Synergetic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: a bio-historical perspective with updates

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B[α]P (benzo[α]pyrene) has been used as a prototype carcinogenic PAH since its isolation from coal tar in the 1930’s. One of its diol epoxides, BPDE-2, is considered its ultimate carcinogen on the basis of its binding to DNA, mutagenicity and extreme pulmonary carcinogenicity in newborn mice. However, BPDE-1 has a similar binding to DNA and mutagenicity but it is not carcinogenic. In addition, BPDE-2 is a weak carcinogen relative to B[α]P when repeatedly applied to mouse skin, the conventional assay site. Its carcinogenicity is increased when applied once as an initiator followed repeatedly by a promoter. This indicates a major role for promotion in carcinogenesis by PAHs. Promotion itself is a 2-stage process, the second of which is selective propagation of the initiated cells. Persistent hyperplasia underlies selection by promoters. The non-carcinogenicity of BPDE-1 has yet to be resolved.

PAHs have long been considered the main carcinogens of cigarette smoke but their concentration in the condensate is far too low to account by themselves for the production of skin tumors. The phenolic fraction does however have strong promotional activity when repeatedly applied to initiated mouse skin. Several constituents of cigarette smoke are co-carcinogenic when applied simultaneously with repeated applications of PAHs. Catechol is co-carcinogenic at concentrations found in the condensate. Since cigarette smoking involves protracted exposure to all the smoke constituents, co-carcinogenesis simulates its effects. Both procedures, however, indicate a major role for selection in carcinogenesis by cigarette smoke. That selection may operate on endogenous mutations as well as those induced by PAHs. There are indications that the nicotine-derived NNK which is a specific pulmonary carcinogen in animals contributes to smoking-induced lung cancer in man. Lung adenoma development by inhalation has been induced in mice by the gas phase of cigarette smoke. The role of selection has not been evaluated in either of these cases.

Modern research on chemical carcinogenesis began with the isolation of benzo[α]pyrene (B[α]P) from coal tar in 1930, and the demonstration that B[α]P and several synthetic polycyclic aromatic hydrocarbons (PAHs) induced tumors upon repeated painting on mouse skin. Many fruitful years of biological and chemical experimentation followed but a new era was marked by the finding that some PAH metabolites, namely their vicinal bay region diol epoxides, formed covalent adducts with DNA. It was generally agreed that the resultant mutations in DNA were the essential initiating steps in carcinogenesis and that one of the diol epoxide enantiomers was the ultimate carcinogen. The most carefully studied ultimate carcinogen was B[α]P diol epoxide-2 (BPDE-2), one of four enantiomeric diol epoxides of B[α]P, which lived up to expectations by being far more effective than its parent hydrocarbon in the induction of lung tumors in newborn mice upon intraperitoneal injection. However, BPDE-2 was far less effective than B[α]P in producing epidermal tumors on repeated topical application to mouse skin despite the demonstration that it bound to DNA as well after its application, as did BPDE-2, which was formed metabolically after applying B[α]P. Although the tumorigenic activity of a single topical application of BPDE-2 was enhanced by following it with repeated treatment of the skin with the promoting agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in an initiation–promotion sequence, it was still less effective than promotion after B[α]P initiation. Furthermore, the enantiomer BPDE-1, which had a similar overall binding capacity to epidermal DNA as BPDE-2, was far less active as either a complete carcinogen or an initiator. These and other results suggested that other metabolites of B[α]P may have participated in skin tumor induction, perhaps in a manner synergistic to that of DNA adduct formation.

The foregoing inconsistencies occasioned the present re-examination of a wide range of results in the biology of carcinogenesis by PAHs, including early pathogenesis of PAH-induced skin tumors, localization and persistence of PAHs in skin, early chromosomal changes, transformation of cells in culture, cytotoxicity and mutagenicity. Detailed consideration is given to the role of the two-stage initiation–promotion sequence (later evolved into an additional stage of promotion) which exhibited synergistic interactions between the stages, and first raised the possibility that some metabolites of B[α]P other than the diol epoxides act as promoters which introduce a large element of selection in the carcinogenic process. Promotion by chemicals other than PAHs in coal tar is also considered, based on findings with cigarette smoke condensate which exhibits strong promotional activity in fractions that contain no PAHs. It is also likely that PAHs and their metabolites have indirect mechanisms of carcinogenesis, exemplified by the production of reactive oxygen and nitrogen species, that are complementary to the mutations induced by the diol epoxides. Several possible directions for future research are indicated with emphasis on the disposition of the PAHs and their metabolites, which could help to clarify the mechanisms involved in skin carcinogenesis by the PAHs.

Abbreviations: 4-α-PDD, phorbol-12,13-didecanoate with the 4-hydroxyl in the α position; B[α]P, benzo[α]pyrene; B[ε]P, benzo[ε]pyrene; BPDE, B[α]P 7,8-dihydrodiol-9,10-epoxide; BPDE-1, also called the syn or cis isomer and diol epoxide I; BPDE-2, also called the anti or trans isomer and diol epoxide II; DBA, dibenz[a]anthracene; DB[a,c]A, dibenz[a,c]anthracene; DBP, dibenzol[a]pyrene; DMB, 7,12-dimethylbenz[a]anthracene; HeLa, human cervical cancer cells; MCA, 3-methylcholanthrene; MMS, methyl methane-sulfonate; NNK, nicotine-derived nitrosamine ketone; PAHs, polycyclic aromatic hydrocarbons; PDD, phorbol-12,13-didecanoate; RPA, phorbol-12-tetradecanoate-13-acetate (originally called PPA but later and here called RPA); TPA, 12-O-tetradeconylophorbol-13-acetate, also known as phorbol 12-myristate-13-acetate (also called PMA).
Most studies of carcinogenesis by tobacco smoke have used mouse skin as the target organ. The concentrations of PAHs in tobacco smoke are far too low to be carcinogenic in skin by themselves, and require the presence of promoters and/or co-carcinogens to induce tumors. The presence of these cofactors in effective concentrations in cigarette smoke condensate indicates that selection of mutated cells makes a significant contribution to carcinogenesis by tobacco smoke. Recently, a reproducible method for producing lung tumors in mice by inhalation of tobacco smoke has been developed. The results indicate that volatile pulmonary carcinogens are present in the gas phase of tobacco smoke.

There is currently sharp disagreement about the relative roles in human pulmonary carcinogenesis of the direct induction of mutations by PAHs in tobacco smoke versus the selection of endogenous mutations. The disagreement is based on differing interpretations of base changes in codons of the p53 gene of human lung cancers. It is unlikely that the issue can be fully resolved without taking into account the presence in tobacco smoke of (i) grossly subcarcinogenic concentrations of active PAHs, (ii) PAHs that are initiators but have little or no activity as complete carcinogens, (iii) effective concentrations of promoters and co-carcinogens for selective clonal expansion, (iv) N-nitrosamines which selectively induce lung tumors in rodents regardless of the route of administration, and (v) gas phase components that induce lung tumors in susceptible mice by inhalation according to a particular regime. The situation is reminiscent of the finding in carcinogenesis of rabbit skin that unrefined coal tar induces tumors that appear earlier, enlarge much faster and are more likely to develop carcinomatous characteristics than those induced by concentrations of pure PAHs far higher than their concentration in the coal tar. The overall results indicate that many diverse substances in coal tar and in tobacco smoke interact via synergistic mechanisms in producing tumors. Selection of endogenous and smoke-induced mutations is likely to play a significant role in human lung carcinogenesis.

**Binding of PAH metabolites to purine residues in DNA**

The polycyclic aromatic hydrocarbons (PAHs) were the first pure compounds of known composition that were shown to cause cancer experimentally (1). This was definitively achieved with dibenz[a,h]anthracene (DBA) (Figure 1E) and its 3-methyl derivative, which had been synthesized and tested for tumor production in mouse epidermis because they had a fluorescence spectrum similar to the dominant spectrum associated with carcinogenic fractions of coal tar (1). Hieger then purified an alcohol extract of coal tar that was highly carcinogenic and exhibited the correct fluorescence (2,3). This product was identical with synthetic B[a]P (Figure 1A), a previously unknown PAH, which was also highly carcinogenic for mouse skin (2). Soon thereafter, many more PAHs were synthesized, a number of which were highly carcinogenic (3). The Kenaway group sought endogenous biochemical pathways for generation of carcinogenic hydrocarbons and the only endogenous compounds that appeared to be chemically related were the recently discovered steroids (4). The idea that a steroid-like hormonal action was responsible for the carcinogenic action of the PAHs gained credence with the finding that the steroidal ovarian hormone estrone induced mammary cancer in male mice. It was decided, however, that conversion of PAHs to steroids was unlikely to occur in vivo (5) and the matter was largely dropped for many years. There was a renewed interest when careful examination showed a remarkable steric resemblance between the carcinogenic PAHs, most of which contain four to five condensed aromatic nuclei, and the steroids (6). The authors’ analysis indicated a direct increase in carcinogenicity as the PAHs become stericly more similar to steroids (6). Interest in this relationship, however, virtually disappeared from sight when it was found that a small fraction of several radioactively labeled PAHs applied to mouse skin was firmly bound to DNA (7). The time course of the binding suggested that metabolism of the compounds was necessary to enable their reaction with DNA. Although there was also binding to RNA and protein, only the binding to DNA was correlated with the carcinogenicity of the PAHs for mouse skin as determined many years earlier (8). The binding to DNA lent weight to the widely held view that the essential step in carcinogenesis is a mutation or series of mutations.

It was then confirmed that the binding of PAHs represents the true extent of metabolic reaction between the hydrocarbons and DNA (9). However, the significance of the correlation between the extent of binding to DNA of mouse skin and carcinogenicity at that site was called into question by the observation of two instances in which the binding of a PAH to DNA was not associated with carcinogenicity. The binding of the non-carcinogenic compound DB[a,c]A (Figure 1F) to DNA of mouse skin was greater than that of its carcinogenic isomer DBA (9). And the binding of 7,12-dimethylbenz[a]anthracene (DMBA) to DNA of a strain of hairless...
mice, in which it produced no tumors, was identical to that in normal mice, in which carcinogenesis does occur. The authors suggested several alternative explanations for the discrepancy between DNA binding and carcinogenesis, including the possibility that the binding is necessary for carcinogenesis but some additional reaction or environmental condition modified by the carcinogen and its metabolites must contribute to eventual tumor formation. This introduced the possibility that some degree of selective growth is required before mutational change can be expressed in tumor formation.

A major clue about the PAH metabolites that bound covalently to DNA came from the report that B[a]P-7,8-diol, a metabolite of B[a]P, bound to DNA extracted from Syrian hamster cells to a 10-fold greater extent than did B[a]P itself (10). The precise identification of the metabolites bound to DNA of intact mammalian cells was revealed by experiments in which radioactively labeled B[a]P was incubated with primary cultures of Syrian hamster cells (11). The elution profiles of DNA hydrolysates from these cells had peaks that were coincident with those of hydrolyzed DNA obtained from incubation of the cells with B[a]P-7,8-dihydrodiol and of DNA from hamster cells incubated with B[a]P-7,8-dihydrodiol-9,10-epoxide. The conclusion was that the coincident peaks from the four treatments were B[a]P-7,8-dihydrodiol-9,10-epoxide-deoxyribosides. A second product was not further investigated, nor was a peak from incubation of the cells with B[a]P-9,10-dihydrodiol. The results indicated that B[a]P is metabolized in cells to the 7,8-dihydrodiol which is a precursor to the 7,8-dihydrodiol-9,10-epoxide (BPDE) (Figure 1B and C) that reacts with cellular DNA. Preliminary experiments indicated that analogous metabolic activation mechanisms applied to other PAHs. The B[a]P diol epoxides bind extensively to guanine residues of DNA (12) (Figure 1D) and the diol epoxides of 7,12-dimethylbenz[a]anthracene (DMBA) and of DB[a,l]P bind mainly to adenine residues of DNA (13,14).

Carcinogenesis and mutagenesis by PAHs and their metabolites

The great bulk of studies in the 1930s on the carcinogenic activity of PAHs was done by repeated painting on the skin of mice to produce papillomas and carcinomas (15). Some auxiliary studies, such as the persistence of the carcinogens in tissue were mainly done by single subcutaneous inoculation to produce sarcomas. In the 1940’s it was discovered that although a single painting of a low dose of the carcinogenic PAHs on mouse skin produced few if any tumors, subsequent repeated painting with croton oil, which by itself appeared to be non-carcinogenic, resulted in efficient tumor production (16). This method was used extensively by Berenblum and Shubik (17–19) to develop the two-stage concept of carcinogenesis. The first or initiating stage brought about by a PAH consists of a specific and irreversible conversion of normal cells to latent tumor cells that lie dormant until stimulated by croton oil in the promoting phase to become visible tumors (20). The majority of tumors resulting from the initiation–promotion procedure were benign papillomas. The promoting agent croton oil itself was initially thought to be non-tumorigenic in repeated painting even if followed by a single initiating dose of a carcinogenic PAH. Subsequent work to be considered later has changed this point of view and resulted in a more complex picture of promotion. The active ingredient of croton oil is TPA. Many of the papillomas in the initiation–promotion scheme eventually regress.

Since the repeated application of the carcinogenic PAHs alone results in production of tumors, many of which are malignant carcinomas (20), they are considered complete carcinogens (15). Complete carcinogens carry out both initiating and promoting functions. When used in both initiating and promoting stages, they yield a higher incidence of carcinomas than does initiation with a PAH and promotion by TPA (20). It was suggested that malignant tumor formation results from two or more carcinogen-induced mutations and that the role of promotion is to enlarge the size of the target cell population available for the second mutation (21). Papillomas induced by repeated carcinogen applications arise from significantly more cells than those induced by the carcinogen-promoter sequence (22). Since many of the papillomas in the initiation–promotion sequence regress when TPA applications cease, it was suggested that TPA allows the expression of the neoplastic phenotype in the initiated cells by a selective (23) or epigenetic mechanism (22). A selective role of TPA in neoplastic development was unequivocally demonstrated in spontaneous transformation of an established line of mouse fibroblasts in which the TPA promotional activity was effective only when continuously applied for at least 4 weeks to cells maintained under strong regulatory conditions (24).

Early testing of PAHs for mutagenic capacity in Drosophila, mice and microorganisms failed to yield consistent evidence of mutagenesis (25). Later results indicated that the active forms of the PAHs are generated by mammalian metabolism, in particular by the TPNH-dependent microsomal enzymes of liver (cited in ref. 26). Mixing of the PAHs with liver homogenates yielded products that were potent frameshift mutagens in certain mutants of Salmonella typhimurium (26).

Bacteria do not duplicate mammalian metabolism in activating carcinogens, so they can be used to test the mutagenic capacity of diverse pure compounds of the type that are formed during the metabolism of the PAHs in mammalian cells. Likewise, the V79 line of Chinese hamster cells does not have the capacity to metabolically activate PAHs, so it can also be used to test various PAH metabolites for mutagenic activity (27). The two mutagenesis test systems were used in combination with carcinogenic capacity in repeated application of B[a]P and its metabolites to mouse skin for 60 weeks to determine the relation of mutagenesis to carcinogenesis for each compound. The results, adapted from the extensive work of Conney and colleagues (28–31) are summarized in Table I. B[a]P, B[a]P-7,8-dihydrodiol and 2-HOBP are equally strong complete carcinogens on mouse epidermis but have no significant mutagenic power in either V79 hamster cells or S. typhimurium. B[a]P-7,8-oxide is a less active carcinogen with slight mutagenic power in S. typhimurium and none in V79 cells. B[a]P-7,8-diol-9,10-epoxide-2 (Figure 1C) (BPDE-2, also called the anti or trans isomer and diol-epoxide I, is a vicinal, bay region diol epoxide) has slight carcinogenic power on mouse epidermis and high mutagenic capacity in both test systems. Its isomer BPDE-1 (also called the syn or cis isomer and diol-epoxide II) (Figure 1B) produced no tumors even at the highest dose tested, but is highly mutagenic. B[a]P-4,5-oxide and 11-HOBP have slight carcinogenic activity and little or no mutagenic activity. The rest of the metabolites tested for both activities are non-carcinogenic and are at best slightly mutagenic in S. typhimurium. Thus there is a striking disjunction between strong epidermal carcinogenicity of B[a]P and its
Table I. Carcinogenicity mutagenicity of B[a]P and its metabolites*

(A)

<table>
<thead>
<tr>
<th>Amount per applicationb (µmol)</th>
<th>Compound</th>
<th>Skin carcinogenicity</th>
<th>Relative mutagenicityd</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Iball indexc</td>
<td>V79 cells</td>
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<tr>
<td>0.4</td>
<td>B[a]P</td>
<td>250</td>
<td>&lt;0.1</td>
</tr>
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<td>&lt;0.1</td>
</tr>
<tr>
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<td>&lt;0.1</td>
</tr>
<tr>
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<td>0.6</td>
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<td>&lt;0.1</td>
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<tr>
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<td>40</td>
<td>100</td>
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<tr>
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<td>0.6</td>
</tr>
<tr>
<td>B[a]P-9,10 and 11,12 oxide</td>
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<td>&lt;0.1</td>
<td>&lt;0.1</td>
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<tr>
<td>1 HOB[a]P 3-, 4-, 5-, 7-, 8-, 9-, 10- and 12-HOB[a]P</td>
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<td>B[a]P</td>
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*Adapted from Levin et al. (30).

(B)

<table>
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<tr>
<th>Compound</th>
<th>Amount per applicationb (µmol)</th>
<th>Mice with tumors (%)</th>
<th>Relative mutagenicityd</th>
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<tr>
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<td>100</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>H4 9,10 epoxide</td>
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<td>4</td>
<td>40</td>
</tr>
<tr>
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<td>0</td>
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*Compounds were applied topically on mouse skin once every 2 weeks for 60 weeks.

Iball index of carcinogenicity = Percentage of mice with tumors at 60 weeks × 100.

Mutagenicity is relative to that of one of the B[a]P 7,8 diol-9,10-epoxides rated as 100 in either V79 or Salmonella typhimurium.

metabolites on the one hand and strong mutagenicity on the other. The strong carcinogenicity of B[a]P-7,8-dihydrodiol, which is the precursor to the BPDEs, combined with the binding of the latter to DNA and their mutagenicity is the rationale for considering the B[a]P-7,8-dihydrodiol as the proximate carcinogen (32). The binding of BPDEs to DNA and their mutagenic potency qualify them as ultimate carcinogens, but their weak or non-carcinogenic capacity for the standard test as complete carcinogens on mouse epidermis has yet to be accounted for, as does the strong carcinogenesis by 2-HOBP, which is not a known precursor to the BPDEs, nor is it a known metabolite of B[a]P (33).

The capacity of B[a]P dihydrodiols to initiate tumor formation was tested in a single application followed by repeated treatment with the promoter TPA for 52 weeks (34). B[a]P-7,8-dihydrodiol was almost as active as initiator as B[a]P itself when measured by the number of mice that developed tumors, but significantly less active when measured by the number of tumors per mouse. However, in another study, B[a]P-7,8-dihydrodiol was as potent an initiator as B[a]P itself (35). The 4,5- and 9,10-dihydrodiols did produce tumors but were less active than the 7,8-dihydrodiol (34). When enantiomers of B[a]P-7,8-dihydrodiol were separately tested, the (−) enantiomer was more active and the (+) enantiomer was considerably less active than B[a]P (28). One of the diol epoxides had 20–30% the initiating activity of B[a]P and the other had only 1% the activity of B[a]P (36). The 9,10- and 11,12-oxides had 2 and 10% the tumor-initiating activity of B[a]P. Hence, both diol epoxides were somewhat more active as tumor initiators than as complete carcinogens, but considerably less active than B[a]P itself in both activities. The only metabolite found to be more potent as a tumor initiator than B[a]P was the (−) enantiomer of trans 7,8-dihydrodiol, which recommended it as a proximate carcinogen requiring further metabolism to an ultimate carcinogen, presumably the diol epoxides.

In a later study, one of four enantiomers of BPDE, designated (−)-BPDE-2, was ~60% as active a tumor initiator as B[a]P (37). The (−)-BPDE-2 and both enantiomers of BPDE-1 had
The diols of 3-methylcholanthrene (MCA) and DMBA capable of conversion to bay region vicinal diol epoxides (MCA-9,10-diol and DMBA-3,4-diol) were not as active in tumor initiation as their parent hydrocarbons when measured in terms of the total number of tumors produced (38). The vicinal diol epoxides of DMBA applied at 100 nmol were less active as tumor initiators than one-tenth that concentration of DMBA itself, especially when evaluated as tumors per mouse rather than percent of mice with tumors (39). Surprisingly, the K-region 5,6-diol of DMBA, which cannot be converted directly into a vicinal diol epoxide, was more active in producing tumors than the 3,4-diol. No satisfactory explanation for the tumor-initiating activity of the DMBA-5,6-diol could be offered in view of the generally accepted importance of the bay region vicinal diol epoxide in DMBA initiation and mutagenesis (40). Indeed, the disagreement in tumor-initiating activities of DMBA derivatives between different laboratories is problematic (38,40). If the initiating activity is thought to be mediated exclusively through the DMBA diols and their epoxides, there is an apparent anomaly in the very small amount of those derivatives present in tissues after application of the parent hydrocarbon in view of the fact that the application of large amounts of the diols or their epoxides is less effective as tumor initiators than the parent hydrocarbon. A similar picture was observed for the very highly carcinogenic dibenz[a,l]pyrene and its fjord-region diol and diol epoxides (41). This can be only partly explained by the claim that PAHs have an alternate mechanism of activation in the form of radical cations (42) which is, in any case, offset by evidence against such a claim (43).

The derivatives of a number of other hydrocarbons have been tested in the initiation–promotion sequence. While the dihydrodiol precursors of bay region diol epoxides are as active as the parent hydrocarbons in most cases, several other derivatives also exhibit significant activity, especially for the weak carcinogens chrysene and benz[a]anthracene (44). The dihydrodiol epoxides were generally less active than the parent hydrocarbons, leading to the suggestion that ‘the tumor initiation requires more from a hydrocarbon than the generation of a reactive dihydrodiol epoxide metabolite’ (44).

**Cytotoxicity of B[a]P and its metabolites**

The designation of BPDE-2 as the ultimate carcinogen for B[a]P leads to the expectation that it would be at least as carcinogenic as the parent hydrocarbon, which it is obviously not on mouse skin, either as a complete carcinogen or as an initiator. One conceivable explanation for this failure that has received little attention is that BPDEs are extremely cytotoxic and destroy the cells in which they are most concentrated. These are presumably rapidly dividing stem cells found in the hair follicles (45–47).

BPDE-1 is at least 60-fold more cytotoxic than B[a]P-4,5-oxide in V79 Chinese hamster cells (48). Although BPDE-1 was highly mutagenic at very low concentrations, there was a sharp reduction in mutations at concentrations higher than 1.0 nmol. In contrast, mutagenesis by B[a]P-4,5-oxide rose continuously far beyond concentrations of the diol epoxide that reduced BPDE-1-induced mutations per surviving cell to less than the control level. Further testing showed that BPDE-2 was even more cytotoxic to V79 cells than BPDE-1 and almost 100-fold more cytotoxic than the moderate carcinogen H4-7,8-epoxide (49). Non-carcinogenic H4-9,10-epoxide was almost as cytotoxic as BPDE-2. In another study (50), BPDE-2 was also highly cytotoxic at low concentrations to V79 cells, although BPDE-1 was much less cytotoxic (Table II). The parental hydrocarbon BP displayed no cytotoxicity, nor did most of the phenolic derivatives, of which only one fairly representative member is shown. There were discrepancies between the two laboratories about the relative cytotoxicities of BPDE-1 and B[a]P-4,5-oxide, which were attributed to differences in methodology and specific V79 clones used as target cells, but there was agreement on the extreme cytotoxicity of BPDE-2 (49). It is noteworthy that both BPDEs are highly toxic to newborn mice upon intraperitoneal injection (51). It has since been shown that the toxicity of dibenz[a,l]pyrene, the most carcinogenic PAH known, interferes with its carcinogenicity at higher doses (52). Therefore the toxicity of BPDEs should be taken into account as a possible explanation for their low carcinogenicity for mouse skin. Particular attention should be paid to their effect on the stem cells of the hair follicles. However, the reduction in tumor production by BPDE-2 with dilution (Table IB) argues against this interpretation.

**Binding of diol epoxides to DNA**

If, as widely believed, metabolic alteration of B[a]P is necessary for carcinogenesis and BPDE-2 is the ultimate carcinogen, then one might expect the application of the pure compound to be much more active in tumor production than the parent hydrocarbons. The fact that it was much less active on mouse
skin calls for an explanation. BPDEs are much more reactive than other B[a]P derivatives and much less stable, with a half-life in phosphate buffered saline of at most a few minutes (49). It therefore seemed perfectly reasonable to attribute their low carcinogenicity to their inability to reach their target DNA when applied directly to skin (29).

The first breach in this reasoning came with the report that the amount of BPDE-2 combined with epidermal DNA after application of the pure derivative was the same as that bound after application of equal amounts of B[a]P or B[a]P-7,8-dihydrodiol (53). It was then suggested that metabolites derived from B[a]P and B[a]P-7,8-dihydrodiol other than BPDE-2 may be involved in skin tumor initiation, or that in vivo metabolically generated diol epoxide reacts more specifically with DNA than does topically applied BPDE-2 (53).

There remained the question as to whether the diol epoxides were actually bound to the DNA of basal cells of the epidermis, which were assumed to be the target cells for initiation. It was found that both BPDE diastereomers bound equally to DNA of the basal layer, as well as to RNA and protein (54). This indicated a lack of correlation between extent of binding and carcinogenicity since BPDE-2 is a far more active initiator than BPDE-1. There is the additional problem that although the (+) enantiomer of BPDE-2 is ~66-fold more potent an initiator than the (−) form, the rate of formation and disappearance of individual adducts was similar over a 72 h period (55). The possibility was suggested that there might be a small subpopulation of stem cells in which the specific binding of the two enantiomers of BPDE-2 is correlated with their tumorigenicity, but there has been no substantiation of this hypothesis.

In later studies it was found that BPDE-2 penetrates rapidly through the epidermis after its topical application and that the bulk of it is rapidly removed (56). The relatively low carcinogenicity of BPDE-2 for mouse skin might partly be accounted for by its rapid passage through the epidermis. However, the levels of total BPDE-2 present in the epidermis after application of BPDE-2 or B[a]P-dihydrodiol were 50–100-fold greater than after application of B[a]P. Despite the differences in total BPDE-2 in the epidermis after application of the three compounds, the extent of formation of BPDE-2 adducts to DNA was about the same and therefore once again not correlated with their tumorigenicity in skin. It was suggested that the ‘disposition’ of the metabolites when applied externally may differ quantitatively—and perhaps, qualitatively—from the disposition of metabolites generated intracellularly from B[a]P (56). It is noteworthy in this regard that BPDE-2 is bound 7–8-fold more efficiently to DNA than BPDE-1 when the parental hydrocarbon is topically applied to mouse skin (57), in contrast to the similar binding of the diol epoxides when the derivatives themselves are applied (54–56). A precise account of the distribution of diol epoxide adducts at specific nucleotide positions of DNA would help to resolve the relationship between binding and carcinogenesis.

The relation between carcinogenicity and DNA binding has also been studied with DMBA and its bay region diol epoxides (DMBADEs). Both syn and anti DMBADEs initiate papillomas in mouse skin when followed by repeated application of TPA, but are less active than DMBA (39). The parent hydrocarbon produces ~6-fold more papillomas per mouse than a 10-fold higher concentration of either the syn or anti DMBADE, although the sum of the binding of the diol epoxides to DNA when they are applied at those high concentrations is the same as that of the derivatives of DMBA when the latter is applied at one-tenth the concentration. It was suggested that the lower efficiency of DNA binding by the topically applied diol epoxides is the result of their high reactivity toward nucleophiles, plus the need to pass through the stratum corneum and granular spinous layers of the epidermis before reaching the target cells in the basal layer (39).

However, mouse epidermis consists of only one or in some places two cell layers (58,59), unlike the multilayered epidermis of humans. Measurement shows that large amounts of topically applied diol epoxides reach the basal cells (56). It was also contended that extrapolation of the ‘tumor-initiating potential’ for a given DMBA adduct from the maximum number of tumors initiated by the same adduct produced by the DMBADEs at the doses used (10 nmol for DMBA, 100 nmol for the DMBADEs), one could fully account for all the tumors formed by DMBA and by the DMBADEs in terms of the measured DNA adducts (39). However, there is no evidence that extrapolation of the low tumor yield from applied DMBADEs to the high tumor yield from DMBA application is warranted, since there is no indication of a linear relation between major DNA adducts and tumor development. All that can be said is that the number of papillomas formed by low doses of DMBA is much higher than 10-fold as much of the DMBADEs when the opposite relation would be expected if the latter accounted for the full tumorigenic potential of DMBA in mouse skin. This discrepancy remains despite the fact that tumor initiating activity, as used in this case, is a more efficient method for production of tumors by diol epoxides than is the method of complete carcinogenesis (28,30,35–37) and therefore presents the best case for considering the diol epoxides as the ultimate carcinogens of PAHs in mouse skin.

**Tumor production in newborn mice**

Unlike the equivocal findings with mouse skin, a pivotal role for the diol epoxides was strongly supported by a series of articles on tumorigenesis induced by three weekly intraperitoneal injections into newborn mice of step-wise increases of B[a]P or its metabolites (Table III). The diol epoxides were

<table>
<thead>
<tr>
<th>Tumors per mouse</th>
<th>1400(^b)</th>
<th>28(^b)</th>
<th>14(^b)</th>
<th>7(^b)</th>
<th>0(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.04; 0.12</td>
<td>0.13; 0.14</td>
</tr>
<tr>
<td>B[a]P</td>
<td>6.3</td>
<td>0.24</td>
<td>0.15</td>
<td>0.12</td>
<td>−</td>
</tr>
<tr>
<td>B[a]P 7,8-dihydrodiol</td>
<td>75.0</td>
<td>1.77</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BPDE-1</td>
<td>−</td>
<td>0.14</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(−)BPDE-1</td>
<td>−</td>
<td>0.15</td>
<td>0.22</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(+)BPDE-1</td>
<td>−</td>
<td>0.25</td>
<td>0.15</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BPDE-2</td>
<td>−</td>
<td>4.4</td>
<td>4.9</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(−)BPDE-2</td>
<td>−</td>
<td>0.13</td>
<td>0.09</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(+)BPDE-2</td>
<td>−</td>
<td>7.67</td>
<td>1.72</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B[a]P tetraols (from B[a]PDE-2)</td>
<td>−</td>
<td>0.05</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

\(^a\)Abstracted from Kapitulnik et al. (51,60) and Buening et al. (61).
\(^b\)Sum of nmoles injected intraperitoneally at 0, 8 and 15 days of age.
\(^c\)Control tumors from four trials.
\(^d\)Not enough survivors of toxic BPDE-1 in one of two experiments to record tumors.
highly toxic to mice even at 1/50th the dose of B[α]P, but BPDE-2 produced about the same incidence of pulmonary adenomas as the 50-fold higher but non-toxic dose of B[α]P (51). B[α]P-7,8-dihydriodiol produced even more pulmonary adenomas per mouse than B[α]P at the same high dose and also produced a high incidence of malignant lymphoma, a lesion not produced by the other compounds.) BPDE-1 was so toxic in these experiments that it left too few survivors for accurate evaluation of its carcinogenicity. BPDE-2 and B[α]P-7,8-dihydriodiol were, respectively, about 40- and 15-fold more active than B[α]P in causing pulmonary adenomas in newborn mice (60). The BPDE-1 and B[α]P tetratol products of BPDE-2 produced no more adenomas than the control level of 0.13 per mouse. When BPDE was separated into its two optical enantiomers and tested at extremely low doses (7 and 14 nmol) in newborn mice, only BPDE-2 produced significant increases in pulmonary adenomas over the control value, and B[α]P was ineffective at these doses (61). The extremely powerful capacity of the (+)-BPDE-2 enantiomer to induce pulmonary tumors in newborn mice when compared with its parental hydrocarbon supports its role as an ultimate carcinogen in the newborn mouse, but raises questions about the ultimate carcinogenic designation of BPDE-2 in skin, where it is less carcinogenic than B[α]P.

It should be noted that an average of ~10% of the newborn controls spontaneously develop pulmonary adenomas. A large increase in pulmonary tumor incidence can be induced in newborn mice with only a single low subcutaneous dose of B[α]P, DMBA or 3-MCA within 24 h of birth (62). A single subcutaneous inoculation into 8-week-old mice of a 20-30-fold greater amount of DMBA produced only one-fifth the number of pulmonary tumors as the lower dose in the newborns. The high spontaneous incidence of pulmonary adenomas in the newborns and the susceptibility to low doses of BPDE-2 suggests that only a single step is required for more tumors to develop, but it also emphasizes that tumor formation in the adult involves more variables.

Pre-neoplastic effects of PAHs in cultures of mouse epidermal cells

Mouse basal epidermal cells or keratinocytes multiply continuously in cell culture if the concentration of calcium in the medium is reduced to 0.1 mM or less (63). At higher concentrations of calcium, proliferation ceases and the vast majority of the cells terminally differentiate. A few cells continue to proliferate and form colonies in the high calcium. The proportion of colony-forming cells increases with the time the cells are kept in low calcium before switching to high calcium. A distinction of malignant epidermal cells is that they continue to proliferate when switched to high calcium. One day treatment of normal epidermal cultures with DMBA, followed by several weeks of continued incubation in low calcium results in a 4-10-fold increase in the number of colonies formed after switching to high calcium (63). However, the altered cells do not exhibit the full range of characteristics of the malignant cells, which include growth in agar and tumor production in mice. There is a correlation between the initiating potency of skin carcinogenesis in vivo and capacity to induce resistance to terminal differentiation (64). The results suggested that the carcinogens induce a pre-neoplastic state in the epidermal cells.

BPDE-2 was ~5-fold more effective than B[α]P in the induction of transformed foci in the epidermal cells and this difference correlated with the quantity of DNA adducts formed (65). The apparent transforming capacity of BPDE-2 for the cultured epidermal cells is in marked distinction to its poor tumorigenic capacity on mouse skin. A similar distinction from mouse skin is the high capacity of BPDE-2 to produce pulmonary adenomas in newborn mice. There are other biological features of carcinogenesis in these two BPDE-2 sensitive systems that distinguish it from carcinogenesis in mouse skin. One is the fact that some keratinocytes resistant to terminal differentiation occur in control cultures, as do some pulmonary adenomas in control newborn mice, while spontaneous skin papillomas are extremely rare in controls. A second difference is that there is no requirement for an extended promotional treatment in the two systems in contrast to that in the skin. A third difference is the plateau in binding of BPDE-2 when it is applied in high doses to mouse skin in contrast to the absence of such a limit when it is applied to the cultured epidermal cells (65). Information about the basis of the difference in DNA binding in the two cases could be revealing about the difference in biological response.

In vitro malignant transformation of rodent cells by PAHs

Exposure of primary or secondary cultures of Syrian hamster embryo cells to PAHs led to their morphological transformation associated with a capacity to induce tumors when inoculated into adult hamsters (66). Small numbers of cells were seeded for cloning and left for 8 days in the presence of PAH. Up to 25% of the clones that developed had a transformed morphology. Mass cultures treated for 8 days with carcinogenic PAHs and cultured in the absence of carcinogen for several months produced sarcomas in hamsters. The high percentages of transformed clones may be deceptive because it was later found that continued incubation after removal of the carcinogenic PAH from the low cell density procedure resulted in a marked reduction in the number of morphologically transformed colonies, indicating that most were only phenotypically transformed (67).

Before the discovery that the vicinal bay region diol epoxides were the major PAH derivatives that bound covalently to DNA (11), much attention was focused on the K-region of the PAHs as the likely active site for carcinogenesis (68). This was based on the false premise that the parent hydrocarbons were biologically active as such and that their metabolism was a purely detoxifying process (3). When it was found that many compounds did not display the carcinogenicity expected on the basis of the reactive K-region, the idea that chemically unaltered PAHs were the carcinogens was undermined.

In 1950, however, it was suggested that the carcinogenicity of PAHs was mediated through metabolically formed epoxides (69). All the PAHs examined formed epoxides at the K-region, which focused attention on the possibility that the K-region epoxides are the ultimately reactive forms in vivo. Synthetic K-region epoxides reacted with nucleic acids and proteins (70). They were highly mutagenic in the Chinese hamster V79 cell line without affecting the modal diploid chromosome number (71) and produced frameshift mutations in S. typhimurium (72). Interest in the K-region epoxides had earlier been tempered by their low to moderate activity as complete carcinogens in either mice or rats when inoculated subcutaneously or applied topically to the skin (73,74) and moderate activity as initiators on mouse skin (73). The findings of their
binding to DNA and mutagenicity warranted examination of the in vitro transforming activity of the K-region epoxides for cells in culture. They proved to be more active in transforming Syrian hamster embryo cells than the hydrocarbons or corresponding K-region phenols (75). Oddly enough, the K-region epoxide of BA was a more efficient transforming agent than the K-region epoxides of DBA and MCA, although the parent hydrocarbon of BA is a much weaker carcinogen than DBA or MCA. Similar effects were produced in a line of cells derived from mouse prostate although the K-region epoxides of MCA were more active in these cells than those of BA and DBA (76). The results in the mouse cells were extended to the K-region epoxides of other PAHs (77). In both sets, cells from the morphologically transformed foci induced anaplastic soft tissue sarcomas in isologous mice.

Subsequently it was found that the hydrocarbon–deoxy-nucleoside products isolated from mouse embryo cells treated with PAHs differed from those of cell-free DNA treated with K-region epoxides of PAHs (78). It was then apparent that the major metabolites responsible for the covalent modification of DNA were not K-region epoxides and this path of research reached a dead end (3). About the same time it was shown that the chromatographic profiles of DNA digests from hamster cells treated with B[a]P matched those of DNA reacted with the bay region 7,8-diol-9,10-epoxide of B[a]P which suggested that the 7,8-diol-9,10-epoxide of B[a]P was the major DNA-bound adduct formed from B[a]P metabolism (11). The bay region diols of 7-methyl BA, DMBA and B[a]P were more potent in the transforming of mouse fibroblasts than the parental hydrocarbons, and much more potent than K-region diols (79). The K-region epoxides ranged from weakly (7-methyl BA and B[a]P) to moderately (DMBA) transforming but always less so than either the bay region diols or the parental hydrocarbons. When the transforming capacity of B[a]P and its metabolites was tested on normal embryo Syrian hamster cells, the bay region trans 7,8-diol was more active than the other non-K-region 4,5- or 9,10-diols and B[a]P (80). BPDE-2 was more active than BPDE-1, which was slightly more active than the K-region epoxide of B[a]P.

In summary, the results in cell culture, along with the high mutagenicity and DNA binding of BPDE-2, supported its status as the ultimate carcinogen of B[a]P. The results agreed in part with the capacity of BPDE-2 to produce both the lung adenomas in newborn mice and the differentiation resistant state of mouse keratinocytes in culture. But they differed from the findings on mouse skin in vivo where BPDE-2 is a poorer carcinogen and initator than B[a]P or B[a]P-7,8-diol, and BPDE-1 is virtually non-carcinogenic in either mouse skin or newborn mice. When combined with the moderate tumor-initiating activity in mice of K-region epoxides (73), which could not be detected as adducts to DNA when the parental hydrocarbons were administered, the results call for a fuller analysis of the mechanisms of transformation and carcinogenesis by PAHs. To carry out such an analysis, it is important to consider the relevant biological aspects of PAH-induced skin tumor development that have to be accounted for by any proposed mechanisms. Many of these were established in the pre-molecular era and are rarely acknowledged in present day discussions.

Requirement for long-term repeated application of PAHs for skin carcinogenesis

Yamagiwa and Ichikawa (81) had succeeded in producing cancer with coal tar because they applied it (on rabbit ears) more persistently over a longer period of time than had their predecessors. The interest in coal tar arose from the high incidence of skin cancer among workers in the coal gasification industry which had coal tar as a by-product. It was natural therefore that application to the skin of experimental animals—more particularly mice because of their convenience—should be the method of choice for testing components of coal tar for carcinogenicity, as well as for related synthetic chemicals. A consistent finding, first with B[a]P from coal tar and synthetic DBA, then with other synthetic PAHs such as DMBA and MCA, was that they had to be applied repeatedly to mouse skin on a weekly or more frequent basis for months before papillomas appeared, and even longer for carcinomas to appear. For example, Hieger (82) found that painting mouse skin bi-weekly for 8 weeks with 0.3% of either DBA or B[a]P yielded no tumors, but painting for 8 weeks with DBA followed by 8 weeks of B[a]P was tumorigenic. A single subcutaneous inoculation of B[a]P in a variety of vehicles rarely produced sarcomas if disappearance of the particular combination was complete in 3 or 4 months but was highly tumorigenic in other combinations which remained detectable by fluorescence in ultraviolet light for more than 5 or 6 months (83).

Tumors were common at the site of subcutaneous inoculation of B[a]P where it persisted for a long time, but no tumors appeared at the site of intraperitoneal inoculation where the rate of disappearance was rapid (84). Rabbits, which were very susceptible to skin carcinogenesis by topical application of DMBA, were highly resistant to subcutaneous inoculation, unlike mice which were susceptible by both routes (85). The resistance of rabbits to subcutaneous inoculation was correlated with the much faster disappearance of DMBA from the subcutaneous tissue of rabbits than of mice.

In one of the first examples of PAH persistence, tumors induced by a lard solution of DBA contained an appreciable amount of the carcinogen 6–8 months after its subcutaneous inoculation (86). Another example of extreme persistence was seen after subcutaneous inoculation of DB[a]P in tricaprylin which produced 98% sarcomas in 24 weeks (87). No metabolites of DBP could be detected in the feces collected for a month after inoculation and the unaltered carcinogen could be found in the tumors months later. The low solubility of DBP was thought to account for the slowness of its action as compared with MCA, and the persistence at the inoculation site was believed to explain the equivalent tumor yield to MCA at one-third the dose of MCA.

The dynamics of disappearance of PAHs could be studied when radioactive labeling of the carcinogens was introduced. The results with three PAHs after subcutaneous inoculation in mice and with two of them after painting on skin are shown in Table IV. DBA disappeared from the subcutaneous site most slowly of the three with a half-life of ~12 weeks, followed by MCA with a half-life of 3.5 weeks and B[a]P with an initial half-life of 1.75 weeks (88,89). DBA had about the same half-life after skin painting as it did in subcutaneous inoculation, but B[a]P disappeared much more rapidly in skin painting with an initial half-life of 1.7 days and 4.3 days after the second day. Excretion was studied only with B[a]P, which appeared entirely in the feces as metabolites (89). The ratio of the neutral fraction (which consists mainly of unchanged hydrocarbon plus the diols) to the fraction bound to macromolecules remained high for subcutaneously inoculated B[a]P up to 8 weeks and decreased sharply at 12 weeks. That shift occurred within days when B[a]P was applied to skin. The
The fate of B[a]P and DBA in subcutaneous tissue and on skin

<table>
<thead>
<tr>
<th>Days</th>
<th>Percent remaining of original dose</th>
<th>Total remaining</th>
<th>Unchanged + neutral metabolites</th>
<th>Tightly bound to macromolecules</th>
<th>Ratio unchanged/bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>B[a]P subcutaneous&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.4</td>
<td>26.5</td>
<td>0.80</td>
<td>33.5</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>26.0</td>
<td>24.4</td>
<td>0.48</td>
<td>50.8</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>18.7</td>
<td>13.6</td>
<td>0.65</td>
<td>20.9</td>
</tr>
<tr>
<td>56</td>
<td></td>
<td>2.0</td>
<td>1.5</td>
<td>0.09</td>
<td>16.7</td>
</tr>
<tr>
<td>84</td>
<td></td>
<td>0.32</td>
<td>0.21</td>
<td>0.06</td>
<td>3.5</td>
</tr>
<tr>
<td>B[a]P on skin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.17</td>
<td>99.0</td>
<td>98.6</td>
<td>0.24</td>
<td>410.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>90.6</td>
<td>39.5</td>
<td>0.65</td>
<td>62.7</td>
</tr>
<tr>
<td></td>
<td>7–9</td>
<td>11.6</td>
<td>10.7</td>
<td>0.51</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>4.7</td>
<td>4.1</td>
<td>0.56</td>
<td>7.3</td>
</tr>
<tr>
<td>DBA on skin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td>95.5</td>
<td>91.5</td>
<td>0.18</td>
<td>508.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>86.8</td>
<td>86.1</td>
<td>0.45</td>
<td>191.3</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>84.3</td>
<td>83.8</td>
<td>0.19</td>
<td>441.1</td>
</tr>
<tr>
<td></td>
<td>20–21</td>
<td>87.8</td>
<td>86.15</td>
<td>0.17</td>
<td>73.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abstracted from Heidelberger and Weiss (89).
<sup>b</sup>A single dose of 0.5 mg of 14C B[a]P was injected subcutaneously.
<sup>c</sup>B[a]P-5-C14 or DBA-9,10-C14 (0.05 mg) was applied once to the skin.

Table V. Effect of oxidative metabolism of B[a]P on logP values of B[a]P (lipophilicity)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>logP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Skin carcinogenicity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative mutagenicity, TA100 S.typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P</td>
<td>6.42</td>
<td>+++++</td>
<td>–</td>
</tr>
<tr>
<td>8-HOBP</td>
<td>5.87</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B[a]P-7,8-epoxide</td>
<td>5.22</td>
<td>+ +</td>
<td>±</td>
</tr>
<tr>
<td>B[a]P-9,10-epoxide</td>
<td>5.22</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>B[a]P-7,8-dihydrodiol</td>
<td>4.54</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>B[a]PDE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.06</td>
<td>–to +&lt;sup&gt;d&lt;/sup&gt;</td>
<td>++++</td>
</tr>
</tbody>
</table>

<sup>a</sup>LogP is the log of the octanol:water partition coefficient (90).
<sup>b</sup>See Table I for more quantitative values of carcinogenicity and mutagenicity.
<sup>c</sup>Instability of the diol epoxides makes a precise determination of their octanol:water partition coefficients difficult if not impossible. No attempt was made to separately test the enantiomers of B[a]PDE.
<sup>d</sup>The two enantiomers differ in carcinogenicity (see Table I).

Neutral to bound ratio was much higher with DBA applied to skin and only showed a significant decrease at 20–21 days. Obviously the metabolism of B[a]P is much faster than that of DBA, with the latter persisting largely unchanged for extended periods of time as previously indicated using the method of fluorescence under ultraviolet light (86).

Localization of PAHs in tissues and cells

Striking features of PAHs are their low chemical reactivity and high lipophilicity. The PAHs which are created by treating organic material at extremely high temperatures are not chemically reactive but are altered under physiological conditions by enzyme metabolism. They are also very insoluble in water as they are very reactive but are altered under physiological conditions by organic material at extremely high temperatures are not chemically reactive. They are also very insoluble in water as indicated by the logarithm of their octanol:water coefficients as shown for B[a]P in Table V (90). Even most of the B[a]P metabolites, which have added polar groups to the parental hydrocarbon, are >10<sup>4</sup>-fold more soluble in octanol than in water. It is only when conjugated with glucuronic acid or glutathione that the B[a]P derivatives become soluble in water and are excreted (91,92). Only a very small fraction of labeled B[a]P appears bound to macromolecules (89) including DNA (53,65).

Localization was studied mainly by observing fluorescence. A large fraction of MCA and B[a]P applied once to the skin is found in sebaceous glands and keratin (93,94). Many epidermal cells are killed by the carcinogenic PAHs, but resistant cells appear in a hyperplastic response to repeated application and the PAH can be seen in the cytoplasm, but not in the nucleus. B[a]P fluorescence was used as a method for detecting 'masked' lipid not seen with conventional stains for lipid like osmic acid (95). B[a]P was associated in these early studies with nuclear membranes, regions rich in mitochondria and regions with an ultrastructure of 'oriented proteins and lipids', i.e. presumably membranes (95). Later work in cell culture as well as epidermis using both fluorescence and autoradiography of radioactively labeled PAHs reported the bulk of the PAHs in lysosomes (96). None of these studies detected PAHs within the nucleus, but this is understandable in light of the small fraction bound to DNA (53,65) and the insensitivity of the techniques used in localization (97). Obviously, improved methods for isolating DNA and detecting radioactively labeled adducts of diol epoxides demonstrated that the diol epoxides were bound to nuclear DNA, but the localization studies emphasize that the great bulk of PAHs and their derivatives were associated with extranuclear structures and could be contributing to the carcinogenic process at those locations. One possible role of the unaltered hydrocarbon is to act as a reservoir for long-term production of diol epoxides.

Tissue response to PAH application on mouse epidermis

Application of 0.6% MCA in benzene three times a week to the backs of mice results in the first appearance of small wart-like swellings at 2 weeks, papillomas beginning at 4 weeks and increasing in number to 12 weeks and carcinomas beginning at 12 weeks and involving 100% of the mice at 24 weeks (58).
Microscopically, after a single application there is widespread death of keratinocytes in the central area of direct application in the first few days with cellular and nuclear swelling accompanied later by cell proliferation in the peripheral areas (98). This response includes the hair follicles which help to repopulate the damaged areas. There are multiple layers of epidermal cells in the marginal areas at 3 days with further multilayering at 6 days. When lower concentrations of either MCA or DMBA are applied only once, there is less destruction in the central area but there is cell swelling at 2 days (Figures 2A and B). There is a mild hyperplasia at 6 days (Figure 2C), which decreases somewhat by 30 days but treated epidermis is still clearly distinguishable from controls (Figure 2D) (59).

With multiple applications (six times a week) of the lower doses of MCA and DMBA, there is an extremely high degree of hyperplasia at 30 days, irregularity of shape and variability of size of both cells and nuclei, and abnormality of chromatin localization (Figure 2E) (59,98). There are also pronounced irregularities in the arrangement of various cell layers and a high degree of mitotic activity. After 12 weeks of MCA painting three times a week, a diverse reaction can be seen in a broad, low power field consisting of hyperplastic epidermis, regenerating hair follicles, benign and pre-cancerous papillomas and an invasive carcinoma (Figure 2H). The full reaction described above, including its persistence, is considered specific for carcinogenic PAHs (59,98,99). The shorter the latent period for tumor production, the smaller is the dose required to provoke the specific hyperplasia (100). This indicates that the carcinogenic potency of a PAH, which takes months for its full expression is anticipated by the nature of the early tissue reaction.

**Table VI. Chromosome breakage and rejoining in DMBA treatment of Chinese hamster cells in culture**

<table>
<thead>
<tr>
<th>DMBA (µmol/ml) Exposure (h)</th>
<th>Period after exposure (h)</th>
<th>Percentage of cells with &gt;1 break</th>
<th>&gt;7 breaks Rejoining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>1.0</td>
<td>12</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>24</td>
<td>55</td>
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</tr>
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<td>24</td>
<td>74</td>
<td>24</td>
</tr>
<tr>
<td>1.0</td>
<td>24</td>
<td>94</td>
<td>87</td>
</tr>
<tr>
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<td>52</td>
</tr>
<tr>
<td>1.0</td>
<td>24</td>
<td>44</td>
<td>20</td>
</tr>
</tbody>
</table>

*Abstracted from Kato (103).

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**Early chromosomal responses to PAHs**

The abnormal distribution of chromatin described above (98) was the first direct indication that the genetic material of the cell might be involved in the carcinogenic process. At the time there were no adequate techniques for characterizing individual chromosomes in tissues but squashes were made of epidermal cells at various times after multiple MCA applications were begun and the chromosomes were stained (101).

Beginning at 3 days, an approximate doubling in size of chromosomes could be seen in ~8% of the cells, with an indication that this reflected an increase in chromosome strands rather than a mere swelling. The proportion of cells with this alteration remained about the same for 2 months and then rose to 20–30% at 72 days. In contrast, only 0–2% of the cells in papillomas had chromosome abnormalities, indicating they were selected against in development of the tumors. However, the later developing carcinomas had much higher percentages (40–76%) of chromosomally abnormal cells than any in the hyperplastic epidermal cells. It is noteworthy that subsequent improvements in chromosome analysis of papillomas after an initiation–promotion sequence confirmed that the great majority of them had a diploid karyotype that only changed to aneuploidy during the development of the carcinomas (102).

A quarter of a century after the initial relatively crude observations, improvement in cytogenetic techniques warranted further investigation of early chromosomal changes in cells treated with PAHs. Chinese hamster cells with their easily identified set of 22 metacentric chromosomes were treated with DMBA for 24 h (103). Single chromatid and chromosome breaks were seen in 23% of the cells at 12 h, with the proportion rising to 55% at 24 h (Table VI). The rise increased to >90% at 24 h after removal of DMBA with a gradual decrease in the next 72 h. Rejoining of the broken ends of chromosomes started after removal of DMBA at 24 h and was seen in 77% of the cells in the next 24 h, with a subsequent decline paralleling that of the chromosome breaks. At the fifth passage after treatment, the majority of DMBA-transformed cells and control cells were normal diploid, but the frequency of cells with structurally new chromosomes, presumably arising from rejoining between different broken chromosomes, was ~4-fold higher in the transformed than in the control cells.

Chromosome breaks were also seen in bone marrow cells of Chinese hamsters after subcutaneous or intraperitoneal inoculation of DMBA (104). The percentage of DMBA-treated cells with breaks in the hamster was generally lower than those found in cell culture, indicating greater stability of the genetic apparatus in the normal environment of the body than in cell culture. This point was reinforced by the low percentage...
of 0.4% breaks in the control marrow cells in the hamster in contrast to the 8% with breaks among the control cells in culture. Interchromatid exchanges occurred frequently, often involving interchanges between chromosomes. Multiple chromosome breaks were commonly seen in cells both in vitro and in vivo, but such cells were probably selected against in the long-term. Intercromosomal exchanges, of course, allow for gene rearrangements that might affect gene regulation and increase the likelihood of transformation.

There are a number of possible mechanisms by which PAHs could produce chromosome breakage. One is, of course, the binding of the bulky diol epoxides to DNA. A second involves the induction of a prooxidant state in PAH-treated cells (105). Evidence for a role of reactive oxygen species in chromosome breakage was found in human leukocyte cultures treated with DMBA (106). DMBA induced about a 3-fold increase in chromosome breaks and these could be reduced by as much as 64% by the presence of antioxidants during DMBA treatment. The antioxidant effect is corroborated by the observation that peroxidation of unsaturated lipids increases in mouse skin ~3-fold by 20 days after a single application of DMBA (107).

The predominant localization of PAHs in lysosomes provides another opportunity for chromosome aberrations (96). Lysosome membranes are extremely labile to peroxidative damage as indicated by the release of bound enzymes (108). Lysosomal DNase, which has two active sites and can cut both strands of DNA, could be among the enzymes leaked and thereby initiate chromosome breaks (109). It has in fact been shown that cathepsin D protease is released to the cytosol under stressful conditions (110) and nucleases might be similarly released. Other forms of stress increase the expression of an endonuclease associated with DNA breakage (111) which might be another source of chromosome damage by PAHs.

**Initiation and promotion of carcinogenesis: a synergistic interaction**

The carcinogenic PAHs are known as complete carcinogens because they can induce tumors in the skin without the intervention of other treatments. But as emphasized above, tumor production requires repeated application of the PAHs over extended periods of time for maximum effect. That raises the question of whether there is more than one stage in the tumorigenic process. In the early 1940s, that question was answered positively in studies of skin carcinogenesis in rabbits and mice. Rabbit ears were painted repeatedly with coal tar for several months resulting in the appearance of some papillomas. In contrast, application of croton oil before the PAH treatment without loss of tumor production was induced quickly and irreversibly, while promotion required repeated application of croton oil until tumors appeared. Initiation step was induced quickly and irreversibly, while promotion required repeated application of croton oil until tumors appeared. Initiation had the characteristics of mutation or chromosomal alteration, while promotion allowed the neoplastic expression of the altered state. Since the combined effect of initiator and promoter is far greater than additive of separate applications, it can be considered synergistic (117).

The active promoting ingredient in croton oil proved to be TPA (118). This compound is a diester of the alcohol phorbol, with one short- and one long-chain fatty acid. Maximum promoting action of the class of phorbol diesters is achieved when the long-chain fatty acid has 14 carbon atoms. TPA can substitute for croton oil in its promotional activities, which are associated with the induction and maintenance of epidermal hyperplasia through the entire period of repeated applications. It was reported that not all agents which induce hyperplasia act as promoters, but that hyperplasia is an essential component of promotion (119). Croton oil, and presumably TPA, does not promote skin tumor development in rabbits, guinea pigs and rats. As noted earlier, wound healing is an effective promoter in rabbits (113), but it was inactive in mice (119) unless...
preceded by enough carcinogen treatment to be almost adequate for high tumor yield (114). However, extending the area of wounding in repeated abrasion of conventionally initiated mouse skin resulted in ample production of tumors, though not as many as induced by TPA (120). The regenerative hyperplasia for effective promotion must be intense enough to produce substantial epidermal thickening since the mild hyperplasia induced by plucking hair or repeatedly rubbing the skin does not act as a promoter. Acetic acid, which produced a low degree of hyperplasia that is not increased in multiple applications, acts as a weak promoter (121).

Although ‘membrane active’ agents such as sodium lauryl sulfate and Tween 60 are sometimes classified as weak promoters in mice (122), others have found them to offer a wide range of promotion (59). The degree of promotion of these agents was correlated with the degree of hyperplasia they produced in mouse skin. A single application of Tween 60 produced intense cell multiplication and high degree hyperplasia which commenced immediately upon application. Repeated application of Tween 60 maintained the hyperplasia unchanged (Figure 2F). It caused neither the cellular nor nuclear atypia, nor disturbances in cell–cell organization characteristic of the application of carcinogenic PAHs. It was concluded that tumor promotion is comparable to intense, continuous, simple reparative cell multiplication (59). If a single initiating dose of MCA was followed 30 days later by 30 days of repetitive treatment with Tween 60 promoter, there was a pronounced irregular hyperplasia consisting mainly of basal cells with small spindle shaped nuclei at right angles to the dermo–epidermal junction (Figure 2G). The pretreatment with MCA resulted in abnormalities in distribution and arrangement of cells by the promoter (compare Figure 2G with F); promotional treatment of initiated skin also produced much more hyperplasia and irregularity of the epidermis than a single initiating dose of MCA alone (compare Figure 2G with D). The irregular hyperplasia induced in initiated epidermis presumably allows selection of rogue clones and the development of discrete neoplastic lesions.

Twice weekly treatment of uninitiated mouse skin with the strong promoter TPA produced a reactional hyperplasia during the first week characterized by cell damage, edema and acute inflammation in both epidermis and dermis (123). Unlike skin treated with the weak promoter Tween 60, there was nuclear pyknosis, cytoplasmic vacuolization and epidermal degeneration concomitant with epidermal growth (124). This picture changed gradually during the following 3 weeks leading to epidermal hyperplasia with mild chronic inflammation of the dermis and hyperplasia of the hair follicles (123). After TPA treatment stopped, the hyperplasia regressed abruptly during the first 2 weeks. Within 2–4 months after ceasing TPA, the epidermis became thinner than normal, as did the rate of proliferation. It was suggested that the treatment selected for cells particularly sensitive to the stimulating effect of TPA (123) which, in skin pretreated with a carcinogenic PAH, presumably would favor the growth of initiated cells.

From the early days of croton oil use in studies of promotion, occasional occurrence of tumors in mice receiving croton oil alone raised the question of whether it was a weak carcinogen (15). The same question arose again with TPA, especially in the Oslo strain of hairless mice (125). Repetitive painting of these mice twice a week with 17 nmol TPA led to tumors in 28% of the mice in 39 weeks, and similar painting with 10 nmol TPA gave 9% tumors in 55 weeks. Smaller yields of tumors were produced with fewer TPA paintings. An argument against the weak carcinogen status of TPA was that it was merely promoting cells that were spontaneously initiated by endogenous mutations. This argument was widely accepted because TPA is non-mutagenic in hamster cells (126) and does not appear to require metabolic activation for its promoting effect in mouse skin (127). Most human cancers are, however, thought to arise from selection of endogenous mutations (128), so it may be a semantic problem to make a qualitative distinction between carcinogens and strong promoters like TPA. All one can say is that TPA is a weak carcinogen in comparison to some PAHs, and that its activity in the initiation–promotion sequence far outweighs its action alone, or as the sum of the independent actions of TPA and initiators. Indeed, it has been estimated that it requires >10-fold as much PAH acting as a complete carcinogen in repeated applications to produce as many papillomas as PAH plus TPA in an initiation–promotion sequence (R.Boutwell as quoted in ref. 129).

The initiation phase of two-stage carcinogenesis in mouse skin can be replaced by activated V-ras genes of the mouse sarcoma virus (130). Virus was introduced by scarification of the skin and twice weekly painting with TPA was begun either immediately or 4 months later. Papillomas were produced in almost all of the mice treated with the activated viral genes and TPA, but none developed in those receiving only the former. The latent period for papilloma appearance was considerably shorter than for those initiated with DMBA. This was thought to reflect the higher levels of expression of the mutated ras genes driven by viral promoters than are observed in chemically-induced tumors associated with activated ras genes. It had previously been shown that the mere establishment of an activated ras gene is not sufficient for transformation of cultured cells and that cooperation with other transfected genetic elements, such as the early region of adenovirus, is needed to provide complementing biochemical activities (131).

Since tumor development in the skin is more difficult to induce than transformation of cells in culture, the requirements of more than one event for transformation indicates that several events are required for papilloma formation.

**Two stages of promotion**

It was originally thought by some that promotion by TPA was a single process related to the strong and persistent hyperplasia induced by repeated application of the compound after initiation by a carcinogen. This view began to change as a result of the following experiments. Application of croton oil to initiated skin for only 6 weeks resulted in few tumors but extension of the croton oil treatment to 14 weeks completed the promotional process with the appearance of many papillomas (132). Substitution of turpentine for croton oil in the last 8 weeks of the promotional period also resulted in many papillomas, but exclusive use of turpentine during the full 14 week period produced none. The incidence of carcinomas, although higher than that of papillomas, followed the same general pattern in response to the three agents. The combined treatment of limited croton oil followed by turpentine was synergistic in the sense that the tumor response was much greater than that engendered by either agent alone. This synergism suggested that there was a qualitative difference between the croton oil and turpentine treatments.

A similar conclusion arose from the substitution of wound healing for turpentine after croton oil treatment of initiated
mice (132). Wound healing alone in the initiated mice yielded no papillomas, but many of the mice treated with croton oil for 5 weeks after initiation developed one or more papillomas along the line of an isolated wound. The three-stage sequence of events was described as (i) initiation to a pre-cancer state carried out by a single small dose of a carcinogen which, in the case of PAHs, could carry out all three stages by itself if it were given repeatedly or in large doses, and probably involves an irreversible change in DNA; (ii) conversion, the first part of promotion induced by croton oil which converts the initiated cell into a dormant tumor cell; (iii) propagation, the second part of promotion, induced by turpentine or wound healing, which is dependent on cellular proliferation (132). There is overlap in these stages in the sense that carcinogenic PAHs can induce all three and croton oil can induce the latter two. In fact, croton oil may be able to induce all three since it does produce some tumors in normal skin and turpentine does promote some tumors in initiated skin without croton oil if it is continued beyond 14 weeks. The possibility should be considered that there is a variety of interactions, cellular events and processes at the molecular and biochemical levels that are driven to different extents by all these treatments, which gives the appearance of synergy when favored by the particular experimental setup.

Croton oil is a complex mixture of substances, and when TPA was shown to be the active ingredient, experiments on promotion were refined and extended while eliminating extraneous effects due to other components. Turpentine also is not a pure chemical, so better defined substances were tested for their ability to carry out the second stage of progression. Mezerein is a diterpene similar to TPA which brings about most of the biochemical changes and the hyperplasia seen with TPA but is 1/50th as active as a promoter (133). Treatment of DMBA-initiated mouse skins with TPA for 20 weeks yielded a large number of papillomas, but treatment for 2 weeks yielded none at 20 weeks (133). If a high dose of mezerein was continuously applied after 2 weeks of TPA, almost as many papillomas appeared by 20 weeks as in continuous treatment with TPA. Mezerein by itself was only a very weak promoter. Hence, mezerein was acting chiefly as a propagator or a second stage promoter.

The weak promoting activity of mezerein by itself complicates the stage-wise analysis of promotion, so a second stage promoter or proliferogen devoid of full promotion activity was sought by substituting a retinoic acid residue for the long fatty acid chain of TPA in synthesizing phorbol-12-retinoate-13-sulfonate (MMS) (140). MMS induced chromosome breaks and gaps in epidermal cells when topically applied to mouse skin but did not exhibit any initiating activity. It did prove to be a rather powerful agent of conversion or stage I tumor promotion. In contrast to TPA though, MMS is a weak inducer of DNA synthesis, which is necessary for conversion (141), so it had to be accompanied by RPA in order to exert a converting effect comparable with that of TPA (140). The results are consistent with a role for chromosome aberrations in the conversion stage of skin tumor development. It should be borne in mind, however, that initiating PAHs also induce chromosome aberrations (103,104) and that wound healing, not usually considered a clastogenic process, induces a moderate degree of conversion (142). It has been noted, however, that wound healing stimulates leukocytes present in the wounded area to give rise to a burst of oxygen radicals presumably to sterilize the area (143). The oxygen radicals might then act on the dividing cells of regenerating epidermis and cause
chromosome aberrations. This is an obvious area for study to test the validity of the chromosome aberration hypothesis of conversion.

Another question about the hypothesis arises from the reversibility of conversion, however slow it is. The reversibility is one reason it has been suggested that conversion is the result of selective growth of a class of initiated cells particularly sensitive to the converting agents (144,145). An argument against this interpretation is that it cannot explain the inversion of the converting process by which it is effective before application of the initiating agent (146). However, there may be considerable heterogeneity of capacity for a proliferative response to converting stimuli among normal keratinocytes. That would give particularly sensitive cells a head start in clonal expansion and increase epidermal sensitivity to tumor formation in subsequent initiating and promoting treatments. Also to be taken into consideration in the analysis of tumor promotion is the observation that DMBA can act as a strong promoter with a course of very small doses (147). Since DMBA is a strong inducer of chromosome aberrations, it would presumably be a convertogenic stimulus in the view that chromosome aberrations underlie conversion (143). It is also noteworthy that benzo[\(e\)]pyrene (B[\(e\)]P), which is a very weak initiator, is a moderately strong promoter of skin initiated by DMBA (148).

The blurring of lines of distinction between various categories of carcinogenesis can be seen in tracing the designation of RPA as a stage 2 promoter in successive papers from one laboratory. It was introduced as a pure second stage promoter, i.e. it failed to exhibit any promoting activity in initiated NMRI mouse skin (134). A few years later, it elicited tumors in 30% of the mice if applied both before and after DMBA initiation (with no prior TPA treatment) and in 15% of initiated mice if applied only after initiation (Table I) (136). Despite the observation of RPAs complete but weak to moderate promoting capacity in NMRI mice, it was later stated that ‘RPA, which is non-converting for NMRI and CD-1 mice has been found to be a converting agent for Sencar mice’ (146) based on observations of RPA effects in Sencar mice (149). In some strains of mice and in some other species, TPA has virtually no promoting activity (150), in others it is a weak but complete carcinogen (151,152). The effectiveness of wounding as a complete promoter increases with the time elapsed from the initiation event (153). Classification of the various compounds and of wounding in a three stage model of carcinogenesis therefore depends on the genetic background of the organism, the experimental set-up and the intervals between the various stages of treatment.

Tumor promotion in connective tissue and cultured fibroblasts

The basic foundation of the initiation–promotion model was established in carcinogenesis of the skin, first in rabbits and then, more systematically and extensively, in mice (15). The skin was convenient for this purpose because it was easy to control the timing of applying different substances and to continuously observe the results. However, a significant fraction of the early studies on PAH carcinogenesis, such as persistence of carcinogen, employed subcutaneous injection which resulted in sarcomas without a noticeable benign intermediate stage. It was therefore of interest to determine whether a promotional stage occurred in this form of carcinogenesis.

A single subcutaneous injection of croton oil produced no tumors in mice in over a year (154). The injection of 0.1 or 0.6 mg MCA induced 28 and 50% tumors, respectively, while the co-injection of croton oil in the inoculum with MCA doubled the incidence of tumors. The latent period of the tumors was also significantly reduced as was the average age of death. Although the promoting effect of croton oil was not as impressive as it was in skin, where it was usually combined with a preceding subcarcinogenic dose of initiator, there was a clear cut increase in the carcinogenicity that might be interpreted as a promoter effect. However, there was only a single application of croton oil, it was simultaneous with MCA application and it increased the tumor incidence in the same way as an increase in MCA concentration would, so it might just as well be considered a co-carcinogen (15). This is another example of how the experimental set-up influences the interpretation of the results.

A possible promoting effect of TPA was studied with the established C3H 10T1/2 line of mouse fibroblasts in cell culture (155). A small number of the fibroblasts was exposed to a sub-effective concentration of MCA, B[\(a\)]P or DMBA for 24 h. After removal of PAH, TPA was added immediately or 4 days later and maintained during the entire 6 weeks of the experiment. In the case of MCA and B[\(a\)]P alone, there was no significant transformation with the low dose used nor was there any significant effect of TPA added immediately after removal of the PAH. However, there was a significant increase in the number of transformed cultures when TPA was added 4 days after removal of the PAH. When higher concentrations of MCA or B[\(a\)]P alone were used, there was significant transformation, but the incidence was reduced by the immediate treatment with TPA. There was a slight increase in transformation over that produced by the PAHs alone when TPA was added at 4 days. The low dose of DMBA, a more potent subcutaneous carcinogen than the others, produced some transformation, that was reduced by treatment with TPA begun immediately after removal of DMBA and not significantly increased by TPA begun 4 days later. The higher dose of DMBA alone produced some foci in the majority of cultures. That number was significantly reduced when TPA was begun immediately after DMBA removal and was unaffected by its addition at 4 days. The experiment showed that low doses of carcinogen that produce no foci by themselves are promoted to produce foci if TPA treatment is begun 4 days after removal of the PAH, but not if it is begun immediately.

TPA was then added at daily intervals after low dose MCA treatment for 1 day which produced no foci by itself (155). Foci did appear when TPA was added 0–4 days after MCA removal. At a higher MCA concentration, which did produce foci by itself, there was inhibition of focus formation by TPA addition immediately or 1 day after MCA removal, and a slight increase in focus formation by TPA addition at 3 and 4 days after MCA removal. Unpublished experiments were cited that showed TPA alone produces an immediate inhibition of DNA synthesis which might have prevented the early divisions required to fix the transformed state after carcinogen treatment. After 2 days of TPA treatment, there was an increase in DNA synthesis. The experiment confirms that TPA promotes focus formation if added after a period in which fixation of the carcinogen-induced lesion is thought to occur.

If cells were allowed to grow to confluence after treatment with low doses of MCA, B[\(a\)]P or DMBA, then treated with or without TPA on day 10, there was no significant focus.
formation (155). The same was true if the cells were replated at 10 days after carcinogen treatment without TPA treatment. But treatment continuously with TPA after replating of the initiated cells resulted in a highly significant increase in transformation. Therefore, treatment with TPA of confluent contact-inhibited cells that had been initiated with sub-transforming doses of PAHs did not promote transformation, but replating them 10 days after initiation so they were allowed to multiply in the presence of TPA up to 6 weeks allowed significant transformation. If TPA was not added after replating there was no significant transformation. Thus mere multiplication of the initiated cells did not result in transformation but some multiplication in the presence of TPA did so. Hence, TPA was doing something besides stimulating cell division to promote transformation.

Although these results in cell culture of fibroblasts exhibited the characteristics of initiation and promotion similar to those in mouse epidermis, there is one result that is quite different (155). The compound 4-α-PDD (phorbol-12-13-didecanoate with the 4-hydroxyl in the α position), which does not act as a promoter in mouse skin, does so with cultured cells, although not as strongly as TPA. It may be that the multiplication of the initiated cultured cells seeded at low densities enhances the effectiveness of 4-α-PDD to promote transformation; in contrast to initiated mouse skin, where there might not be sufficient sustained proliferation in the presence of 4-α-PDD to allow promotion to occur.

The effects of another promoter, phorbol-12-13-didecanoate (PDD), was studied in the Balb/c3T3 line of mouse fibroblasts (156). The promoting activity of two polypeptide growth factors was included in the study, but the effects of PDD are of particular interest in the present context. The initiating agent MCA alone produced very few or no transformed foci in successive experiments, but PDD alone was far more active in transformation, and the combination still more active. The considerable activity of PDD alone suggests that the Balb/c3T3 line contained initiated pre-neoplastic cells. The fact that the two polypeptide growth factors also produced foci in the absence of MCA treatment supports this explanation. When MCA treatment was combined with PDD or the growth factors, the number of foci was increased in every case indicating that MCA treatment added to the number of initiated cells.

The original NIH 3T3 line of mouse fibroblasts undergoes spontaneous transformation if kept under contact inhibition at high population density for 2 weeks, then subcultured to confluence (157). Repeated passage of the original line at low population density resulted in a subline that did not exhibit transformation despite prolonged incubation at high density followed by a second round at high density (158). Addition of TPA during the first round of incubation at high cell density led to transformation after about 4 weeks of contact inhibition at confluence, with no evidence of transformation even after 8 weeks in the untreated control. If TPA was added continuously to cells that were frequently passaged at low population density and aliquots of the cells repeatedly tested at high density for transformed focus formation, the cultures remained negative throughout the 8 weeks of the experiment. The implication of these results is that TPA only acted as a promoter when it was applied to a population of cells that was maintained under the selective condition of contact inhibition at high density. One effect of TPA in the NIH 3T3 experiments (158) was to increase the saturation density of the cultures, an indication of continuing cell multiplication at high population density, which would provide the opportunity for mutation in large populations and selection of growth variants.

Examining the effects of promoters on initiated and non-initiated cells of the three lines described above reveals considerable differences in their responses to treatment with the initiators and promoters. The C3H 10T1/2 cells underwent substantial transformation with 0.25 µg/ml MCA alone, and little or no transformation with 0.1 µg/ml TPA alone (155). Balb/c 3T3 cells underwent minimal or no transformation with a much higher dose of MCA (1.0 µg/ml) but considerable transformation with the strong promoter PDD at the same dose as TPA that was used with the C3H 10T1/2 cells (156). The original standard line of NIH 3T3 cells underwent spontaneous transformation without treatment with either PAH or phorbol ester after one round of prolonged selection at high density (157). A subline of the NIH 3T3 cells failed to undergo transformation after a very extended period of selection at high density, but did transform with TPA treatment under the same condition of selection (24). Sublines of NIH 3T3 cells that readily undergo transformation after a single 2 week incubation at high population density respond to TPA with a suppression of transformation, presumably because the extra growth stimulation by TPA diminishes the extent of selection required for the development of transformed foci (158).

Another example of variation in transforming behavior within and between fibroblast cell lines is seen in comparing the relative responsiveness of C3H 10T1/2 and NIH 3T3 cells to transfection with c-Ha-ras oncogene. One laboratory found the two cell lines equally sensitive to transformation by the transfected oncogene (159); another laboratory reported that the NIH 3T3 line was 50-fold more sensitive than the C3H 10T1/2 line to transformation by the transected Ha-ras genes, although both cell lines had the same transfection competence (160). Further inter-laboratory differences in the C3H 10T1/2 line are implicit in the findings that transformation is induced only by adding PAHs in one laboratory (161) and that it undergoes spontaneous transformation with no additives under prolonged selective conditions in another laboratory (162).

Variation among mouse strains in sensitivity to tumor promoters is not restricted to cell culture. Wide variation in response of mouse skin in the living animal to TPA is seen in degree and persistence of hyperplasia and in sensitivity to promotion (150). RPA, which was said to have only second-stage promotion (150), had substantial complete promoting activity in SENCAR mice (149); however, it is likely that there is less within-strain variation in the intact organism than there is in its cell cultures because of the novelty to normal cells of the culture environment and a number of uncontrolled variables.

Role of PAHs and promoters in carcinogenesis by tobacco smoke

A real association between smoking, especially of cigarettes, and lung cancer was established on the basis of clinical and statistical investigations (163–165). Early attempts to produce lung or skin cancer in experimental animals by tobacco smoke produced results of questionable significance, but a sustained, successful study of skin carcinogenesis in mice by tobacco smoke condensate was begun in the early 1950s (166). Three times a week painting of mouse skin with cigarette tar produced papillomas in 50% of the mice in an average of 56 weeks and carcinomas in 44% of the mice in 71 weeks. Maximum
tumor production required 12 months of three times a week application of high concentrations of the tar (167). Condensate had to be obtained from tobacco heated to at least 720°C to produce papillomas and at 800°C or higher to produce both papillomas and carcinomas, the latter being close to the measured temperatures of burning cigarettes (168).

The neutral fraction obtained from condensed cigarette smoke contained most of the PAHs of tar, including B[α]P, and it had the strongest tumorigenic activity of all the fractions (169). The B[α]P content of total tar, as well as that of the active fractions, was far too low by itself to account for their tumor producing activities (170). In fact, no carcinogens were identified in sufficient quantity to account for the positive results in tobacco tar or its fractions. Most of the tumor-producing activity of the neutral fraction appears in a subfraction eluted from chromatograms with carbon tetrachloride (169). This subfraction contains about eight identified PAHs, including B[α]P, as well as some unidentified PAHs. The amount of B[α]P is less than 1/25th the amount of pure B[α]P required to account for the tumor-producing activity of the fraction. The neutral plus acidic fraction was more active than the neutral fraction alone suggesting the presence of promoting activity in the acidic fractions. The water soluble fraction of the tar had no tumorigenic activity.

Further studies of the PAHs with four, five and six condensed nuclei in tobacco smoke condensate indicated they are the major but not the only skin carcinogens in the condensate (171). B[α]P could account for only 1.6–2.4% of the tumorigenic activity of tar. The carbon tetrachloride fraction, which amounts to only 1.7% of total tar, has most of its carcinogenic activity. That activity is the result of at least the sum of all the PAHs, including some unknown. Experiments in which two weakly carcinogenic PAHs were injected together subcutaneously yielded more tumors than the sum of their individual yields, indicating a synergistic action (172). The tumor-inducing effects of two strongly carcinogenic PAHs were additive, but not perfectly so. Other non-PAH tumorigenic substances, especially promoters, were thought necessary to account for the activity of tobacco smoke. Estimates based on subcutaneous injection of condensate into mice indicated that >0.5% of the sarcoma-producing activity could be accounted for by the amount of B[α]P present (173). It was concluded that >99% of the tumor-producing activity of the tar consisted of co-carcinogenic or promoter-like substances.

Tar obtained by the combustion of cigarette tobacco at 500–700°C failed to induce tumors in more than a small percentage of mice when applied frequently to the skin (174). Painting with a threshold concentration of B[α]P as initiator produced few tumors but a large number of papillomas appeared when the B[α]P followed by frequent applications of cigarette tar (174). Carcinomas also appeared some time after the papillomas. The number of tumors produced by the initiation–promotion sequence of B[α]P and tar far exceeded the number produced by the sum of tumors produced by B[α]P alone and tar alone. A similar picture was produced by B[α]P and croton oil. Although the author felt the results qualified cigarette tar as a co-carcinogen, they equally qualify it as a tumor promoter. In contrast, the combination of cigarette tar and croton oil produced no more tumors than the sum produced by each alone, indicating that the tar had minimal initiating activity under the conditions of the test.

Reagent grade phenol and commercially obtained derivatives of phenol were reported to promote the formation of papillomas and carcinomas when repeatedly applied after initiation with DMBA (175). There had to be at least one unsubstituted carbon ortho to the phenol group for promoting effect. The results were entirely analogous to those obtained with croton oil, although the effective dose of phenol was 10-fold higher than that of croton oil and many times higher than that of TPA, the active component of croton oil. It was clear, however, that pure phenol, as well as croton oil (176), were weakly carcinogenic on their own when adequately tested in susceptible mice.

The phenolic fraction of cigarette smoke condensate was then tested for its capacity to promote tumor formation after initiation by DMBA in a strain of mice inbred for susceptibility to skin tumor production by carcinogens and promoters (177). The smoke phenolic fraction itself produced no tumors when repeatedly applied three times a week, and the single dose of DMBA itself produced a few papillomas (6 papillomas in 37 surviving mice) but the combined treatment produced 65 papillomas with 1 and possibly 2 carcinomas in 30 survivors. The results clearly indicated that the phenolic fraction of tobacco smoke condensate had considerable tumor-promoting activity in susceptible mice, although it had little carcinogenic activity under the experimental conditions described.

As noted by others, it was concluded that the amount of B[α]P plus the other known carcinogens in the smoke condensate was far from enough to account for the complete carcinogenic effect of tobacco condensate on mouse skin. The concentration of B[α]P alone was thought to be in the range that might initiate tumors with the aid of promoters. However, that was on the assumption that B[α]P was as active an initiator at low doses as DMBA when, in fact, B[α]P has to be used at much higher concentrations than DMBA to initiate tumors and binds less well to DNA (148). Some of the PAHs in smoke condensate that are borderline or negative as carcinogens, such as BA or DB[αc]A, are relatively potent initiators (178). In addition, there might also be present in the condensate incomplete carcinogens such as urethane (179,180), which are able to produce tumors of the skin only with the aid of promoters. The relative weakness of the carcinogenic effect of the cigarette smoke condensate was attributed to the paucity of initiators based on the observation that adding initiators to the condensate increases its carcinogenic effect; but adding promoters does not (177). The authors considered it not improbable that the correlation between smoking habits and lung tumor incidence is determined not primarily by the carcinogenic effect of tobacco smoke but by its predominantly tumor-promoting action on the bronchial epithelium. The relatively high concentration of phenolic compounds in the smoke condensate could account for most of its promoting activity. This projection was supported by the finding that the phenolic fraction promotes tumor formation at the same concentration as its components are present in the original condensate (181,182). Since phenol itself is present in the phenol fraction at only one-tenth the amount at which pure phenol promotes, there must be other promoters present in the phenol fraction. There is also some promotional activity in the acidic fraction of the condensate as well as in the neutral fraction which contains most of the PAHs, and in the residue of the phenolic fraction.

Further experiments with whole cigarette smoke condensate showed remarkable promoting activity and no tumorigenicity without initiation at the dose levels used (183). Attention was again drawn to several PAHs that were weakly- or non-carcinogenic but were active initiating agents, with the implica-
tion that there may be more such agents in tobacco smoke (178,184,185). Although the results were somewhat clouded by some tumor production by croton oil itself at the dose used, the conclusions were reinforced by demonstrable initiating activity of the non-carcinogenic PAHs with a much lower dose of the croton oil that by itself produced no tumors whatever (185). Thus, while $<3\%$ of the carcinogenic activity of cigarette smoke condensate could be accounted for by the activity of the known carcinogens present (181,186), their initiating activity plus that of the non-carcinogenic initiators in concert with the strong promoting activity of the condensate could reasonably be expected to account for its full carcinogenic activity.

It is of interest to note that some of the tumor-promoting agents in cigarette smoke may have their source in unburned tobacco. Both an aqueous alkaline extract and an acetone–benzene extract of unburned cigarettes tobacco produced skin tumors when painted on mice previously initiated with DMBA (187). The extract from as little as 0.5 cigarettes a day was effective, which raises the question of their role in oral cancer associated with tobacco chewing. Polyphenolic compounds are thought to be among the promoters of unburned tobacco (188).

Low concentrations of anthralin, a polyphenolic derivative of anthracene, promotes mouse skin tumors (189). Phenolic metabolites of PAHs might act as promoters and 2-HOPB is itself a complete carcinogen in mouse skin (29,30), which indicates it has promotional activity. However, no clear relationship between aqueous extracts of unburned tobacco and cigarette smoke promoters was apparent (190).

The multifactorial basis for carcinogenesis by tobacco smoke has interesting parallels in epidermal carcinogenesis by coal tar. Once B[a]P, which was isolated from coal tar, and a number of synthetic PAHs were found to be carcinogenic on mouse skin, carcinogenic studies in mice were largely confined to the use of pure PAHs. There was little further study in mice of carcinogenesis by coal tar itself since it was generally assumed that B[a]P was the major and perhaps only carcinogen present. However, several fractions of coal tar entirely free of B[a]P were found to be carcinogenic, one of them with the unusual property of high carcinogenic potency for rabbit skin and none for mouse skin (191). Although the final active material of the fractionation represented more than a 200-fold concentration of coal tar, it still consisted of a mixture of substances and the active constituents were not identified. Almost half a century later, 19 major PAHs were identified in pharmaceutical coal tar (192). At least seven of the PAHs were known to be carcinogenic while there was only limited or inadequate evidence of carcinogenicity for the remaining 12. Even this study represented only a small fraction of the hundreds of PAHs in coal tar (193). Many of the 19 PAHs exhibited binding to DNA, which was taken as an indicator of their biological activity.

While little work continued with coal tar itself on mouse skin, observations were made on its application to rabbit skin which gave rise to the terms initiation and promotion (114). It was observed that tumors induced by B[a]P and MCA remained small, dry and indolent for long periods whereas many of the tar tumors were fleshy, vigorous and rapidly enlarging. This difference was attributed entirely to the superior promotional activity of the coal tar. It is possible therefore that the PAHs themselves in coal tar, like those in cigarette tar, are present in too low a concentration to be complete carcinogens and produce their effect through initiation combined with a large contribution from promoters. One reason for the diversion from work on coal tar is probably related to its diminished medical importance with the virtual cessation of coal gas production, while cigarette smoking has remained a major medical problem. It should be informative to compare the PAH concentrations in coal tar and cigarette smoke condensate because they have much in common regarding skin carcinogenesis.

Co-carcinogenic and anti-carcinogenic compounds in cigarette smoke condensate

Co-carcinogenesis differs from tumor promotion operationally in that co-carcinogens are applied repeatedly in the same solution as carcinogens, rather than as promoters which are repeatedly applied alone after a single initiating dose of carcinogen. A series of 21 constituents of tobacco smoke and related compounds were tested for co-carcinogenic activity in the presence of a low dose of B[a]P (194). The classes of compounds were phenols, aliphatic hydrocarbons, non-carcinogenic aromatic hydrocarbons, long-chain acids and alcohols and TPA. The results of testing some of the compounds are shown in Table VII. Catechol, the most abundant phenol in tobacco smoke, had strong co-carcinogenic activity but no tumor-promoting activity. It increased the number of papillomas and carcinomas induced by B[a]P alone by 3–5-fold. Similar increases were shown by the other co-carcinogens shown in Table VII. The classic promoter TPA had strong co-carcinogenic activity but there was, in general, no correlation between promotion and co-carcinogenesis. Seven of the 21 compounds tested exhibited strong co-carcinogenic activity: in addition to the four shown in Table VII, the others were undecane, pyrene and fluoranthene. None of the co-carcinogens were significantly carcinogenic by themselves. The aliphatic hydrocarbons, decane and tetradecane, as well as the two most powerful promoters known, TPA and anthralin, displayed both promoter and co-carcinogenic activity; neither of the latter two is a component of cigarette smoke condensate. TPA, although used at a dose several orders of magnitude lower than the tobacco co-carcinogens, caused far more papillomas by itself than any of them; when combined with B[a]P, it only marginally increased the number of carcinomas produced by B[a]P alone. The 2 mg dose of catechol used in the co-carcinogenic test is equal to the amount in 4–5 non-filter cigarettes and in 8–10 filter cigarettes. These results take on particular significance because the co-carcinogenic methodology simulates the simultaneous exposure to components over extended periods of time that is experienced by smokers. The mechanism of action of the co-carcinogens is unknown. It is notable that the co-carcinogens represent different classes of compounds and are therefore likely to have different mechanisms of action.

Four of the compounds that were tested completely inhibited the carcinogenic activity of B[a]P. They were the phenols esculin and quercetin, the aliphatic hydrocarbon squalene and the long-chain fatty acid, oleic acid. It could not be determined at the time whether the co-carcinogenic and anti-carcinogenic effects of tobacco smoke as tested on skin (194) would have the same effect when tested by inhalation since there was no reliable inhalation test for lung carcinogenicity. Now that there is such a test (195–197), re-examination of the question might be feasible.
Direct mutation versus selection by cigarette smoke in human lung cancer

Recently disagreement has arisen between those who view the main effect of cigarette smoke in the etiology of lung cancer as the result of direct targeted mutation by B[a]P metabolites or structurally related compounds and those who offer a selection-based explanation of the mutations in tumors. The advocates of direct mutation by B[a]P of cigarette smoke in lung tissue base their claim on the apparent coincidence of mutational hot spots in the p53 gene of lung cancer among smokers and adduct hot spots induced by B[a]P in the p53 gene of human cervical cancer (HeLa) cells and bronchial epithelial cells (198). This conclusion was criticized because of the absence of a critical control group of non-smoking lung cancer patients and the contention that the data were insufficient in general to prove that the p53 mutations in lung cancer are anything other than predominantly endogenous in origin (199). Another group did compare both the p53 mutations in lung cancer of smokers and non-smokers using recently updated data (200). These authors concluded ‘that physiological stresses (not necessarily genotoxic) aggravated by smoking is the leading risk factor in the p53-associated etiology of lung cancer’. There followed a rebuttal with a more complete analysis of the data, which concluded that the p53 mutations in lung cancer are from direct damage from cigarette smoke carcinogens rather than from selection of pre-existing endogenous mutations (201) and a reply to the rebuttal (S.N.Rodin and A.S.Rodin, in preparation).

None of the above took into consideration the historical experimental evidence considered earlier of the relative roles of initiation and promotion by cigarette smoke condensate in skin carcinogenesis of mice. The relevant evidence is as follows. The concentration of B[a]P in the condensate is 40-100-fold too low to account for direct carcinogenesis by the condensate on mouse skin (171) and in the subcutaneous tissue of rats, respectively (173). The combined concentration of known PAH carcinogens in the condensate is unlikely to be responsible for >3% of its carcinogenic potential (171,181). Some of the PAHs that are ineffective as skin carcinogens, however, act as initiators, as do the known carcinogens at lower concentrations than required for their complete carcinogenic activity. Initiation, of course, requires promotion to elicit tumor formation and the predominant role of promotion is to select the initiated cells. The initiating activity of the condensate is relatively weak and is bolstered by the action of strong carcinogens (177). In contrast, the promoting (and therefore selective) activity of the condensate is strong (183) and is not increased by adding exogenous promoters (177). Hence, a major pathway of cigarette smoke condensate in skin carcinogenesis is likely to be selection. The selected cells would have to be initiated by the condensate or arise endogenously. Since the phenolic fraction of the condensate does not, by itself, produce skin tumors (177,181), the promoters in whole smoke condensate must be acting on mutations induced by B[a]P and other initiating agents of the condensate rather than acting on endogenous mutations.

Also ignored in the disagreement about the relative roles of direct targeted mutations in tobacco-related lung cancers is the well-authenticated presence of carcinogenic N-nitrosamines in tobacco and tobacco smoke (202). They are specifically associated with tobacco and are present only in environments polluted by tobacco smoke. They are formed by nitrosation of nicotine and structurally related alkaloids. The amount of N-nitrosamines in mainstream cigarette smoke is hundreds of times higher than their regulated level in cooked bacon or beer. The strongest carcinogen of the tobacco-specific N-nitrosamines is called NNK for nicotine-derived nitrosamine ketone, which produces lung tumors in mice plus other respiratory tract tumors in rats and Syrian golden hamsters by various routes of administration, but has not been tested by local administration in the respiratory tract (203). It is relatively specific for the lung and is a weak carcinogen in skin. It requires metabolic activation for binding to DNA where it forms methyl adducts to guanine and pyridyloxobutyl adducts. Of the 20 carcinogens in tobacco smoke that have convincingly been shown to cause lung tumors in laboratory animals, the PAHs and NNK are thought most likely to play major roles (203). In view of the number of PAHs and N-nitrosamines in cigarette smoke that cause G→T transversions, the assignment of the G mutations in the p53 gene from human lung cancers to specific carcinogens (198) has been termed at best speculative (203).

The obvious inadequacy of the foregoing results for understanding the role of smoking in lung cancer is that none of

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**Table VII. Co-carcinogenesis by some representative constituents known or suspected to be in cigarette smoke condensate**

<table>
<thead>
<tr>
<th>Type of carcinogen</th>
<th>Carcinogen (5 µg B[a]P)</th>
<th>Co-carcinogen (dose)</th>
<th>Mice with papillomas</th>
<th>Total papillomas</th>
<th>Squamous carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>–</td>
<td>Acetone</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Acetone</td>
<td>14</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Phenols</td>
<td>–</td>
<td>Catechol (2 mg)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Catechol (2 mg)</td>
<td>36</td>
<td>90</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Pyrogallol (5 mg)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Pyrogallol (5 mg)</td>
<td>40</td>
<td>95</td>
<td>33</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>–</td>
<td>Decane (25 mg)</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hydrocarbon</td>
<td>+</td>
<td>Decane (25 mg)</td>
<td>44</td>
<td>105</td>
<td>41</td>
</tr>
<tr>
<td>Aromatic Hydrocarbon</td>
<td>–</td>
<td>B[e]P (15 µg)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>B[e]P (15 µg)</td>
<td>33</td>
<td>79</td>
<td>27</td>
</tr>
<tr>
<td>Phorbol ester</td>
<td>–</td>
<td>TPA (2.5 µg)</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>TPA (2.5 µg)</td>
<td>25</td>
<td>58</td>
<td>15</td>
</tr>
</tbody>
</table>

*aAbstracted from Van Duuren and Goldschmidt (194).
B[a]P (5 µg) was applied in the same solution as the co-carcinogen in 0.1 ml acetone three times a week to the dorsal skin of 50 female Swiss mice for 368 or 440 days. Controls of B[a]P or co-carcinogen alone in acetone, or acetone alone, were similarly applied.*
them used inhalation as the means of exposure to produce tumors of the respiratory tract. Early positive results on lung neoplasms in mice of the A strain based on inhalation of whole cigarette smoke (204) were dismissed as ‘unimportant’ partly because they produced adenocarcinoma rather than bronchiolar cancer (166), at that time the major form of lung cancer in humans. Since then adenocarcinoma has become the most common type of lung cancer in the United States (205). In the meantime, there was another report of lung adenocarcinomas produced by inhalation of fresh cigarette smoke, this time in Snell’s strain of mice that, like the A strain, had a background of spontaneous lung tumors (206). No lung tumors were produced in C57 Black mice, a strain that had no spontaneous lung tumors (206). A particularly interesting aspect of this report was the observation that the gas vapor phase of cigarette smoke was at least as carcinogenic as the whole smoke in the Snell’s strain, as indicated by an increased frequency and earlier occurrence of lung adenocarcinomas than controls. Although the C57 Black mice developed no lung adenocarcinomas after inhaling cigarette smoke, they did develop a higher frequency of vascular changes in lung and heart than occurred naturally in this strain. The results indicated an enhancing effect of cigarette smoke on existing abnormalities and suggest that the differential response of humans to cigarette smoking has a genetic explanation.

The study of tumorigenesis in lung by inhalation of cigarette smoke has been carried further in recent years with the same highly susceptible A strain of mice used by Essenberg (204). The A strain spontaneously develops increasing numbers of lung tumors with age. These numbers and the response of strain A mice to carcinogens have been stable and reproducible for many years and it is considered the preferred strain for study of lung carcinogenesis (207,208). Tumor development in these mice shares many features with the development of adenocarcinomas in humans (209). Although the A strain mice developed marked increases in lung tumors within 6–8 months of a single injection of a variety of PAHs (207,210), they generally failed to do so when chronically exposed to cigarette smoke by inhalation over an extended period of time (211,212). It was then found that exposure to environmental tobacco smoke of measured nicotine and particulate composition for 5 months followed by 4 months of recovery in filtered air resulted in a significant increase of lung tumors (195). Environmental tobacco smoke is a mixture of 85% sidestream smoke that curls off the end of a lit cigarette between puffs and 15% mainstream smoke that is first inhaled by the smoker and then exhaled (195). Filtration of the smoke to remove particulate material did not reduce the increased number of lung tumors (196). The filtration process markedly reduced the concentration of PAHs and nitrosamines to a level 3 to 6 orders of magnitude below doses required to produce one lung tumor per mouse. Chemopreventative agents that reduced lung tumor multiplicities in mice injected with the tobacco-associated nitrosamine NNK, which is a specific inducer of lung tumors, had no effect on lung tumor multiplicities in mice exposed to tobacco smoke (197). Although the carcinogetic components of the vapor gas phase have not been identified, it is evident they are neither B[a]P nor NNK. Treatment of human tracheobronchial cells with gas phase cigarette smoke led to DNA strand breakage correlated with chemical modification of all four DNA bases involving attack by reactive oxygen and nitrogen species (213). The procedure of 5–6 months exposure of A strain mice to environmental tobacco smoke followed by 4 months recovery in air has been successful in producing a significant increase in lung tumor incidence in seven independent trials in one laboratory (197) and has been confirmed in another laboratory (214). It is noteworthy that the environmental tobacco smoke used in these experiments is largely composed of sidestream smoke, which is a significantly stronger carcinogen for mouse skin than is mainstream smoke (215) and has 10-fold higher amounts of some known cigarette smoke carcinogens than mainstream smoke (193,216). When mainstream smoke was used, it failed to enhance the lung cancer yield (214). Since the untreated A strain mice, like Snell’s mice (206), have a significant frequency of lung tumors, the cigarette smoke may be enhancing a predisposed tendency. This is reminiscent of the lung tumor increase produced in newborn mice by B[a]P and its metabolites (51,60,61) and of the increase in pre-neoplastic foci in epidermal cell cultures produced by several PAHs and other carcinogens (64). Enhancement (206) could be a euphemism for selection, which has been suggested as the leading risk factor of smoking in producing human lung cancer (199,200).

The Syrian golden hamster was considered by some as the preferred animal for inhalation studies on tobacco smoke because it has a low background incidence rate of spontaneous pulmonary tumors and few interfering respiratory infections (217). It has been repeatedly confirmed that inhalation of tobacco smoke causes carcinomas in the larynx of hamsters. In a large study with 4440 hamsters that inhaled smoke from German cigarettes one, two or three times a day for 52 weeks or for a lifespan, 0.62–10.6% developed laryngeal carcinomas (218). No such tumors developed in sham exposed controls nor in animals exposed to the vapor phase, in contrast to the results described above in strain A mice (195,196). In studies with inbred lines of hamsters, 19% of one strain developed laryngeal tumors, while only 4.4% developed in the other strain on inhalation of smoke from Kentucky reference cigarettes for up to 100 weeks (219). With improved methodology and using the susceptible hamster strain, 47.4% developed laryngeal cancer upon inhalation of smoke from reference filter cigarettes from the United Kingdom for 59–80 weeks (220). No laryngeal tumors developed in controls in either study. The only significant results in hamster inhalation studies were obtained with production of laryngeal tumors which differ from the lung carcinomas produced by smoking in humans and the lung tumors produced in A strain mice by inhalation (195). A high incidence of the laryngeal tumors in hamsters was produced by inhalation of filter cigarettes indicating PAHs were not the carcinogetic agents (220). It would seem that the lung tumors produced by inhalation in A strain mice (195) are the closest to those seen in human smokers and that factors other than, or in addition to, PAHs and NNK have to be considered as significant factors in lung carcinogenesis. An important factor in achieving positive results in the mouse inhalation experiments was to allow a 4 month smoke-free period after 6 months of exposure to cigarette smoke, which suggests, paradoxically, that continued smoking might temporarily inhibit the growth of cells initiated for tumor production by that very same smoke.

Most of the experimental work on carcinogetic components of tobacco smoke, however, has been done by painting mouse skin with smoke condensate or its fractions. This has led to much valuable information about the carcinogetic effect of complex mixtures of PAHs and of promoters in skin tumor development, but the results cited above on inhalation of
烟气在A品系小鼠中引发疑问关于皮肤肿瘤的致突变作用。如前所述，一剂一次的烟气引人注意，因为其在肺部造成高突变。这Cab为B[a]P (203)。这些疑虑被加强由事实证明DBA，其在 Tilc exists in mouse mice and，因为DBP-a,P，是肿瘤诱导的。”

在吸烟诱导的肿瘤形成中增加了当用TPA，但不可持续。”


dehydrodiol (222) which is a strong initiator in mouse skin and a carcinogen in rat mammary gland (223). In light of the positive results on lung tumor induction by inhalation in mice of gas phase cigarette smoke，which is almost devoid of PAHs，their long-posed major role in human lung cancer should be stringently re-evaluated.

It seems likely that tobacco carcinogenesis in cigarette smokers is from a complex mixture of initiating PAHs，nitrosamines and probably other agents. The relative importance of multiple initiating events over a prolonged interval and multiple promoting events in combination with initiating events has yet to be determined.

Reprise

The bay region diol epoxides of PAHs are widely accepted as the ultimate carcinogenic forms of PAHs through their covalent binding to DNA. If this were so，one would expect that they would be carcinogenic at lower concentrations than the parental hydrocarbon. This is in fact the case upon intraperitoneal injection into newborn mice where BPDE-2 produces lung adenomas at 1/50th the concentration that B[a]P when repeatedly painted on mouse skin (29-31). Even when applied on skin as a single initiating dose，BPDE-2 was less active than B[a]P (37)，although more active than it was as a complete carcinogen. A strong case can therefore be made for BPDE-2 as the ultimate carcinogen of B[a]P in the lung of newborn mice; the results in mouse skin indicate that additional processes come into play. One of them is，of course，promotion since the effectiveness of BPDE-2 is significantly increased when promoted by TPA，but that is insufficient when it is expected that an ultimate PAH carcinogen would be more effective than its parental hydrocarbon，as it is indeed in newborn mouse lung carcinogenesis. The original thought that application of the diol epoxide might be rendered ineffective because of its high reactivity was ruled out by the finding that as much bound to DNA of mouse skin as when B[a]P was applied (53-56).

One obvious implication of the ineffectiveness of BPDE-2 in skin is that other metabolites of B[a]P contribute to skin carcinogenesis. One of these is BP-7,8-dihydropyrene which is as active as B[a]P in skin carcinogenesis (29-31). The fact that B[a]P-7,8-dihydropyrene is an immediate precursor of BPDE and that H2BP-7,8-diol is completely inactive are reasons for considering the former the proximate carcinogen and BPDE the ultimate carcinogen in skin，but that does not explain the weak carcinogenicity of BPDE. Another possibility is that the great toxicity of the diol epoxides (48-50) interferes with skin tumor development，but that seems unlikely in view of the decrease of their effectiveness upon dilution (29-31).

A chemical derivative of B[a]P that is an active skin carcinogen is 2-HOBP，although it is not a normal metabolite of B[a]P (33). Aside from the untested possibility that 2-HOBP is partly converted to BPDE，its carcinogenic activity suggests that there are routes to skin carcinogenesis other than direct binding to DNA. That certainly seems to be the case for neoplastic transformation of fibroblastic cells in culture since K-region epoxides (70,76,77) and diol epoxides (79,80) were more efficient than the parent hydrocarbon in neo-plastically transforming the cells，although neither group was detectably bound to DNA when the parental hydrocarbons were applied to cells (11,78). The K-region epoxides were mutagenic (71,72) and interacted with nucleic acids and proteins (70)，but were less carcinogenic than their parental hydrocarbons in rodents when injected subcutaneously or painted on the skin (73,74). Hence，there was again a divergence of carcinogenic results between cell culture (or newborn mice) on the one hand and adult skin on the other that indicates that mechanisms in addition to direct binding of metabolites to DNA underlay PAH carcinogenesis in the latter case.

Interestingly，Hieger，who played a key role in the isolation of B[a]P from coal tar and establishing the carcinogenic activity of B[a]P and synthetic PAHs (1,2,5)，later considered the possibility that PAHs might employ carcinogenic mechanisms that are independent of chemical interaction with cellular components when he learned of spontaneous transformation of rodent cells in culture，and sarcoma production by subcutaneously inserted plastic films (224) and had shown that a long persisting deposit of subcutaneously injected cholesterol in mice produced sarcomas (225). It was subsequently shown that the plastic film-induced sarcoma cells arose in the host reaction capsule surrounding the film among cells that were some distance from the film itself (226). These findings raise the possibility that the persistent unmetabolized parental PAHs themselves might contribute to carcinogenesis，perhaps by inciting a supportive dermal reaction or promoting a more permissive environment for clonal expansion of initiated cells.

Synergism in carcinogenic mechanisms first became apparent in the interdependence of initiation and promotion with the realization that promoting agents were not mutagenic (126) and did not require metabolic activation for their promoting effect on skin (127). Initiation required only a single application of an agent，while promotion required repeated application over an extended period of time. Promotion itself consisted of a relatively stable stage of conversion，which probably involved chromosome aberrations (143)，and a stage of proliferation which drove the selection of altered cells in neoplastic development. DNA adduct formation by PAH metabolites does induce mutations but it is not sufficient for initiation of skin tumors，since some PAH metabolites like BPDE-1 form mutagenic adducts with DNA but are not carcinogenic (56). In this context it is instructive to note that the effective application of carcinogenic PAHs to skin results in the binding of ~50,000 diol epoxides to the DNA of each epidermal cell (43)，although only a few are presumably sites of initiation. Since most tumors are of clonal origin，the large number of DNA adducts distributed among many cells raises the possibility that the cells that form the microenvironment of the prospective tumor cell are altered in such a way as to provide a selective microenvironment for development of that cell into a tumor.

The mutation-producing efficiency per DNA adduct is not known but it seems likely that there are multiple mutations per cell and particular combinations of these mutations may
be required for initiation. In addition, multiple mutations in each epidermal cell of a topically treated field may underlie the widespread apoptosis and sublethal damage that characterize the early stages of skin response to PAH application (98,100) and thereby increase the permissiveness of the microenvironment for clonal expansion of initiated cells. Such facilitation is plausible given the observations that (i) thoracic radiation or cyclophosphamide treatment of mice before intravenous injection of tumor cells enhances the number of lung colonies by a factor of up to 100 or more (227) and that (ii) irradiation of cleared mouse mammary fat pads promotes the tumorigenic potential of unirradiated mammary tumor cells that otherwise have low tumorigenic penetrance (228). These observations and others (229,230) point to the hierarchical structural organization of tissue as an important element of maintaining normal cell behavior in the face of considerable atomic and molecular heterogeneity within cells (231) including many mutations (232). The need for repeated application of PAHs to skin over an extended period of time to produce tumors (82,83,85) may be to persistently disrupt the architecture of the epidermis (98,99) and thereby diminish its regulatory power over rogue clones. This disorganizing effect appears to be operative in the development of human colon cancer in which dysplastic adenomas associated with APC mutations are much more likely to progress to carcinomas than are the relatively well ordered polyps which have a high incidence of K-ras mutations (233,234). Promotional treatment may also contribute to the disorganizing effect since it causes the almost immediate shrinking and aggregation of cultured cells (158) and eliminates metabolic cooperation between them (235).

In order to account for the sharp discrepancy between the poor carcinogenicity of BPDEs for skin and their remarkable capacity for lung adenoma formation in newborn mice, one should consider mechanisms for mutagenesis that do not require direct binding to DNA. The need for tumor promotion in skin makes up part of the difference, but promotion requires neither mutation (126) nor binding to DNA (127). In any case promotion could not fully make up for the fact that BPDE-2 is far more active than either B[a]P or B[a]P-7,8-diol in lung carcinogenesis. Perhaps the relatively rapid rate of lung growth in newborns provides its own promotional stimulus. This would still require additional initiating mechanisms in the skin to account for the poor carcinogenicity of BPDE-2 in skin.

One possible indirect mechanism is the production of reactive oxygen species. There is a 3-fold increase in peroxidation of mouse skin within 20 h after a single application of DMBA (107). There is an increase in chromosome breaks in human blood leukocytes treated with DMBA and the incidence of breaks can be reduced >60% by certain antioxidants (106). This indicates that PAHs induce chromosomal damage by forming reactive oxygen species. It had in fact been known that PAHs induce chromosome damage soon after their application (101,103,104). It is noteworthy that the gas vapor phase of cigarette smoke, which is largely free of PAHs and reduces nitrosamine concentrations of the smoke 10–20-fold (196), produces much more damage to DNA by its reactive nitrogen species than by oxygen radicals (213). The role of reactive nitrogen species in PAH and nitrosamine carcinogenesis has not been thoroughly explored. TPA, the most active of tumor promoters, produces chromosome breaks which appear related to changes in lipid metabolism (236) and to the increased production of hydroperoxide in mouse skin after repeated TPA application (237). Reactive oxygen species could be produced by some of the many metabolites of PAHs which could also contribute promotional activity to account for complete carcinogenesis by the PAHs.

Localization of PAHs in lysosomes (96) has suggested that there is a release of DNase to cause double-stranded breaks in chromosomes (109). The steric resemblance of PAHs to steroids suggested that they act on the same sites as steroid hormones (6). Such speculation is bolstered by the activity of B[a]P and its hydroxylated metabolites in an estrogen receptor reporter gene assay (238) and the antiestrogenic responses of cells to PAHs mediated by binding to Ah receptors (239). DMBA and B[a]P cause sustained increases in cellular Ca^{2+} and cell proliferation in primary cultures of human mammary epithelial cells which are not caused by the non-carcinogenic PAHs B[a]P and anthracene (240).

A common feature of cancer cells is a high rate of aerobic glycolysis which was attributed by Otto Warburg to damaged respiration (241). This thesis of damaged respiration was disputed (242) but the high ratio of aerobic glycolysis to respiration was confirmed (243). A possible source of impaired respiration in PAH-induced tumors is implicit in the steric relation between PAHs and steroids and the finding that steroids increase the permeability of phospholipid structures to simple cations in directions similar to biological membranes (244). These phospholipid structures resemble membrane-bounded biological structures like mitochondria, and PAHs localize in lipid-rich membranes (95). The coupling of respiration to phosphorylation is achieved by creating a proton gradient across the inner mitochondrial membrane (245) and it is reasonable to assume that the proton gradient would be impaired by the localization of PAHs in mitochondrial membranes. The resultant decrease in oxidative phosphorylation would favor selection of cells that could substitute glycolysis for respiration in generating ATP. While such a selectionist hypothesis is admittedly speculative, it conforms with the evidence for cellular selection in the development of PAH-induced tumors (246); and with the evidence that selection plays a major role in the origin of human cancers (128,199,200,247) and in the spontaneous transformation of cells in culture (230). In any case, it should be apparent that there are many possible mechanisms in addition to, or complementary to, DNA adduct formation that could come into play and help in the understanding of the discrepancies in PAH induction of tumors in different systems by the parental PAHs and their diol epoxides.

The variety of possible mechanisms would shed light on a number of purely biological observations about PAH-associated tumor development. The best known of these observations is the two-stage initiation–promotion sequence, which has grown to the three-stage initiation–conversion–proliferation sequence and its functional correlates of mutation–chromosome aberration and cellular selection (143). Less well-known is the observation that tumors produced by coal tar are far more vigorous than those induced by high concentrations of pure PAHs, which led to the conclusion that coal tar has more carcinogenic activity of coal tar is usually attributed to its multiple PAH constituents (192,193,248), it is not unlikely that it also contains multiple promotional components like those of tobacco smoke condensate that contribute to carcinogenesis (177,181,183). The capacity of PAHs to act as complete carcinogens implies their action as both initiators and (complete) promoters. A
number of the metabolites of B[a]P can induce hyperplasia, which is an essential component of promotion (249), but not all agents that induce hyperplasia are promoters (119). Since several of the B[a]P metabolites that induce hyperplasia are non-carcinogenic (29–31), it would be of interest to know which of them can act as promoters.

Comprehensive review of the tumor-producing capacity of the PAHs and their metabolites indicates that the term ultimate carcinogen should be qualified by stating the tissue target and the age of the host, etc., in which the criteria of an ultimate carcinogen are met. The evidence for BPDE-2 as the ultimate carcinogen for lung in newborn mice is strong, not only because it is highly mutagenic and binds to DNA, but because it is effective at almost 2 orders of magnitude lower concentration than B[a]P. Even there it is noteworthy that the strain of mice in which the parameters were established (BLU.Ha ICR) has a background incidence of spontaneous lung adenomas (51,60,61), which marks it as particularly susceptible to lung cancer with the possibility that the carcinogenic effect is an enhancement of a pre-existing tendency rather than initiation of new lesions (206). In mouse skin, the relative carcinogenicity of B[a]P and BPDE-2 is reversed with the parental hydrocarbon effective at lower concentrations than is the diol epoxide (29). Tumor promotion reduces but does not eliminate the difference and it certainly does not approach the expectation that an ultimate carcinogen be effective at lower concentrations than its precursors. The fact that the diol epoxides do bind to DNA in mouse skin (53–56) eliminates the possibility that they are metabolized to tetraols before they can form DNA adducts and suggests that mechanisms other than, or in addition to, such binding contribute to carcinogenesis.

The similarity in binding of BPDE-1 and BPDE-2 to DNA when they are applied to mouse skin (54–56) in contrast to the difference in tumor production there requires further investigation. The suggestion that the disposition of the two isomers in skin might account for the large difference in their epidermal carcinogenicity (56) should be pursued experimentally. A similar course of action is warranted to account for the large differences in lung adenoma production in newborn mice by the diol epoxides (51,60,61). If the differences in neoplastic transformation by PAHs in different targets cannot be explained in terms of specific site binding to DNA, then alternative mechanisms should be considered.

It is likely that synergistic mechanisms are involved in lung carcinogenesis by tobacco smoke. Studies on skin carcinogenesis by cigarette smoke condensate indicated that the concentrations of carcinogenic PAHs in the condensate were far too low to be carcinogenic by themselves (171,173). However, some weak or non-carcinogens are active initiators when followed by TPA promotion (178,184,185). Strong promoting activity was demonstrated in cigarette smoke condensate (177,178,183). Catechol proved to be an active co-carcinogen of B[a]P at concentrations approximating those in tobacco smoke (194). The co-carcinogenic procedure of repeated combined treatment of an initiating agent with co-carcinogen approximates the simultaneous long-term exposure of cigarette smokers to both agents (194). The inferred role of promoters and co-carcinogens in tumor production by tobacco smoke has to be considered in the current disagreement as to whether lung carcinogenesis in smokers is the direct result of mutagenesis by B[a]P and related PAHs (198,201), or results from selection by physiological stresses, not necessarily genotoxic, that are aggravated by smoking (200; S.N. Rodin and A.S. Rodin, in preparation). Nor can the role of NNK and other N-nitrosamines in tobacco be ignored, especially since they are relatively specific lung carcinogens in experimental animals regardless of the route of administration (202,203). This dispute is of great significance in understanding tumor pathogenesis, not only in lung cancer, the most common of human neoplasms, but in many other human tumors, most of which bear endogenous mutations (128,199). Synergistic mechanisms and the role of selection should also be kept in mind in evaluating the pathogenesis of lung cancer in mice by inhalation of environmental tobacco smoke (195,196).

Note added in proof

The promoting action of TPA in skin carcinogenesis has been characterized as either (a) an epigenetic event on the basis of the regression of papillomas when TPA applications cease (22); or (b) selective proliferation of initiated cells in a heterogeneous population in which the majority uninitiated cells respond by differentiation (23,145). Both terms assume the non-genetic nature of promotion, and may apply equally to co-carcinogenesis by tobacco smoke (194). However, chromosome aberrations also contributes to promotion (139,143) and perhaps also to tobacco co-carcinogenesis.

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