CARCINOGENICITY OF BENZO[a]PYRENE DERIVATIVES: THE BAY REGION THEORY

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Abstract - A substantial body of carcinogenicity data is available for benzo[a]-
pyrene (BP), benzo[a]anthracene (BA), and numerous known and potential metabolites
of these polycyclic hydrocarbons. The tumor data firmly establish that the 7,8-
dihydrodiol of BP and the 3,4-dihydrodiol of BA are proximate carcinogens and that
the 7,8-diol 9,10-epoxides and the 3,4-diol 1,2-epoxides are ultimate carcinogens
derived metabolically from the respective hydrocarbons. The data support the "bay
region" theory of hydrocarbon induced carcinogenicity which asserts that diol
epoxides on angular benzo rings of polycyclic hydrocarbons, in which the epoxide
ring forms part of a "bay region" of the hydrocarbon are prime candidates for
ultimate carcinogens. Perturbational molecular orbital calculations indicate that
electronic properties of the π-electron system are responsible for the unusually
high chemical reactivity of such diol epoxides.

INTRODUCTION

The polycyclic aromatic hydrocarbon benzo[a]pyrene (BP) is one of the most prevalent envi-
ronmental carcinogens to which man is exposed. As little as 0.10 μmol to 0.15 μmol of BP
applied once every two weeks to the backs of C57BL/6J mice produces a 100% incidence of
Squamous cell carcinoma on the skin of the animals while one 0.4 μmol dose followed twice
weekly by a promoting agent for 20-30 weeks causes a 90% tumor incidence in the animals, and
the tumor yield is 4-8 papillomas per mouse. In the United States alone, over 1300 tons of
this carcinogen are released into the environment each year as an unfortunate by-product of
numerous combustion processes (Ref. 1). Since BP is relatively inert, it is thought to
exert its carcinogenic activity through a metabolically formed reactive intermediate(s)
which covalently interacts with cellular macromolecules such as DNA, RNA, and protein (Ref.
2). BP, like other carcinogenic and noncarcinogenic aromatic hydrocarbons (Ref. 3 & 4), is
first converted to arene oxides at several positions by the cytochrome P-450 mixed function
oxidases in the endoplasmic reticulum of cells (cf. Ref. 5). Once formed, these arene
oxides are subject to spontaneous isomerization to phenols (Ref. 6), chemical or enzymatic
conjugation with the thiol group in glutathione (Ref. 7), enzymatic hydration to trans-
dihydrodiols by the action of epoxide hydrase (Ref. 8 & 9), and covalent interaction with
nucleophiles within the cell (Fig. 1). Formation of arene oxides at the 4,5-, 7,8-, and
9,10-positions are known to represent the substantial precentages of the total metabolism
of BP, and these arene oxides can isomerize spontaneously to phenols. The very unstable
1,2- and 2,3-arene oxides probably account for the 1- and 3-hydroxy-BP (1- and 3-HOBP)
known to be metabolites. In addition, 6-HOBP is formed and autoxidized to quinones
involving the 6-position (Ref. 10 & 11). Since the reactive metabolite(s) responsible for
the biological activity of BP may represent only a very small percentage of the total metabo-
lites and since the reactive species may not be directly detectable due to its reactivity,
metabolism studies may be of limited value in attempting to identify the suspect molecule.
For this reason, our laboratories undertook a systematic evaluation of all the known and
potential metabolites of BP which could be readily synthesized (Ref. 12) in an attempt to
identify the metabolic pathway(s) responsible for the carcinogenic activity. Although other
approaches to the identification of ultimate carcinogens from BP, such as mutagenicity, cell
transformation, and covalent binding to nucleic acid, have been undertaken by other labora-
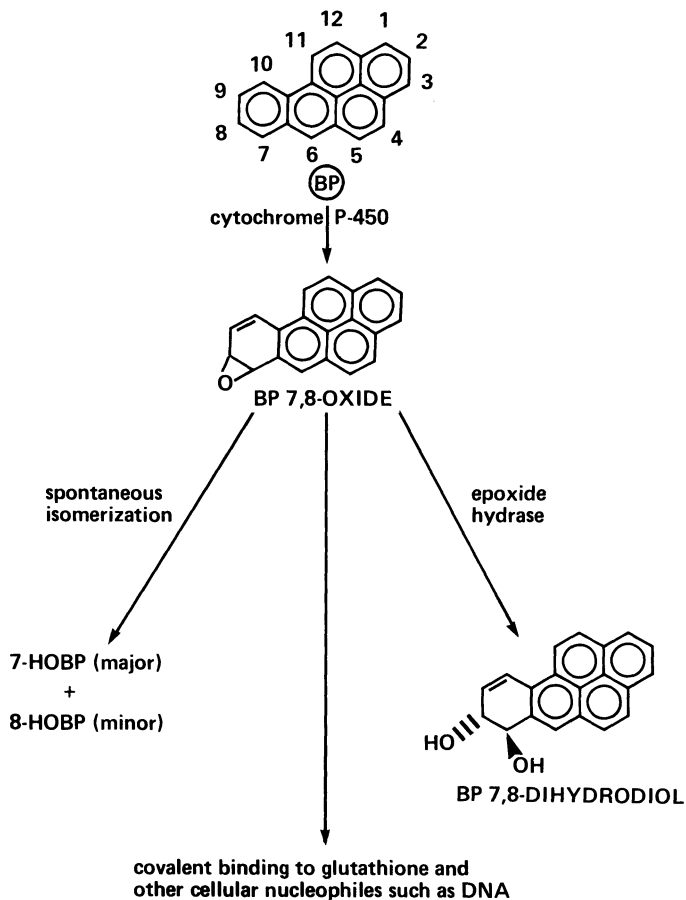tories as well as our own (cf. Ref. 13), only carcinogenicity data will be considered here.

Fig. 1. Selected metabolites of benzo[a]pyrene
which arise from benzo[a]pyrene 7,8-oxide.

CARCINOGENICITY OF BP DERIVATIVES

Carcinogenicity of arene oxides

Carcinogenicity of BP and BP 4,5-, 7,8-, 9,10-, and 11,12-oxides has been tested by applica-
tion of each compound once every two weeks to the backs of C57BL/6J mice for 60 weeks (Ref.
14 & 15). Doses of 0.1 µmol and 0.4 µmol were selected (Fig. 2) in order to be able to
detect carcinogens which are significantly less active than BP, since the dose of 0.1 µmol
causes tumors in nearly all of the animals treated with BP. Both BP 9,10- and 11,12-oxides
were inactive at the doses tested, and BP, 4,5-oxide produced only one skin tumor among the
30 mice tested. In contrast, BP 7,8-oxide was a potent carcinogen at the high dose,
although clearly less active than BP when tested at the lower dose. Histological examina-
tion established that almost all of the tumors produced by BP and BP 7,8-oxide were Squamous
cell carcinomas. Thus, both BP and BP 7,8-oxide are complete carcinogens on mouse skin.
Preliminary data (unpublished) are now available on the carcinogenic activity of these four
arene oxides upon intraperitoneal injection into newborn mice. As in the skin model, BP
7,8-oxide is much more tumorigenic (pulmonary adenomas) than the 4,5-, 9,10-, and 11,12-
oxides of BP. These arene oxides have also been tested for their skin tumor initiating
abilities. In these experiments, a single dose of 0.2 µmol of each compound was applied
once to the backs of CD-1 mice followed by twice weekly applications of 10 µg of the promot-
ing agent 12-O-tetradecanoylphorbol-13-acetate (16,17). The data parallel the chronic
studies on skin and the experiments in the newborn in that BP 7,8-oxide had appreciable but
lower tumor-initiating activity than BP while the other three arene oxides were much weaker
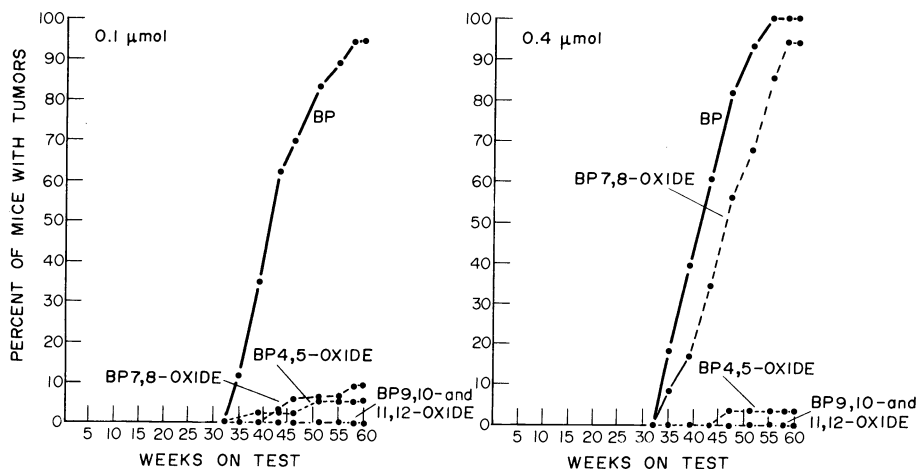in activity.

Fig. 2. Skin tumors produced by application of BP oxides once every two weeks to the backs of male C57BL/6J mice.

Carcinogenicity of phenols

Several experiments have been done to determine whether BP 7,8-oxide acts directly as a carcinogen. Since non-K-region arene oxides readily isomerize to phenols (6), the twelve possible isomeric phenols of BP were tested as complete carcinogens by chronic application to mouse skin at the 0.4 μmol dose (Fig. 3, Ref. 15,18) with the result that 2-HOBP was
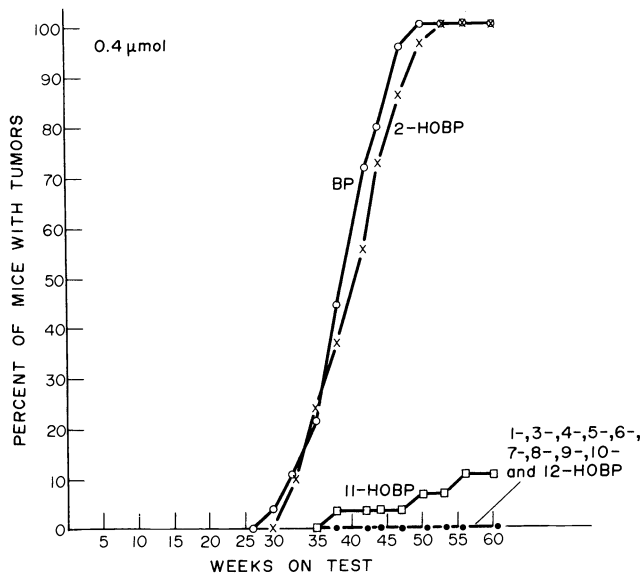

Fig. 3. Skin tumors produced by application of BP phenols once every two weeks to the backs of male C57BL/6J mice.

equipotent to BP, 11-HOBP was weakly active and the remainder of the phenols were inactive. Similar results were obtained from initiation-promotion experiments (19) where 11-HOBP was moderately active, BP and 2-HOBP were strong tumor intitiators, and the remaining phenols had less than 5% of the activity of BP. Clearly, the activity of BP 7,8-oxide cannot be due to its phenolic isomerization products. Although 6-HOBP has been considered as a possible ultimate carcinogen because of its facile conversion to 6-oxy-BP (Ref. 20 & 21), this possibility seems unlikely in light of the present data and its lack of activity in the newborn mouse (unpublished). The surprising high activity of 2-HOBP, which is even more active than BP in the newborn mouse (unpublished), is under further study.

Carcinogenicity of dihydrodiols
Since BP 7,8-oxide is the metabolic precursor of BP 7,8-dihydrodiol (Fig. 1), the dihydro-
diol was tested as a complete carcinogen by chronic application to mouse skin (Fig. 4, Ref.
22). At a dose of 0.15 µmol once every two weeks, both BP and BP 7,8-dihydrodiol caused a
100% incidence of tumors in the animals. At the same dose, less that 20% of the animals
developed tumors when treated with BP 7,8-oxide. BP 9,10-dihydrodiol is practically in-
active as a carcinogen under similar conditions (unpublished). Decreased activity of the
labile arene oxide relative to the dihydrodiol may be due in part to isomerization of the
arene oxide to inactive phenols. Since BP 7,8-dihydrodiol is too stable chemically to be
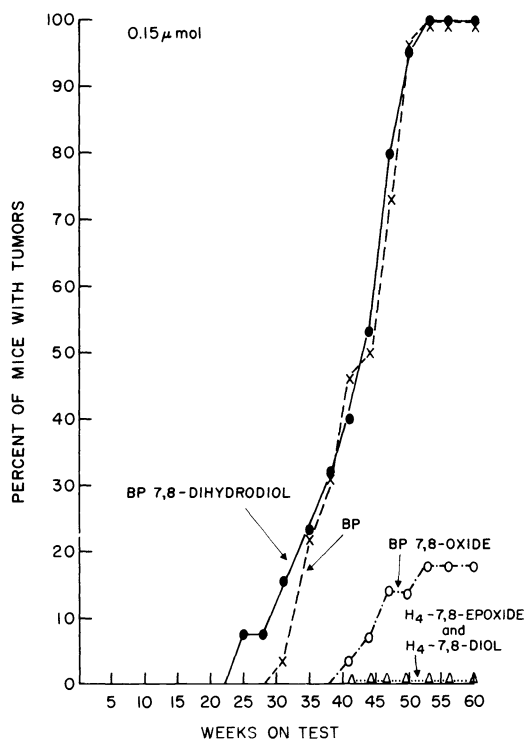

Fig. 4. Skin tumors produced by application of BP 7,8-dihydrodiol and
related compounds once every two weeks to the backs of male C57BL/6J mice.

considered as a reactive metabolite, both BP 7,8-oxide and BP 7,8-dihydrodiol must be con-
sidered as proximate carcinogens. Complete inactivity of 7,8,9,10-tetrahydro BP 7,8-
epoxide and 7,8,9,10-tetrahydro BP 7,8-diol as carcinogens (Fig. 4, Ref. 22,23) indicates
that the 9,10-double bond is important in their sequential metabolic transformation into
ultimate carcinogens. If BP 7,8-dihydrodiol is a proximate carcinogen from BP, it should
be significantly more active than BP as a carcinogen. When BP and the 7,8-dihydrodiol
were compared for tumorigenicity in the dose range of 0.025-0.10 µmol, the dihydrodiol was
indeed more active but only to a small extent (Ref. 23). Attempts to demonstrate higher
activity for the dihydrodiol by initiation-promotion experiments have indeed shown BP 7,8-
dihydrodiol to be a potent initiator although the dihydrodiol was found to be either equal
to (Ref. 16,17) or slightly less active (Ref. 24) than BP. Since all of the dihydrodiols
produced from BP by liver microsomes are highly optically active (25), the optical
enantiomers of BP 7,8-dihydrodiol were examined in an initiation-promotion experiment (26).
The data indicate the the (-)-enantiomer is 5- to 10-fold more potent than the (+)-
enantiomer as a tumor initiator (Fig. 5) and that the (-)-enantiomer caused about 50% more
papillomas per mouse than did BP at an initiating dose of 0.1 µmol. The initiation-
promotion study which found BP 7,8-dihydrodiol slightly less active than BP (Ref. 24) is
somwhat surprising in light of the fact that the dihydrodiol used was biosynthetic. Since
this dihydrodiol was probably the (-)-enantiomer (Ref. 25), much higher activity would be
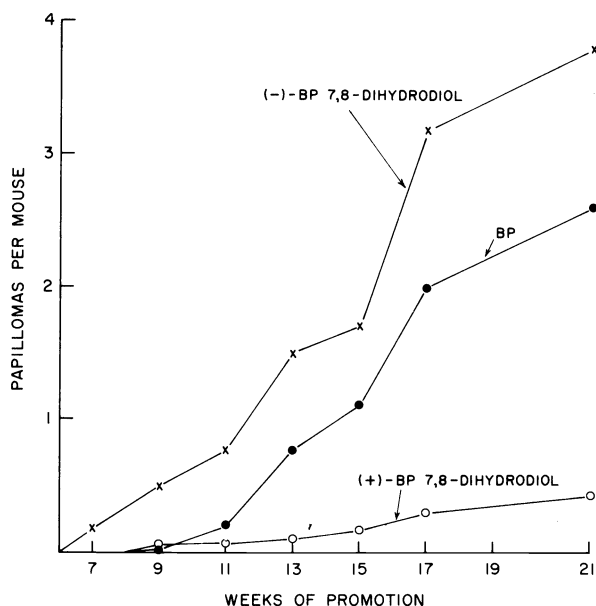expected.

Fig. 5. Papillomas produced on the skin of CD-1 mice after a single initiating
dose of 0.10 µmol of (+)- and (-)-BP 7,8-dihydrodiol followed by twice weekly
application of 16 nmol of 12-0-tetradecanoylphorbol-13-acetate as a promoting agent.

Carcinogenicity of 7,8-diol-9,10-epoxides

During the course of the above tumor studies, Borgen et al. (Ref. 27) found that liver micro-
somal metabolism of BP 7,8-dihydrodiol resulted in more extensive binding to added DNA than
did metabolism of BP or several other metabolites of BP. Secondary oxidative metabolism of
all primary oxidative metabolites of BP was reported (Ref. 28), a "diol oxide" was suggested
(Ref. 3) as one of several secondary oxidative metabolites which might account for the
metabolism-induced binding of BP 7,8-dihydrodiol to DNA, and Sims et al. (Ref. 29) provided
evidence that a 7,8-diol-9,10-epoxide was the metabolite responsible for the binding. Metab-
olism of BP 7,8-dihydrodiol could produce either or both diastereomerically related diol
epoxides in which the epoxide oxygen is either cis (isomer 1 series) or trans (isomer 2
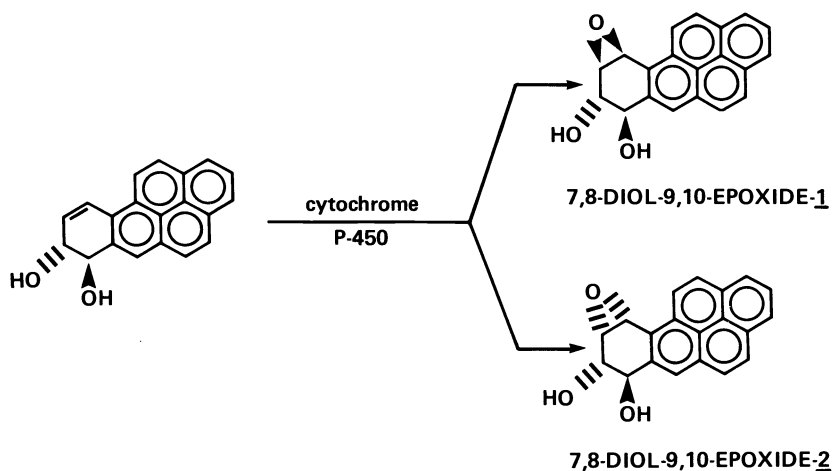series) to the benzylic hydroxyl group at position-7 (Fig. 6). The stereochemical situation

Fig. 6. Structures of the diastereomerically related BP 7,8-diol-
9,10-epoxides which form on metabolism of BP 7,8-dihydrodiol.

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is of particular interest since the close proximity of the 7-hydroxyl group to the epoxide oxygen in isomer 1 causes markedly enhanced chemical reactivity relative to isomer 2, presumably due to anchimeric assistance to attack on the epoxide (Ref. 30,31). When the BP 7,8-diol-9,10-epoxide isomers 1 and 2 were tested as complete carcinogens on mouse skin by application of 0.02 μmol to 0.40 μmol of each compound once every two weeks for 60 weeks (Table 1, Ref. 23), isomer 1 was inactive while isomer 2 caused tumors in only 13% of the

TABLE 1. Carcinogenicity of benzo[a]pyrene and benzo[a]pyrene
7,8-diol-9,10-epoxides by application once every two
weeks to the backs of C57BL/6J mice for 60 weeks.

Compound	Dose every two weeks (μmol)	Percent of mice with tumors
BP	0.40	100
	0.10	50
	0.02	4
BP 7,8-diol-9,10-epoxide-1	0.40	0
	0.10	0
	0.02	0
BP 7,8-diol-9,10-epoxide-2	0.40	13
	0.10	7
	0.02	0

animals compared to a 100% tumor incidence for BP at the high dose. Similarly, isomer 2 had only 20-30% of the initiating activity of BP while isomer 1 had only 1% (Ref. 16 & 17). Failure of the diol epoxides to be more active than the parent hydrocarbon, a requirement for proof of an ultimate carcinogen, in this tumor model may be a consequence of their high chemical reactivity. Although the diol epoxide isomers are potent hyperplastic agents, presumably through interaction at the surface of skin cells (Ref. 32), they may not be effective in reaching receptors for tumor initiation deep within the cells. The newborn mouse has provided a very useful model for studies of the tumorigenic activity of BP 7,8-dihydrodiol and the 7,8-diol-9,10-epoxides (Table 2, Ref. 33 & 34). At a total dose of 1400 nmol, BP caused about 6 lung adenomas per animal while the 7,8-dihydrodiol produced

TABLE 2. Tumorigenicity of benzo[a]pyrene, benzo[a]pyrene 7,8-dihydrodiol and benzo[a]pyrene 7,8-diol-9,10-epoxide-2 in newborn Swiss-Webster mice.[a]

Compound	Total dose (μmol)	No. of animals autopsied	Percent of mice with malignant lymphomas	Percent of mice with lung adenomas	Lung adenomas[b] per animal
Control	0	48	0	4	<0.1
BP	1400	45	0	84	6.3±1.0
BP 7,8-dihydrodiol	1400	50	70	74	75±14
BP 7,8-diol-9,10-epoxide-2	28	37	0	81	4.9±0.8

[a]Compounds were administered in three injections on day 1, 8, and 15 of life.

[b]The experiment was terminated when the animals were 24 weeks of age.

about 75 lung adenomas per animal. At the very low dose of 28 nmol (Ref. 34), animals treated with BP, BP 7,8-dihydrodiol, and BP 7,8-diol-9,10-epoxide-2 developed 0.24, 1.77, and 4.42 pulmonary adenomas per mouse, respectively. This sequential increase in tumorigenic activity clearly establishes BP 7,8-diol-9,10-epoxide-2 as an ultimate carcinogen in this tumor model and provides the first example of an ultimate carcinogen in the polycyclic aromatic hydrocarbon series based on tumor data. Due to very high toxicity, the tumorigenic activity of BP 7,8-diol-9,10-epoxide-1 has yet to be accurately assessed (Ref. 33).

THE BAY REGION THEORY

In an attempt to generalize the concept of BP 7,8-diol-9,10-epoxides as ultimate carcin-
ogens from BP to other polycyclic aromatic hydrocarbons, we proposed a concept (Ref. 35)
now known as the "bay-region" theory. The foundation of this theory rests on our initial
observations (Ref. 30) of remarkably high chemical reactivity for the epoxide ring in the
BP diol epoxides. The fundamental question then became one of identifying those factors
responsible for this unusual reactivity. A unique structural feature of the 7,8-diol-9,10-
epoxides is that the epoxide ring forms part of a "bay-region" in the hydrocarbon. For BP,
the "bay-region" is the hindered area between the 10- and 11-positions. The term "bay-
region" originates from the field of proton magnetic resonance where hydrogens in such
positions have been noted to resonate below the usual aromatic envelope due to edge deshield-
ing by the proximate aromatic ring (Ref. 36). The simplest example of a "bay-region" is
the hindered area between the 4- and 5-positions of phenanthrene. The basic hypothesis of
the "bay-region" theory is that a diol epoxide be formed on a saturated, angular benzo-
ring such that the oxirane ring forms part of a "bay-region" of the hydrocarbon. A
principle role of the dihydrodiol as a precursor is to provide a metabolic pathway to an
aliphatic epoxide rather than an arene oxide since aliphatic epoxides are more susceptible
to nucleophilic attack relative to other solvolytic processes (Ref. 37). This point is
illustrated by the fact that the 7,8-diol-9,10-epoxides of BP hydrolyze to mixtures of
tetraols with only trace amounts of the 7,8-diol-9,10-keto isomerization product produced
in the acidic to basic pH range (Ref. 31). Initial biological support for the "bay-region"
theory came from reinterpretation of existing carcinogenicity data which indicated that
alkyl or halogen substitution on the critical benzo-ring markedly reduced carcinogenicity
relative to the parent hydrocarbon (Ref. 35, 38 & 39). Such substitution is expected to
substantially reduce the rate of enzymatic epoxidation at the formal double bond to which
the substituent is attached.

The fundamental reason for unusually high chemical reactivity of an epoxide on a saturated,
angular benzo-ring which forms part of a "bay-region" of the hydrocarbon derives from the
electronic nature of the π-skeleton which remains after saturation of the benzo-ring.
Pertubational molecular orbital calculations by the method of Dewar (Ref. 40) have been used
to estimate the ease of carbonium ion formation from all of the possible isomeric diol
epoxides on terminal-rings of a large number of polycyclic hydrocarbons (Ref. 41 & 42). The
calculations estimate the energy change in the π-electron system for the conversion
Ar → ArCH$_2$+ as a model for the reactivity of diol epoxides. Application of these calcu-
lations to the hydrocarbon benzo[a]anthracene is illustrated in Fig. 7 (see next page),
where larger values of $\Delta E_{deloc}/\beta$ signify greater ease of carbonium ion formation. As is
shown for benzo[a]anthracene, formation of a carbonium ion on a saturated, terminal ring
is always easiest when this carbonium ion forms part of a "bay-region." The calculations
also allow a qualitative ranking of hydrocarbons for carcinogenicity (Fig. 7) based on the
ease of formation of the "bay-region" carbonium ion (Ref. 39, 41 & 42).

TUMORIGENICITY OF BENZO[a]ANTHRACENE DIHYDRODIOLS

The hydrocarbon benzo[a]anthracene (BA) was selected as the first compound to be examined
as a test of the "bay-region" theory. Even though BA is considered to be a rather weak
carcinogen, the perturbational molecular orbital calculations indicate the ease of carbon-
ium ion formation in the "bay-region" is quite high ($\Delta E_{deloc}/\beta = 0.766$) when compared to
the more potent carcinogen dibenzo[a,h]anthracene ($\Delta E_{deloc}/\beta = 0.738$, Fig. 7, see next page).
Thus, the metabolic precursor of BA 3,4-diol-1,2-epoxides was anticipated to show strong
biological activity. Furthermore, BA provides an excellent model for studies on the much
more potent carcinogens 7-methylbenzo[a]anthracene, 7,12-dimethylbenzo[a]anthracene, and
3-methylcholanthrene since all four hydrocarbons share the same π-electron skeleton. The
five metabolically probable dihydrodiols (Fig. 8) and most of the corresponding diol epoxides
were synthesized for biological testing (Ref. 43 & 44). Initial experiments on the ability
of the cytochrome P-450 system to metabolically activate BA and the dihydrodiols to mutagens
(Ref. 45) and on the mutagenic activity of the diol epoxides toward bacterial and mammalian
cells (Ref. 46) provided strong support for the "bay-region" theory in that the BA 3,4-
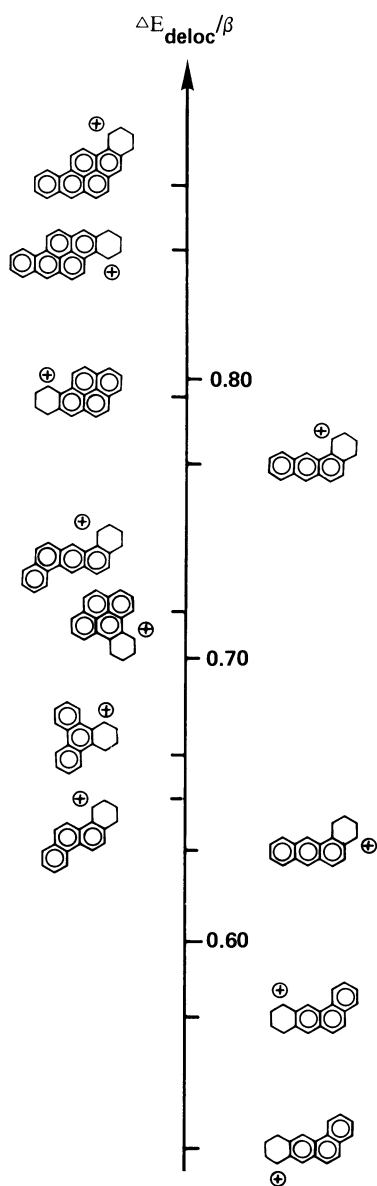dihydrodiol and the diastereomeric BA 3,4-diol-1,2-epoxides had the highest activity.

Fig. 7. Comparisons of the predicted ease of carbonium ion formation
for the four possible benzylic carbonium ions from benzo[a]anthracene (right)
and for the benzylic "bay region" carbonium ions for several hydrocarbons (left).
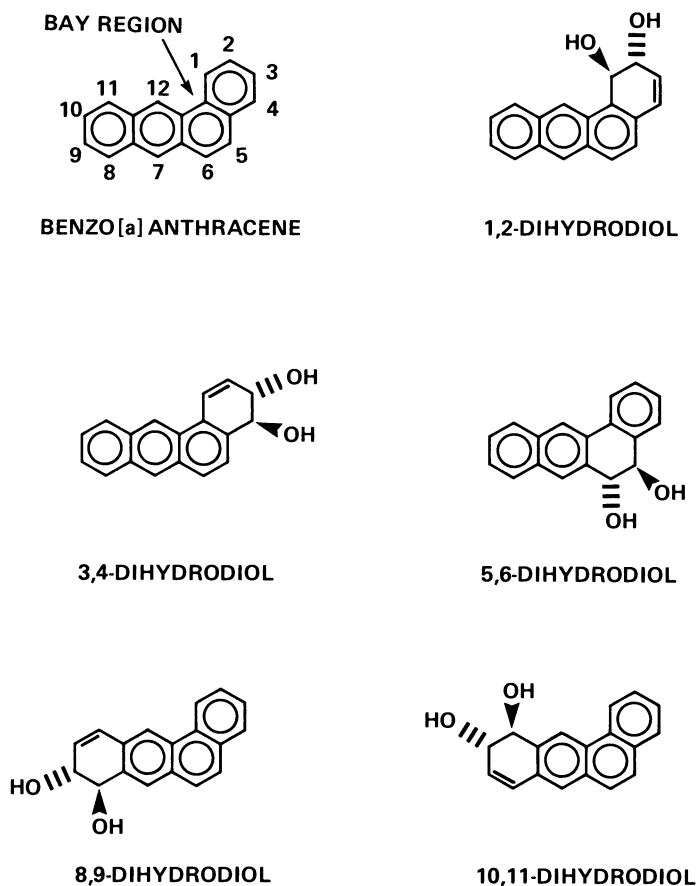
Fig. 8. Structures of the five metabolically probable dihydrodiols of benzo[a]anthracene.

BA and the five dihydrodiols (Fig. 8) were tested for their ability to initiate skin tumors in CD-1 female mice (Ref. 47). Eighteen days after a single topical application of 0.4-2.0 μmol of each compound, the mice were treated twice weekly with the skin promoter 12-0-tetradecanoylphorbol-13-acetate. After twenty weeks, comparisons of latency period, percent of mice with tumors, and number of papillomas per mouse all indicated that the BA 3,4-dihydrodiol was 10- to 20-fold more tumorigenic than the parent hydrocarbon which was more active than the other four dihydrodiols (Ref. 47, Table 3). Experiments in the newborn mouse were even more dramatic (Ref. 48). Animals were given a total dose of 2800 nmol of either BA or each of the dihydrodiols. The BA 3,4-dihydrodiol caused 30-fold more pulmonary adenomas than did BA while the other dihydrodiols had little or no activity. In addition, 28% of the mice treated with the 3,4-dihydrodiol developed malignant lymphoma. Both the promotion experiments (Ref. 47) and the experiments in newborn mice (Ref. 48) provide clear evidence that the 3,4-dihydrodiol is a proximate carcinogen derived from BA. Preliminary results of initiation-promotion experiments with the BA 3,4-diol-1,2-epoxides on the skin of CD-1 mice indicate that these diol epoxides are more active than BA in this tumor model (unpublished). These tumor experiments and the mutagenicity experiments on the diol epoxides (Ref. 46) implicate the BA 3,4-diol-1,2-epoxides as ultimate carcinogens from BA and provide strong support for the "bay-region" theory.

TABLE 3.   Tumor induction in CD-1 mice by benzo[a]anthracene and
           benzo[a]anthracene dihydrodiols after 20 weeks of promotion
           with 12-0-tetradecanoylphorbol-13-acetate.

Compound (2.0 μmol)	Surviving animals	Tumor animals	Total tumors	Tumors per survivor
Control	30	1	1	0.03
BA	30	7	9	0.30
BA 1,2-dihydrodiol	30	2	2	0.07
BA 3,4-dihydrodiol	30	24	144	4.80
BA 5,6-dihydrodiol	30	6	6	0.02
BA 8,9-dihydrodiol	29	5	6	0.21
BA 10,11-dihydrodiol	29	4	4	0.14

CONCLUSIONS

Since definitive proof for the identification of an ultimate carcinogen can only come from
carcinogenicity studies, there are only two examples of polycyclic aromatic hydrocarbons for
which ultimate carcinogens are known: the 7,8-diol-9,10-epoxides from BP and the 3,4-diol-
1,2-epoxides of BA where the epoxide forms part of a "bay-region" in each case.   Additional
indirect support is, however, available in support of the "bay-region" theory.   Studies on
the metabolic activation of chrysene and its three metabolically probable dihydrodiols
(Fig. 9) to a compound mutagenic to bacteria have shown that the 1,2-dihydrodiol which has a

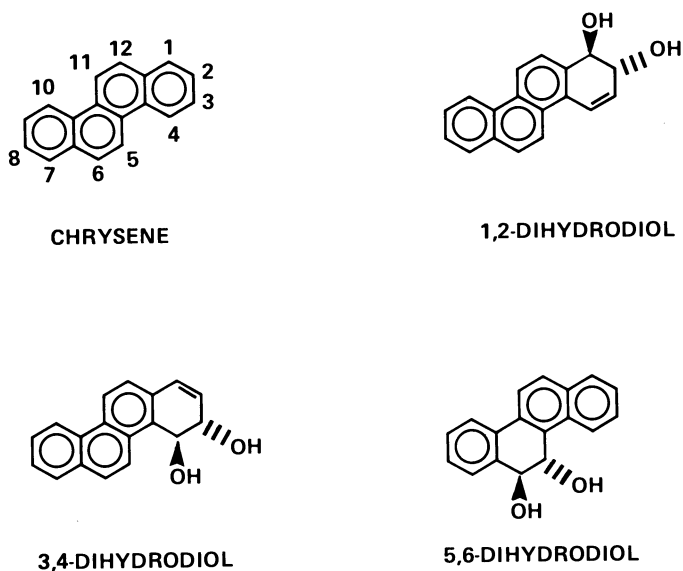CHRYSENE

1,2-DIHYDRODIOL

3,4-DIHYDRODIOL

5,6-DIHYDRODIOL

Fig. 9. Structures of the three metabolically
probable dihydrodiols of chrysene.

"bay-region" double bond produced a 20-fold higher mutagenic response than chrysene or the
other two dihydrodiols (Ref. 49).   Interestingly, attempts to form mutagens from trans-1,2-
dihydroxy-1,2,3,4-tetrahydrochrysene were without success thereby suggesting 1,2-diol-3,4-
epoxides as the probable mutagens formed from the 1,2-dihydrodiol.   Studies on the ability
of drug metabolizing enzymes to convert 7-methylbenzo[a]anthracene and its isomeric
dihydrodiols into bacterial mutagens (Ref. 50) and into compounds which transform cells in
culture (Ref. 51) have demonstrated that the 3,4-dihydrodiol with the "bay-region"
double bond produces the most activity.   Furthermore, studies of the metabolism induced
binding of 7,12-dimethylbenzo[a]anthracene to the DNA of cultured cells have shown a bound

adduct which has a 1,2,3,4-tetrahydro-7,12-dimethylbenzo[a]anthracene chromophore (Ref. 52).
The 3,4-diol-1,2-epoxides of the parent hydrocarbons (Fig. 10) are probably the active
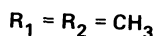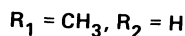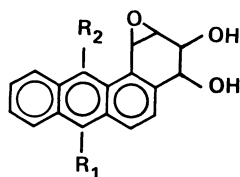

$R_1 = CH_3, R_2 = H$

$R_1 = R_2 = CH_3$

Fig. 10. Structures of the probable carcinogens
formed from 7-methyl-, and 7,12-dimethylbenzo[a]anthracene.

agents in each case. These latter studies are particularly important in that they suggest
that the "bay-region theory applies to substituted as well as unsubstituted polycyclic
aromatic hydrocarbons.

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