of intermediates to macromolecules, and mechanism of action of aromatic amines and amides stems from the work of Miller and Miller[120] and Weisburger and Weisburger[121] and a large portion of that work was carried out with the *N*-acetylarylamine 2-AAF (Figure 13). Several findings[122,123] indicate that the ultimate carcinogen(s) is one or more of the metabolites of *N*-hydroxy-2-AAF rather than 2-AAF or *N*-hydroxy-2-AAF itself. The enzyme sys-

TABLE 2

Polycyclic Aromatic Carcinogens and Aromatic Amines: DNA Binding of Metabolites and Mutagenicity with Bacterial Tester Strains, Catalyzed In Vitro by Liver Subcellular Fractions from MC-Treated B6 or D2 Mice

Compound	Concentration in assay for metabolites binding to DNA[a] (μM)	Total amount presumed to be covalently bound to DNA (pmol/mg DNA)[b]		Estimated percent of total added compound that is bound covalently	Ratio (B6/D2)	Concentration at which genetic differences in mutagenesis were maximal (μg/plate)	Bacterial tester strain in which genetic differences in mutagenesis were maximal	Revertants/plate	
Benzo[*a*]pyrene	6	B6	12	0.30	5.4	1.0	TA98	B6	540
		D2	2.2	0.055				D2	150
3-Methylcholanthrene	6	B6	10.7	0.27	3.0	100	TA1538	B6	180
		D2	3.6	0.089				D2	40
Benzo[*a*]anthracene	60	B6	108	0.27	6.6	10	TA100	B6	830
		D2	16.5	0.042				D2	540
Dibenzo[*a,h*]anthracene	6	B6	6.7	0.17	13	10	TA100	B6	1240
		D2	0.52	0.014				D2	310
Dibenzo[*a,c*]anthracene	6	B6	21	0.47	3.8	10	TA100	B6	1250
		D2	5.6	0.18				D2	410
7,12-Dimethylbenzo[*a*]anthracene	6	B6	23	0.57	2.7	10	TA100	B6	820
		D2	8.6	0.22				D2	580
2-Acetylaminofluorene	60	B6	35	0.088	2.5	10	TA1538	B6	4100
		D2	14	0.036				D2	980
Dopamine	6	B6	3.6	0.090	2.0				
		D2	1.8	0.045					
Benzidine	60	B6	23	0.057	1.0				
		D2	23	0.058					
6-Aminochrysene						10	TA1538	B6	11,900
								D2	850
α-Naphthylamine						100	TA100	B6	750
								D2	500
β-Naphthylamine						100	TA100	B6	650
								D2	320

[a] For technical and practical reasons (such as expense of some of the compounds and relatively low specific radioactivity of ^{14}C-labeled compounds), the test compounds were usd at varying concentrations in the in vitro assay.[99,101] Experiments with benzo[*a*]pyrene concentrations of 0.6, 6.0, and 60 μM, however, showed little difference in the relative heights of all nine peaks.[165] The results in this table are based on two different experiments, except with dopamine and the steroids in which only one experiment was performed. Because two separate experiments never differed more than 20 to 30%, however, we believe these results are accurate.

[b] Values representing "noncovalent" binding (radioactivity bound to DNA in the presence of heat-denatured microsomes) were subtracted from values obtained with active microsomes.

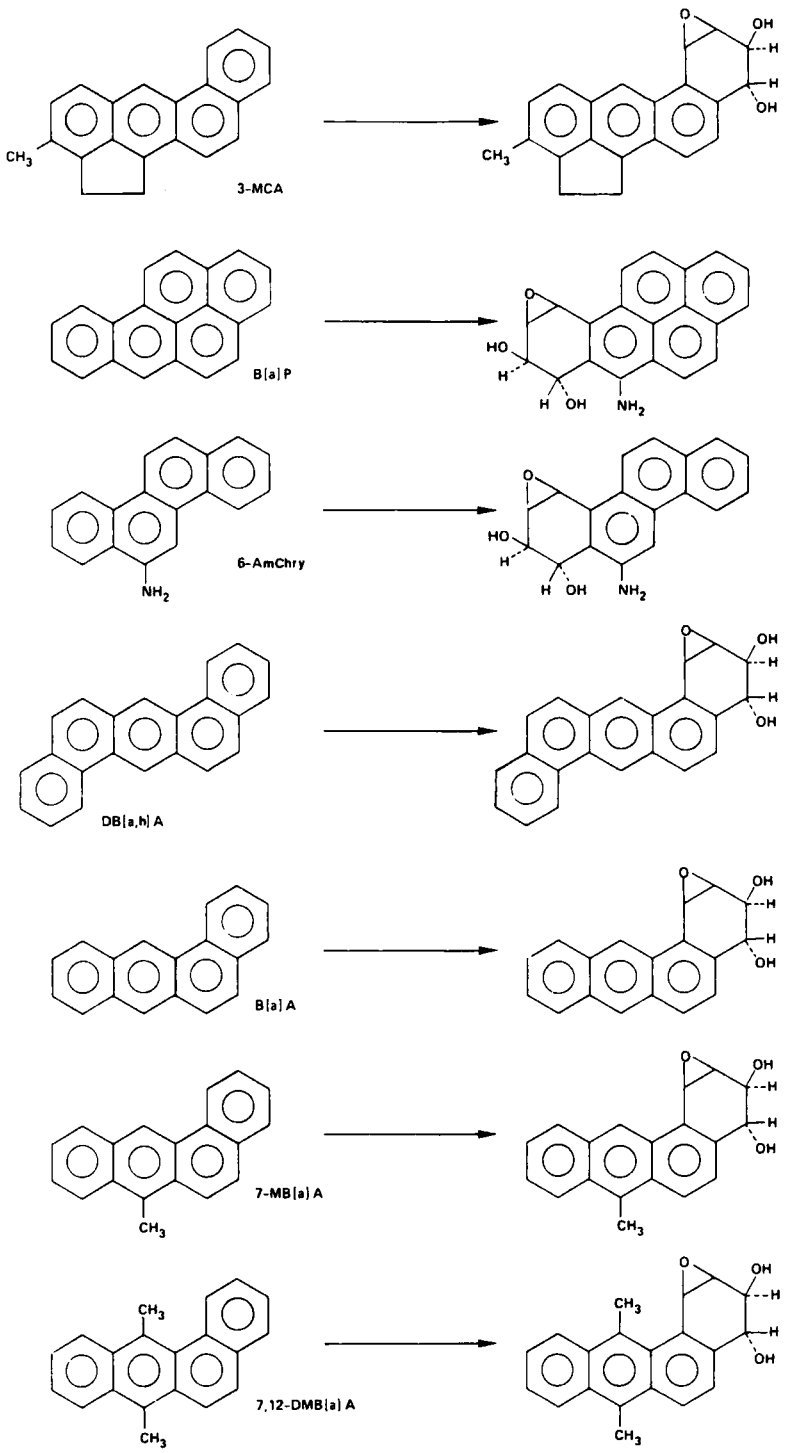


FIGURE 12. Postulated reactive intermediates catalyzed predominantly by cytochrome P_1-450, if the "bay region" theory holds true for a large number of polycyclic hydrocarbons.[79] Other forms of P-450 may metabolize these substrates to these diol-epoxides as well, but perhaps the turnover number is 1/10 or 1/100 that of diol-epoxide formation by P_1-450. From top to bottom, structures of substrates shown are MC, BP, 6-aminochrysene, dibenzo[*a,h*]anthracene, BA, 7-methylbenzo[*a*]anthracene, and 7,12-dimethylbenzo[*a*]anthracene. (From Pelkonen, O., Boobis, A. R., and Nebert, D. W., *Carcinogenesis: a Comprehensive Survey*, Vol. 3, Jones, P. W. and Freudenthal, R. I., Eds., Raven Press, New York, 1978, 383. With permission.)

tems implicated include sulfotransferase(s), deacetylase(s), transacetylase(s), and UDP glucuronosyltransferase(s).[123-125]

Chemical or enzymic one-electron oxidation systems that convert the *N*-hydroxy intermediate to a nitroxide free radical may also be important because the free radical can dismutate to the carcinogenic electrophiles *N*-acetoxy-2-AAF and 2-nitrosofluorene.[126,127] The relative importance of each of these processes in the conversion of *N*-hydroxy-2-AAF to the ultimate carcinogen(s) is not clear and, in fact, may differ among species and among tissues within the same species.

Thorgiersson and co-workers (1973)[37] demonstrated that *N*-hydroxylation of 2-AAF in liver microsomes from mice and hamsters is a cytochrome P-450-dependent oxidation. Subsequently it was shown[70] that the MC-inducible *N*-hydroxylase activity is associated with the *Ah*^{b} allele in mice. From Figure 13, it is obvious that any increased formation of the proximal carcinogen *N*-hydroxy-2-AAF by increased P_1-450 content might be an important mechanism for increased 2-AAF-induced tumorigenesis. This hypothesis is presently under study by Thorgeirsson and co-workers;[71] preliminary data appear to confirm this hypothesis.

Hepatic Necrosis

It now appears that the *N*-hydroxylation of acetaminophen (Figure 14), like that of 2-AAF, is predominantly mediated by P_1-450; the *N*-hydroxylation of several other *N*-acetylarylamines likewise appears to be catalyzed by cytochrome P_1-450.[71] The rearrangement of the *N*-hydroxy derivative to a highly reactive electrophile has been postulated[128] to be the principal mechanism by which GSH conjugation or covalently bound protein or nucleic acid occurs *ortho* to the hydroxyl group. It can be seen in Figure 14 that an increase in *N*-hydroxylation enhances the need for reduced glutathione, and it is known[128] that GSH depletion in the liver precedes marked increases in covalently bound acetaminophen. The amount of radioactive metabolite bound cova-

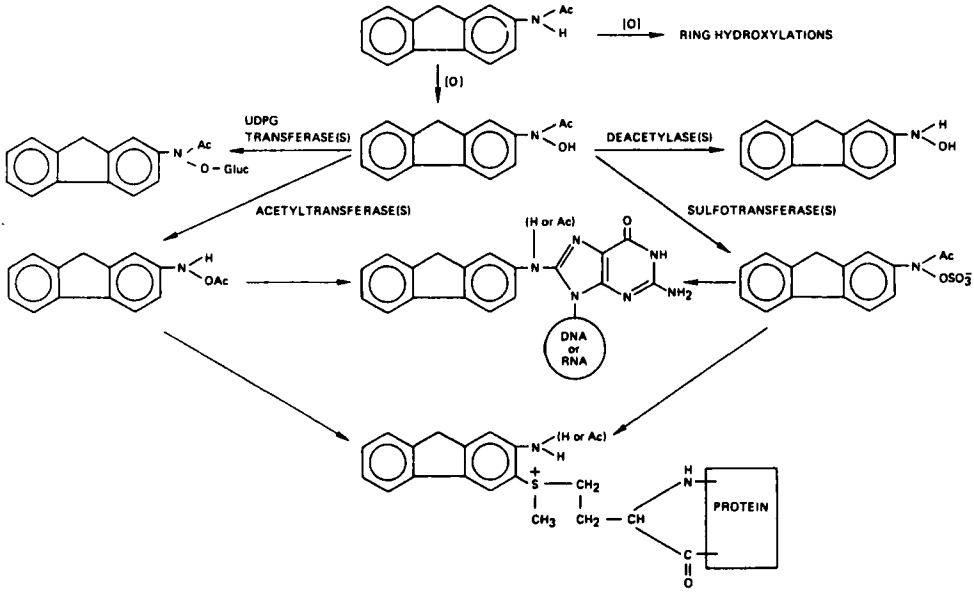


FIGURE 13. Important metabolic pathways for 2-AAF (adapted in part from Miller and Miller (1974)).[120] Whereas ring hydroxylations of 2-AAF occur predominantly via phenobarbital-induced cytochrome P-450,[36] the *N*-hydroxylation appears to be predominantly mediated via the polycyclic hydrocarbon-inducible P_1-450. (Modified from Felton, J. S., Nebert, D. W., and Thorgeirsson, S. S., *Mol. Pharmacol.* 12, 225, 1976. With permission.)

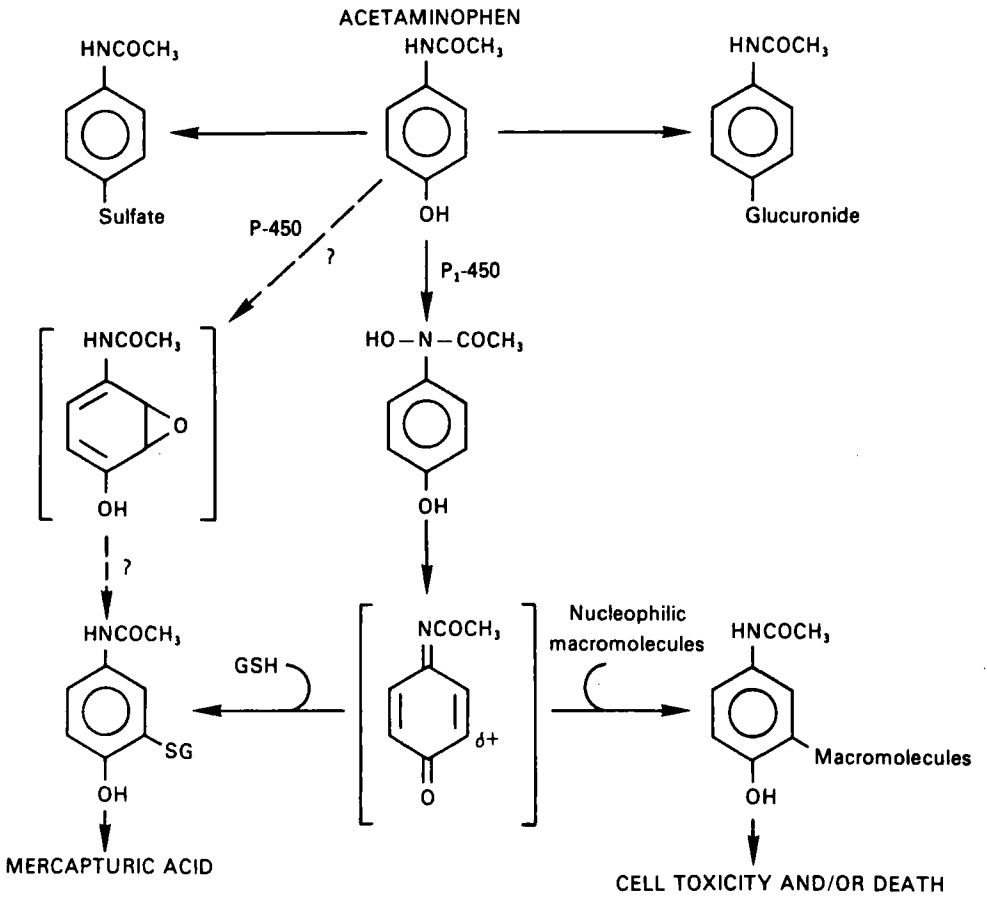


FIGURE 14. Known and postulated metabolic pathways for acetaminophen.[6] The dashed arrows with question marks represent pathways that have not been experimentally substantiated, and the compounds in brackets are postulated intermediates. Increased P_1-450 content will lead to acetaminophen-induced GSH depletion and increased covalent binding of acetaminophen metabolites to cellular nucleic acids and proteins. (From Thorgeirsson, S. S. and Nebert, D. W., *Adv. Cancer Res.*, 25, 149, 1977. With permission.)

lently to acid-precipitable material, following administration of large doses of [^{3}H]acetaminophen, is associated in the hamster and mouse with GSH depletion and the magnitude of hepatic necrosis observed.[129] It recently has been found in this laboratory[71] that the *Ah*^{b} allele is highly correlated with acetaminophen-induced GSH depletion (Figure 15), hepatotoxicity, and increases in covalently bound metabolites of the drug.

Of further interest is the fact that large doses of 2-AAF do not cause hepatic GSH depletion as does acetaminophen, in spite of similar large increases in the rate of *N*-hydroxylation of both of these drugs.[168] The larger number of alternative pathways for *N*-hydroxy-2-AAF (Figure 13), compared with the pathways for acetaminophen (Figure 14), may account for this observation.

Cataract Formation

Lenticular opacification (or cataract) results from senility, congenital defects, viral infections, metabolic disorders, and various types of physical and chemical insult to the lens.[130,131] Cataracts can be induced in lenses in organ culture.[132,133] Because of the diversity in cataractogenic agents, no single mechanism can account for the different forms of cataract. Osmotic imbalance produced by polyol accumulation within the

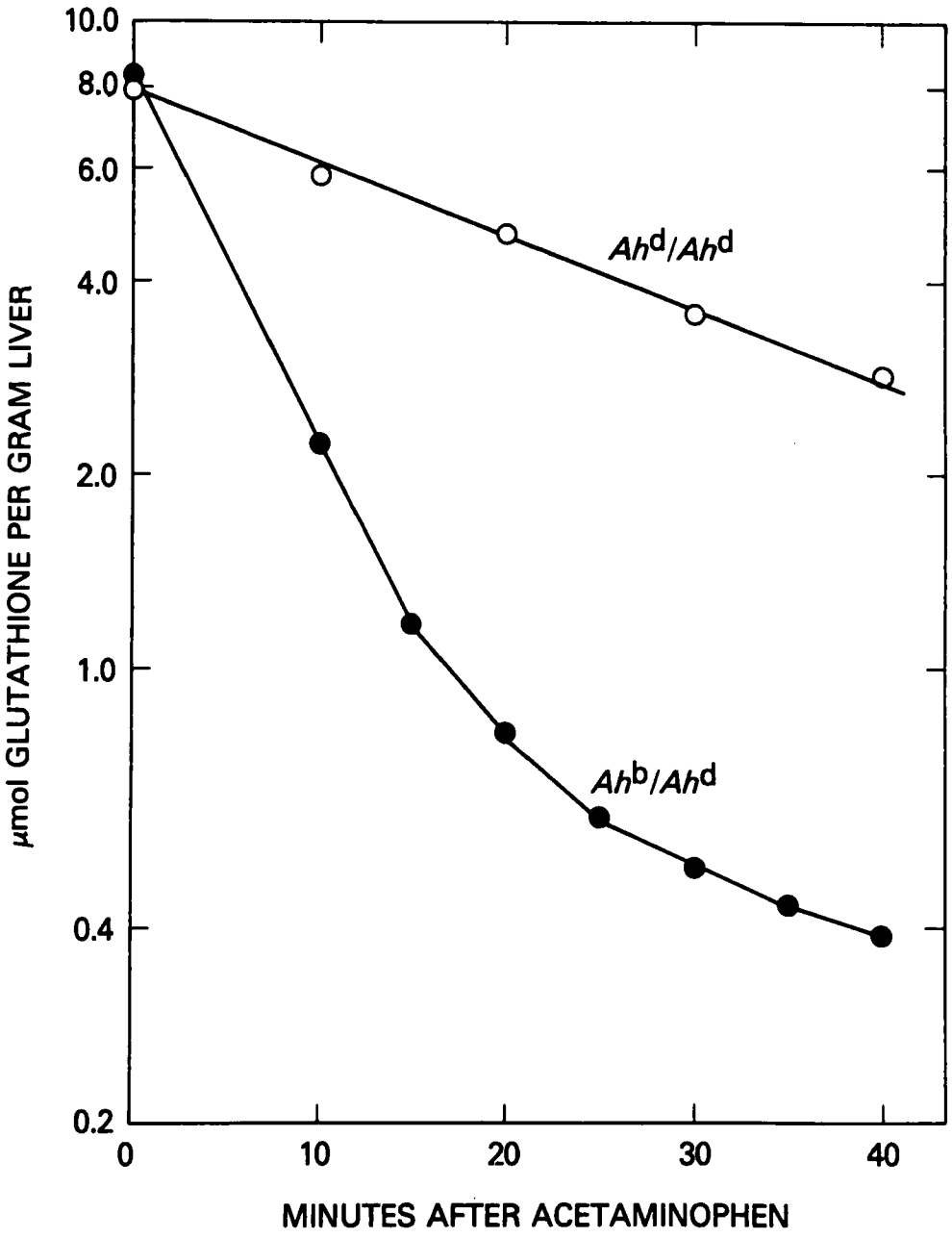


FIGURE 15. Association between acetaminophen-induced GSH depletion and the Ah^d allele in MC-treated (B6D2) D2 mice.[6] The phenotype of these individuals has been determined 2 weeks earlier by the zoxazolamine paralysis time, as previously described.[69] At 48 hr after MC (80 mg/kg body weight), each mouse received 400 mg of acetaminophen per kilogram and was killed at the indicated times. Each point represents the GSH content in three livers combined. (From Thorgeirsson, S. S. and Nebert, D. W., *Adv. Cancer Res.*, 25, 149, 1977. With permission.)

lens was proposed[134] to be responsible for sugar-induced cataract, and light-scattering by aggregated protein was suggested[135-137] as a cause of senile cataracts. Cataracts induced by chemicals and drugs, especially naphthalene, have been extensively investigated because of their similarly to senile cataracts.[138-141]

Figure 16 shows the absolute correlation between the Ah^b allele and acetaminophen-

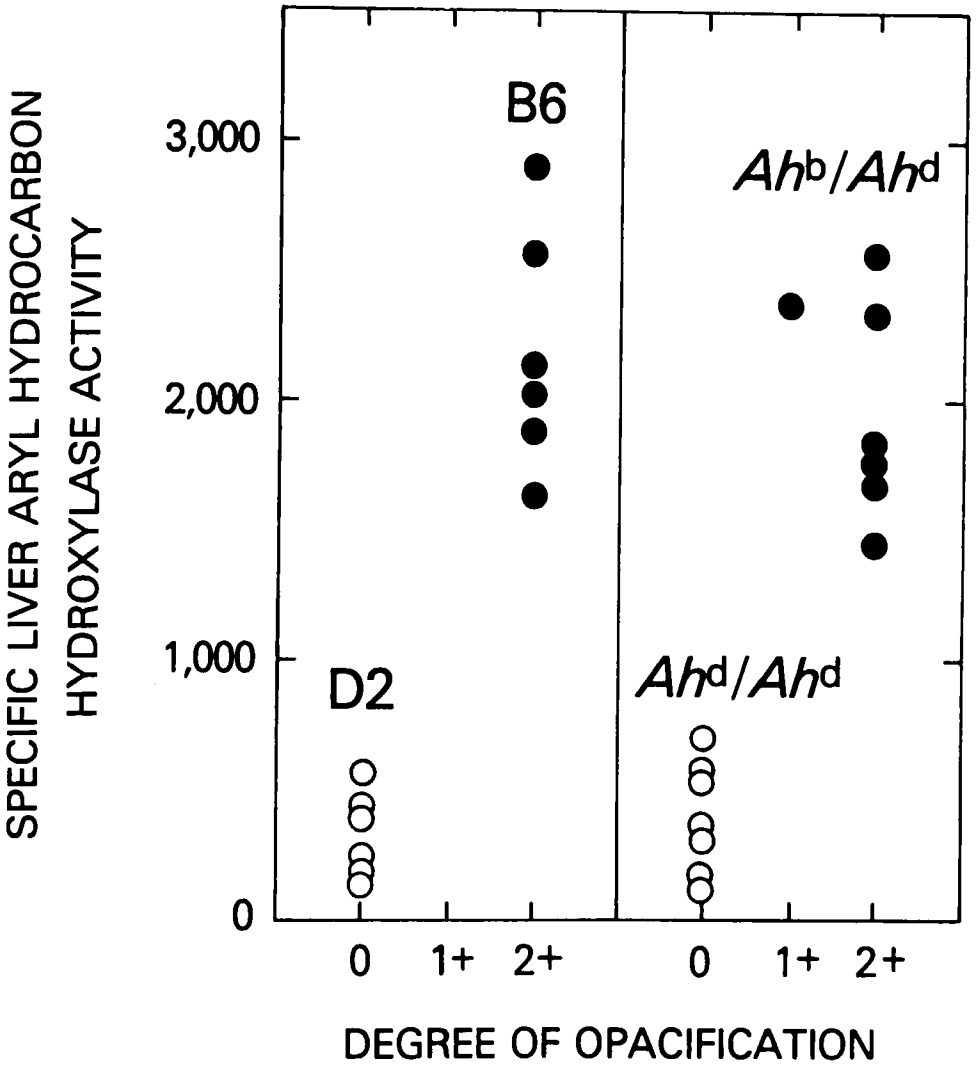


FIGURE 16. Correlation between hepatic AHH inducibility and cataractogenesis in the nonresponsive D2 inbred strain, the responsive B6 inbred strain, and the nonresponsive Ah^d/Ah^d and responsive Ah^b/Ah^d progeny from the B6D2F$_1$ × D2 backcross.[142] Sexually immature (5- to 6-week-old) mice of either sex were always used. The mice were treated i.p. with MC (200 mg/kg body weight) in corn oil (25 mℓ/kg) 48 hr prior to acetaminophen (1000 mg/kg body weight) in warm water (25 mℓ/kg). The acetaminophen was completely dissolved at the time of injection. No mice died before 6 hr at this dose. The eyes were evaluated with an ophthalmology slit lamp 5 hr later:), 0, no signs of opacification; 1+, about 50% opacification; 2+, complete opacification. The mice were then immediately killed and liver microsomal AHH activity was determined by the previously described[45] method, using BP as substrate. Seven nonresponsive Ah^d/Ah^d and seven responsive Ah^b/Ah^d weanlings (either sex) from the B6D2F$_1$ × D2 backcross were genotyped with respect to the *Ah* locus by the zoxazolamine paralysis time, as previously described;[24,69] these mice were then administered the MC and acetaminophen in this experiment 2 weeks later. (Copyright ©1978 by The American Association for the Advancement of Science, from Schichi, H., Gaasterland, D. E., Jenson, N. M., and Nebert, D. W., *Science*, 200, 539, 1978. With permission.)

caused cataracts. Hence, individuals in the same family develop or fail to develop cataracts following the same dose of the same drug, depending upon this single allelic difference. In preliminary studies (data not shown), we found that acetaminophen causes cataracts in other MC-treated responsive inbred strains, such as A/J, CBA/J, and C3H/HeJ, but not in other MC-treated nonresponsive inbred strains, such as RF/J, AKR/J, SJL/J, and SWR/J.

The opacity that we found[142] consisted of a thin opalescent layer just anterior to the equatorial cortex of the lens. The layer of opacity continued anteriorly to the visual axis. Subepithelial vacuolation was seen within the lens fibers of the opaque lens. The cornea remained clear, and the retina and retinal pigmented epithelium were normal via light microscopic examination. No vacuoles in the subepithelial layer of the lens of Ah^d/Ah^d mice were found.

The mechanism of acetaminophen-induced cataract formation is uncertain. However, reactive intermediates of radio-labeled acetaminophen (formed predominantly in the liver) are bound covalently in the lens and in numerous other tissues of the genetically responsive individual at much higher levels than in nonresponsive mice.[169] Also, phenobarbital pretreatment, which enhances glucuronide conjugation of acetaminophen principally in the liver[143-146] and is independent of the *Ah* locus,[6,52,71,161] prevents cataracts in these responsive mice.[169] There is much less acetaminophen-induced hepatotoxicity in phenobarbital-treated than in MC-treated animals.[143-146] A reactive metabolite of acetaminophen may therefore pass into the aqueous humor from the blood stream; the steady-state level of this reactive intermediate may be decreased by phenobarbital in spite of more acetaminophen hepatoxicity in phenobarbital-treated animals than in controls.[143-146] The fact that the anterior portion of the lens shows opacification is consistent with the circulation pathway of aqueous humor from the posterior chamber across from the front of the lens to the anterior chamber.*

EVIDENCE FOR THE *Ah* LOCUS IN THE HUMAN

In 1973 two published reports[148,149] gave initial hope to clinical geneticists and oncologists. The extent of AHH induction in culture mitogen-activated lymphocytes by MC was examined in 353 healthy subjects, ranging in age from 2 to 89 years old and including 67 families with 165 children.[148] The distribution of inducibilities in the patients tested in the Houston area was trimodal, the group being designated as having "low", "intermediate", and "high" inducibility. The data were consistent with a hypothesis of two alleles at a single locus and gave an excellent fit to the Hardy-Weinberg equilibrium, with a frequency of 0.717 for the "low-inducibility" allele and 0.283 for the "high-inducibility" allele (although the sample is biased by including parents and siblings). Fifty patients with bronchogenic carcinoma were then compared with 46 patients having other types of tumors and with 85 healthy controls.[149] The authors concluded that a person having the "intermediate" phenotype has a 16 times increased risk and a person having the "high" phenotype has a 36 times increased risk of developing bronchogenic carcinoma, compared with persons having the "low-inducibility" phenotype.

Because of the variability of the lymphocyte AHH determination and because several laboratories[119,150-153] have been unable to confirm readily the monogenic hypothesis, we undertook a study of twins (Figure 17) in order to assess the extent to which interindividual differences in lymphocyte AHH inducibility are under genetic control, irrespective of the mode of heritability. A heritable trait is measurable if monozygotic twins show a common response more frequently than dizygotic twins. This heritability index H can be quantitated by the equation:

$$H = (V_d - V_m)/V_d$$

in which V_d and V_m denote, respectively, the mean intrapair variance of dizygotic and monozygotic twins.[158] Hence, if V_d and V_m are the same, H is zero and variations in

* A recent clinical report[147] suggests the possibility of a cause-and-effect relationship between cataract formation and acetaminophen overdose.

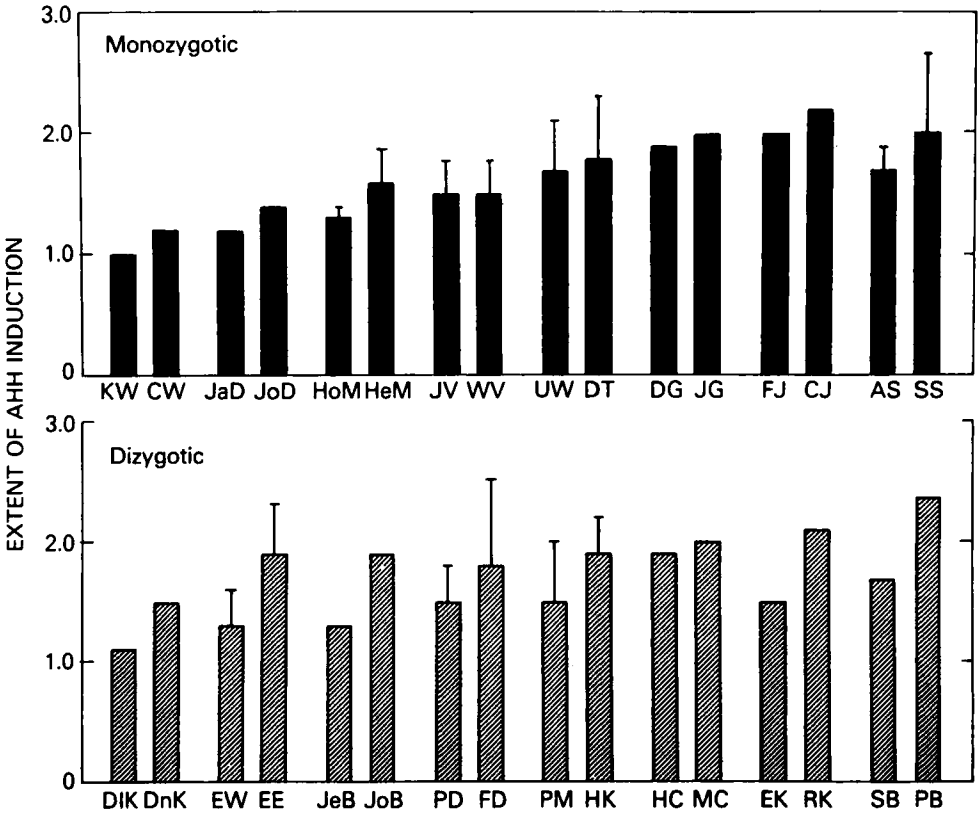


FIGURE 17. Distribution of AHH inducibilities in 54 individuals.[152] Each unit represents the mean induction ration from a single individual studied on 2 to 14 separate occasions. The solid areas represent 34 unrelated individuals and include as single units the intrapair means of 8 monozygotic twin pairs. The stippled areas denote ten sibling pairs (including eight pairs of dizygotic twins), each unit representing a single individual. (From Atlas, S. A., Vesell, E. S., and Nebert, D. W., *Cancer Res.*, 36, 4619, 1976. With permission.)

the trait are said to be due to environment (which includes experimental conditions). On the contrary, if variation between monozygotic twins is small compared with that between dizygotic twins, H approaches unity and the observed variations are said to be principally due to heredity. The H for our study with 16 twin pairs tested two to five times is 0.80 for all experiments and 0.77 using a single lot of fetal calf serum for all cultures, indicating a considerable heritable component for AHH inducibility in human cultured mitogen-activated lymphocytes.[152] Similar results have been obtained more recently with human cultured monocytes from twins.[154] The day-to-day experimental variation of this tissue culture system, however, has made it almost impossible to compare results obtained at different times.[119,150-153]

In conclusion, there exists sufficient evidence that heritable variation of AHH inducibility occurs in man. Experimental difficulties, however, make it impossible at this point in time to be certain whether AHH induction is controlled by a single genetic locus or by two or more loci (i.e., is polygenic). In a recent study[155] in which benzo[*a*]anthracene or aminophylline plus benzo[*a*]anthracene were used as inducers, the gene controlling AHH induction was assigned to human chromosome 2. Yet, it has also been reported[156] that two distinctly different forms of AHH activity — one induced by benzo[*a*]anthracene and another induced by dibutyryl cyclic AMP plus theophylline — are under independent regulation in tissue culture. Moreover, there was no method of telling in this study[155] whether the regulatory gene

cytosolic receptor on chromosome 2, for example, "derepresses" the hamster structural gene for P_1-450. If the regulatory gene for AHH induction is part of the "structural gene cluster" for AHH, as has been shown[157] to be the case for the "β-glucuronidase cluster" in mice, it might be argued[155] that the regulatory and structural genes for AHH activity both exist on human chromosome 2. In view of the *trans* genetic regulation of the *Ah* locus evoking increases in numerous structural gene products[6] (described in this review, for example) in the mouse, however, it is certainly too premature to suggest that the regulatory *Ah* gene and the AHH structural gene (i.e., P_1-450) are closely linked — in either the mouse or the human. At the present time, therefore, AHH inducibility or specific activity in cultured mitogen-activated lymphocytes or any other similar test system is not a reliable enough test to be used as a biochemical marker for determining who is at risk for bronchogenic carcinoma or other cancers.[73,119]

Summary of Conditions in Mouse Associated with the *Ah* Locus

In addition to causing tumors, polycyclic hydrocarbons and other chemicals and drugs cause toxicity under varying experimental conditions, presumably related to metabolic potentiation by cytochrome(s) P_1-450 controlled by the *Ah* locus (Table 3). 7,12-Dimethylbenzo[*a*]anthracene causes skin ulcers more readily in responsive strains than in nonresponsive strains; the offspring of the backcrosses and intercross were never studied,[161] but most likely this condition is associated with the responsive allele at the *Ah* locus. Shortened survival time after large i.p. doses of polycyclic hydrocarbons and birth defects likewise appear to be associated with the responsive allele at the *Ah* locus and are presumably due to increased steady-state levels of toxic polycyclic hydrocarbon reactive intermediates. On the other hand, increased metabolism associated with the Ah^b allele, is responsible for detoxification in the experimental conditions of: shortening the effect of zoxazolamine, protecting the animal from acute lindane tox-

TABLE 3

Tumorigenic or Toxicologic Phenomena Associated with Aromatic Hydrocarbon Responsiveness in Mice

	Ref.
Increased susceptibility to:	
MC-initiated s.c. sarcomas	52,103,159,160
Squamous cell carcinoma of the lung produced by intratracheal instillation of MC	61,102
BP-initiated s.c. sarcomas	52,73,102
Hepatic necrosis due to acetaminophen	70
Skin inflammation due to 7,12-dimethylbenzo[*a*]anthracene	161
Fetal toxicity and malformations due to administration of 3-methylcholanthrene or 7,12-dimethylbenzo[*a*]anthracene to the mother	25,162
Shortened survival time following:	
I.P. adminstration of polycycylic aromatic hydrocarbons or polychlorinated biphenyls	33
Oral administration of polychlorinated biphenyls	33
Increased resistance to:	
I.P. administration of lindane	33
Oral administration of polycyclic aromatic hydrocarbons or lindane	33
Paralysis produced by zoxazolamine	69
Aplastic anemia produced by oral BP	33,97,163
Leukemia induced by topical MC	99

icity, preventing aplastic anemia caused by daily ingestion of BP, and preventing leukemia caused by topical MC. Metabolic potentiation of acetaminophen, associated with an increase in *N*-hydroxylation by cytochrome(s) P_1-450, leads to GSH depletion, increased covalent binding of reactive intermediates, hepatotoxicity, and cataractogenesis.

Specific increases in the mutagenicity of MC, 6-aminochrysene, and 2-AAF in vitro are associated with cytochrome(s) P_1-450 and the Ah^b allele. Although the mutagenicity of BP in TA1535, TA1537 and TA1538 bacterial tester strains was not found[26,27] to be associated with the Ah^b allele, more recent data[164] with TA98 demonstrate such a correlation.

BP metabolite-nucleoside complexes formed by incubating radioactive BP with liver or skin microsomes and DNA in vitro can be separated by column chromatography and in some cases, partially identified; increases in eight out of nine such peaks are associated with cytochrome(s) P_1-450 and the Ah^b allele.[89] If one compares the responsive C3 with the responsive B6 inbred strain, there is a five- to six-fold higher carcinogenic index for s.c. BP in the C3.[52] This finding in vivo is not demonstrable in vitro by either the mutagenesis assay[165] or the binding of BP metabolites to DNA nucleosides,[166] indicating the difficulty that still exists in attempting to predict in vivo carcinogenicity of a compound by in vitro test systems.

SUMMARY

The *Ah* locus controls the induction of at least 20 monooxygenase activities and associated cytochrome(s) P_1-450 by MC and numerous other polycyclic aromatic compounds. AHH induction is associated with P_1-450 induction. *N*-acetylarylamine *N*-hydroxylase induction and the induction of some of the other 20 monooxygenase activities may be associated with other cytochromes since newly refined separatory techniques can resolve increases in more than two (perhaps five) moieties each believed to be a cytochrome associated with the Ah^b allele. One product of the regulatory *Ah* gene is believed to be a cytosolic receptor protein, which has a high affinity (apparent $K_d \cong 1$ n*M*) for polycyclic aromatic inducers and which appears to be defective (i.e., diminished affinity) in nonresponsive mice. Other induced macromolecules that appear to be under the same regulatory control include microsomal UDP glucuronosyltransferase, cytosolic reduced NAD(P):menadione oxidoreductase, and cytosolic ornithine decarboxylase.

Regulation of responsiveness probably involves several alleles at more than one locus, but differences between C57BL/6 (responsive, Ah^b) and DBA/2 (nonresponsive, Ah^d) mice can be almost completely explained by the difference at the *Ah* locus. Heterozygotes (Ah^b/Ah^d) are responsive, but other genetic crosses between appropriate inbred mouse strains can result in the expression of additive inheritance or a situation in which the lack of responsiveness is dominant. Responsiveness occurs not only in liver, but also in numerous nonhepatic tissues such as lung, kidney, bowel, skin, lymph nodes, retinal pigmented epithelium of the eye, bone marrow, ovary, testis, and mammary gland. Compared with Ah^d/Ah^d mice, Ah^b/Ah^b and Ah^b/Ah^d individuals have: a high inflammatory response to topical application of 7,12-dimethylbenz[*a*]anthracene; a high susceptibility to MC- and BP-induced s.c. sarcomas and MC-induced lung tumors; an increased resistance to zoxazolamine-induced paralysis, lindane toxicity, and BP-induced bone marrow toxicity and leukemia; and an increased susceptibility to acetaminophen-induced hepatic necrosis and cataract formation and to polycyclic hydrocarbon-induced birth defects, stillborns, resorptions, decreased birth weight, and ovarian primordial oocyte depletion. The Ah^b allele is associated in vitro with a high mutational rate in *Salmonella* by metabolic activation

of several chemical carcinogens and increases in numerous specific metabolites of chemical carcinogens bound to DNA nucleosides.

There exists sufficient evidence that heritable variation of AHH inducibility occurs in man. Experimental difficulties in the day-to-day variability of the AHH assay with cultured lymphocytes or monocytes, however, make it impossible at this time to be certain of whether this induction process is controlled principally by a single gene. It therefore remains to be determined whether this genotype can ever be used as a biochemical marker for predicting increased susceptibility to certain types of environmentally caused cancers or toxicity in man.

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