# MULTISTAGE CHEMICAL CARCINOGENESIS IN MOUSE SKIN[1]

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## SUMMARY

Skin tumors in mice can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter. The initiation phase requires only a single application of either a direct acting carcinogen or a procarcinogen which has to be metabolized before being active and is essentially an irreversible step which probably involves a somatic cell mutation, as ~~evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities~~. There is a good correlation between the skin tumor initiating activities of several polycyclic aromatic hydrocarbons (PAH) and their ability to bind covalently to epidermal DNA. Results ~~from our~~ laboratory ~~as well as others~~ suggest that "bay region" diol-epoxides are the ultimate carcinogenic form of PAH carcinogens. Potent inhibitors and stimulators of PAH tumor initiation appear to affect the level of the PAH diol-epoxide reacting with specific DNA bases. Recent data suggests that the tumor promotion stage involves at least three important steps: 1) the induction of embryonic looking cells (dark cells) in adult epidermis; 2) an increased production of epidermal prostaglandins and polyamines; 3) sustained proliferation of dark cells. Retinoic acid specifically inhibits step two whereas the anti-inflammatory steriod fluocinolone acetonide is a potent inhibitor of steps one and three. The mechanism and the importance of a specific sequence for each step in chemical carcinogenesis in mouse skin ~~will be discussed in~~ are detailed.





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[3]Abbreviations used: PAH, polycyclic aromatic hydrocarbon; TPA, 12-0-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCB, polychlorinated biphenyl; BP, benzo(a)pyrene; BP-7,8-diol-9,10-epoxide or BP-diol-epoxide, (±)-trans 7β; 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BA, benz(a)anthracene; 7-meBA, 7-methylbenz(a)anthracene; DMBA, 7,12-dimethylbenz(a)anthracene; DB(a,h)A, dibenz(a,h)anthracene; DB(a,c)A, dibenz(a,c)anthracene; MC, 3-methylcholanthrene; B(e)P, benzo(e)pyrene; Mez, mezerein; 4-0-meTPA, 4-0-methyl-12-0-tetradecanoylphorbol-13-acetate; EPP, ethylphenylpropiolate; Poly I:C, polyriboinosinic:polyribocytidylic acid; 7,8-BF, 7,8-benzoflavone; FL, Friend erythroleukemia cells; HPLC, high pressure liquid chromatography.

## SUMMARY

Skin tumors in mice can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter. The initiation phase requires only a single application of either a direct acting carcinogen or a procarcinogen which has to be metabolized before being active and is essentially an irreversible step which probably involves a somatic cell mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities. There is a good correlation between the skin tumor initiating activities of several polycyclic aromatic hydrocarbons (PAH) and their ability to bind covalently to epidermal DNA. Results from our laboratory as well as others suggest that "bay region" diol-epoxides are the ultimate carcinogenic form of PAH carcinogens. Potent inhibitors and stimulators of PAH tumor initiation appear to affect the level of the PAH diol-epoxide reacting with specific DNA bases. Recent data suggests that the tumor promotion stage involves at least three important steps: 1) the induction of embryonic looking cells (dark cells) in adult epidermis; 2) an increased production of epidermal prostaglandins and polyamines; 3) sustained proliferation of dark cells. Retinoic acid specifically inhibits step two whereas the anti-inflammatory steriod fluocinolone acetonide is a potent inhibitor of steps one and three. The mechanism and the importance of a specific sequence for each step in chemical carcinogenesis in mouse skin will be discussed in detail.





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# INTRODUCTION

Skin tumors in mice can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter. The initiation phase requires only a single application of either a direct acting carcinogen or a procarcinogen which has to be metabolized before being active and is essentially irreversible, while the promotion phase is initially reversible but later becomes irreversible. This system has not only provided an important model for studying carcinogenesis and for bioassaying carcinogenic agents, but it is also one of the best systems for investigating the effects of inhibitors of chemical carcinogenesis. Recently, the generality of the two-stage system of carcinogenesis has been shown to exist in a number of systems besides the skin such as the liver, lung, bladder, colon, esophagus, mammary, and diaplacental as well as cells in culture (1). This report describes the important events we believe to be associated with tumor initiation by polycyclic aromatic hydrocarbons (PAH)[3] and with promotion by phorbol esters. The data presented suggests that: (1) The "bay region" diol-epoxides of PAH are the ultimate tumor initiating forms. (2) There is a good correlation between the abilities of PAH to inhibit epidermal DNA synthesis, to covalently bind to DNA and to initiate tumors. (3) There is a good correlation between the promoting abilities of phorbol esters to promote tumors and their abilities to induce ornithine decarboxylase (ODC), cell proliferation and dark cells. However, when other nonpromoting hyperplastic agents are used only dark cell induction correlates. (4) Certain polyamines and

prostaglandins can enhance phorbol ester tumor promotion. (5) Anti-inflammatory steroids, retinoids and protease inhibitors are potent inhibitors of tumor promition. (6) Certain weak promoters such as mezerein which mimics 12-0-tetradecanoylphorbol-13-acetate (TPA) in many biochemical and morphological effects are potent second step promoters in a two-stage promotion regimen.

## TUMOR INITIATION

The tumor initiation phase appears to be an irreversible step which probably involves a somatic cell mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities.(2, 3). Most tumor initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules. (4). The Millers have proposed a significant general theory to explain the initial event in chemical carcinogenesis which states that all chemical carcinogens that are not electrophilic reactants must be converted metabolically into a chemically reactive electrophilic form which then reacts with some critical macromolecule to initiate the carcinogenic process ( 4 ).

Previous studies have demonstrated a good correlation between the carcinogenicity of several PAHs and their ability to bind covalently to DNA (5, 6). Table 1 summarizes our data which shows the strong correlation between binding and initiating activity plus the good correlation between the initiating activities of several PAH and their ability to inhibit epidermal DNA synthesis (See reference 7 and 8 for details). It should be emphasized that the inhibition of DNA synthesis by PAH tumor initiators shown in Table 1 is not followed subsequently by a stimulation of DNA synthesis, any change in protein and RNA synthesis, or any observable morphological change in the skin such as inflammation and epidermal cell proliferation. However, if a complete carcinogenic dose of a PAH is applied to mouse skin, there is also an initial inhibition of DNA synthesis followed by a large stimulation ( 7 ). In addition, there is a stimulation in RNA

and protein synthesis which is subsequently followed by inflammation and epidermal hyperplasia ( 7 ). It appears that as the dose of the PAH carcinogen is lowered to initiating doses one titrates out the macromolecular synthesis, inflammation and epidermal cell proliferation.

In order to help us better understand the mechanism of PAH carcinogenesis, we have been studying many compounds with the capacity to inhibit PAH tumor initiation. Table 2 summarizes various potent inhibitors of skin tumor initiation in mice. In most of our studies we have used PAH carcinogens which must be metabolized by the mixed-function oxidases to active form(s) before they are carcinogenic. Some of the flavones and antioxidants appear to inhibit carcinogenesis by inhibiting the metabolism of the carcinogen to its ultimate carcinogenic form (9,14-17). 5,6-Benzoflavone and quercetin have been found to be inhibitory to skin, lung and mammary carcinogenesis whereas 7,8-benzoflavone inhibits skin carcinogenesis by some polycyclic hydrocarbons and enhances carcinogenesis by others (9,17,18). The antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT),are widely used as food preservatives and have been shown to inhibit skin, lung, mammary, forestomach, colon and liver cancer in experimental animals induced by a wide range of chemicals (17). Similar inhibitory results have been noted for selenium and vitamins C and E. (17). The noncarcinogenic polycyclic hydrocarbons and the environmental contaminants appear to inhibit skin carcinogenesis by inducing the metabolism of the carcinogen to detoxified products, thereby decreasing the binding of the PAH to DNA (19-23).This is epitomized by the environmental contaminants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and

polychlorobiphenyls (PCB) which are extremely potent inducers of polycyclic hydrocarbon carcinogen metabolism and potent inhibitors of their carcinogen effect (22, 23). Although TCDD is one of the most toxic agents known, its inhibitory effect on polycyclic hydrocarbon carcinogenesis is at nontoxic dose levels.

Sulfur mustard inhibits tumor initiation by actually killing the initiated cells (24). The polyinosinic:polycytidylic acid (Poly I:C) and the anti-inflammatory steroids appear to inhibit tumor initiation by slowing down carcinogen metabolism by their anti-growth effect (25, 26). Some of the agents listed in Table 2 have been shown to inhibit carcinogenesis in a number of tissues and by a variety of chemical carcinogens indicating they may be useful agents in the chemoprevention of cancer in man (17). In general, the inhibitors of skin tumor initiation shown in Table 2 inhibit by either (1) alteration of the metabolism of the carcinogen (decreased activation and/or increased detoxification), (2) scavenging of active molecular species of carcinogens to prevent their reaching the critical target site(s) in the cells, or (3) competitive inhibition. In all cases this leads to a decrease in covalent binding to critical targets such as DNA. Table 3 reveals a good correlation between the ability of a number of compounds to inhibit tumorigenesis and their ability to inhibit the binding of the PAH to DNA. The major DNA and RNA adduct formed when benzo(a)pyrene (BP) is exposed to cells in culture, human or bovine bronchus or mouse skin is derived from the interaction of BP-7,8-diol-9,10-epoxide between the C-10 of BP and with the exocyclic amino group of guanine (12, 13). Figure 1 illustrates the effect of pretreating mouse skin with TCDD on the appearance of this BP adduct with DNA (See reference 10 for details). As can be seen TCDD effectively eliminated this

major adduct whereas the early eluting material was unaffected. This suggests that the BP-7,8-diol-9,10-epoxide bound to the exocyclic amino group of guanine and/or to the exocyclic amino group of adenine which comigrates but only represents a small portion of the peak may be the critical interaction or event which initiates the tumorigenic response.

We have recently tested the four possible diol-epoxides of BP for skin tumor initiating activities and have found that only the BP 7$\beta$,8$\alpha$-diol-9$\alpha$,10$\alpha$-epoxide (+-BP-diol-epoxide 2 or +-BP-diol-epoxide anti) has activity comparable to the parent hydrocarbon (27). Figure 2 illustrates the structures of the four possible BP-diol-epoxides.

Jerina and coworkers (28, 29) have proposed a theory which predicts that "bay region" diol-epoxides of PAH are important in their carcinogenic activity. A "bay region" occurs in a PAH when an angularly fused benzo ring is present. There is now direct evidence from tumorigenicity studies that "bay region" diol-epoxides of BP (27, 30) and benz(a)anthracene (BA) (31, 32) are ultimate carcinogenic metabolites. In addition, recent studies have shown that certain benzo-ring dihydrodiols (immediate precursors of "bay region" diol-epoxides) of the carcinogens BP, BA, 7-methylbenz(a)anthracene (7-meBA), 7,12-dimethylbenz(a)anthracene (DMBA), dibenz(a,h)anthracene [DB(a,h)A] and chrysene are tumorigenic in mice (27, 30, 32-35, 37, 38). The "bay region" diol-epoxides of BP, BA and DMBA are shown in Figure 3.

## TUMOR PROMOTION

In addition to causing inflammation and epidermal hyperplasia, the phorbol ester tumor promoters have been shown to have several other morphological and biochemical effects on the skin. These responses to phorbol ester tumor promoters are summarized in Table 4. Of all the observed phorbol ester related effects on the skin, the induction of epidermal cell proliferation, ODC and dark cells appear to correlate the best ( 40-43, 48). Also, the tumor-promoting potency in vivo of a series of phorbol esters correlated well with their ability to stimulate ODC activity and DNA synthesis in epidemal cells in culture ( 53). Furthermore, the phorbol esters have been shown to have several other effects on cells in culture. These include: induced changes in the phenotype of normal cells that mimic features of transformed cells (54); an increased frequency of cell transformation by chemical carcinogens and radiation in cell culture (55, 56); comitogenic activity in lymphocytes (57); induced plasminogen activator production in cultured cells ( 53 ); inhibited terminal differentiation in Friend erythroleukemia cells (54, 58), chicken myoblasts (59), neuroblastoma cells (60), chondroblasts ( 61), adipose cells ( 58 ); stimulated terminal differentiation in human myeloid leukemia cells and human melanoma cells ( 62, 63); increased Epstein Barr Virus (EBV) expression in lymphoblastoid cell lines and EBV transformation of human cells (64, 65); decreased large external transformation-sensitive glycoprotein (LETS), inhibited collagen synthesis and stimulated 2-deoxyglucose transport in chicken embryo fibroblasts (66, 67); and membrane changes (68).

Unlike the PAH carcinogens, the phorbol ester tumor promoters do not have to

be metabolized by the skin or cells in culture to an active form (69). Instead, metabolism to phorbol and phorbol monesters leads to decreased activity (69).

It is difficult to determine which of the many phorbol ester tumor promoter related responses are essential components of the promotion process. There is a good correlation between the promoting abilities of a series of phorbol esters and their ability to stimulate epidermal hyperplasia (40); however, the correlation fails if one looks at nonphorbol ester hyperplastic agents (70). Later O'Brien et al. (48) reported an excellent correlation between the tumor promoting ability of various compounds (phorbol esters as well as nonphorbol ester compounds) and their ability to induce ODC activity in mouse skin. Raick found that phorbol ester tumor promoters induced the appearance of "dark basal cells" in the epidermis, whereas ethylphenylpropiolate, a non-promoting epidermal hyperplasic agent, did not (41, 71). In addition, wounding induced a few dark cells but seemed to correlate with its ability to be a weak promoter (42, 43, 71). In addition, a large number of these dark cells are found in papillomas and carcinomas (42, 43).

Various modifiers of the tumor promotion process have been very useful in our understanding of the mechanism(s) of tumor promotion. Table 5 summarizes the potent inhibitors of skin tumor promotion in mice by phorbol ester tumor promoters.

The anti-inflammatory steroid, fluocinolone acetonide, was found to be an extremely potent inhibitor of phorbol ester tumor promotion in mouse skin (72). Repeated applications of as little as 0.01 μg almost completely counteract the skin tumorigenesis. Fluocinolone acetonide also effectively counteracts the tumor

promoter induced cellular proliferation. Certain retinoids have also been found to be potent inhibitors of mouse skin tumor promotion (73). In addition, Sporn and coworkers have found that certain retinoids are potent inhibitors of lung, mammary, bladder and colon carcinogenesis (74). Verma and coworkers (73) have shown that certain retinoids are potent inhibitors of phorbol ester induced epidermal ornithine decarboxylase activity. This plus their effect on epithelial differentiation appears to be related to their anti-carcinogenic effect. We have recently found that a combination of fluocinolone acetonide and retinoids produces an inhibitory effect on skin tumor promotion greater than that produced by each separately (75). Troll and Belman (76) have found that protease inhibitors, cyclic nucleotides, DMSO and butyrate also inhibit mouse skin tumor promotion by phorbol esters. Schinitsky and coworkers (77) reported the inhibitory effect of Bacillus Calmette-Guerin (BCG) vaccination on skin tumor promotion. It has been shown that Poly I:C has an inhibitory effect on carcinogenesis and tumor promotion (25). This appears to be mediated by its inhibition of promoter and carcinogen induced cell proliferation (25).

Other modifiers of tumor promotion have allowed us to better understand the involvement of certain mediators of promotion and characteristics of the putative TPA receptor. The effects of these modifiers are shown in Table 6. When phorbol or phorbol 12,13-diacetate (non-promoting) were given simultaneously with TPA following DMBA initiation, they had no effect on TPA promotion. However, when given simultaneously with TPA, the non-promoter 4-0-methyl-TPA and the weak promoter mezerein were found to inhibit TPA promotion in a dose-dependent

manner. Although 4-0-methyl TPA is non-promoting, its three dimensional structure is very close to that of TPA (78) which may allow it to compete with the putative TPA receptor. Figure 4 shows the similar structures of TPA and mezerein. Mezerein, a plant-derived ester of 12-hydroxy-daphentoxin, is also a diterpene containing cyclopentenone rings and a long chain lipophilic group at position 12.

Cotreatment of prostaglandin $F_{2\alpha}$ or $E_2$ with TPA was found to significantly enhance tumor promotion by TPA whereas $E_1$ had an inhibitory effect. None of the prostaglandins tested ($F_{2\alpha}$, $E_2$ and $E_1$) had promoting activity when given repetitively after DMBA initiation. It was recently reported that certain prostaglandins caused an enhancement of complete skin carcinogenesis by 3-methycholanthrene (MC) (79). Prostaglandin involvement in tumor promotion and carcinogenesis, in general, was suggested not only because of its direct function in inflammation but also because of the stimulation of prostaglandin production in some cell types by phorbol ester tumor promoters (80) and its structural similarities with TPA (81). However, a very recent report showed that TPA did not compete for prostaglandin $E_1$ binding to specific receptors in adipose cells (82).

The interaction of TPA with other membrane receptors as well as with intracellular receptors has been reported. Several investigators have shown that TPA can interact with epidermal growth factor (EGF) receptors (83, 84). A recent study convincingly presented results that TPA specifically competes with EGF receptors and not with other growth factor receptors (85). TPA has also been shown to physically associate with all the intracellular fractions of epidermal cells such as microsomes, mitochondria, cytosol and chromatin fraction (86). Although the

binding of TPA to the mitochondrial and microsomal fractions was greater than to the cytosol and chromatin fractions, dexamethasone , a potent anti-promoter selectively decreased the in vivo binding of TPA to the cytosol and chromatin fractions (86). Nevertheless, at present, no one has reported a specific high affinity receptor for TPA in the cell or on the membrane.

Also shown in Table 6 is the enhancing ability of putrescine and diacetylputrescine on tumor promotion when injected intraperitoneally into mice a few minutes before topical treatment with TPA. Putrescine, deacetylputrescine or spermidine were ineffective as tumor promoters when given repetitively after DMBA initiation. The involvement of polyamines in tumor promotion was suggested by the 100 to 400 fold induction of epidermal ODC by TPA (48). Table 7 shows that although TPA induces a many fold induction of ODC, there is only a 2 to 3 fold induction of putrescine and spermidine without any increase in spermine. It is of interest to point out that retinoic acid is a potent inhibitor of the TPA induced ODC activity as well as putrescine level but not of spermidine and spermine levels (73). We have found that fluocinolone acetonide has very little effect on TPA-induced ODC activity and putrescine and spermine levels but effectively inhibits spermidine accumulation.

Importance of Dark Basal Keratinocytes in Tumor Promotion

As stated previously, the induction of dark basal keratinocytes and ODC by phorbol ester tumor promoters appears to correlate better with tumor promotion than the other phorbol ester related skin responses. It has recently been reported

that mezerein, a diterpene similar to TPA (Figure 4), was capable of bringing about most of the morphological and biochemical changes in skin and in cells in culture that TPA does, but TPA was at least 50 times more active as a tumor promoter (87). A comparison of these TPA and mezerein responses are shown in Table 8. As can be seen, mezerein is as potent or more potent than TPA. This is especially true regarding the induction of epidermal ODC and epidermal hyperplasia. The effect of mezerein on ODC activity suggests that ODC induction is not a critical event in tumor promotion (87). It should be emphasized that this is also true for the other morphological and biochemical responses to mezerein.

Since the induction of dark basal keratinocytes by phorbol ester tumor promoters was the only other response that correlated well with promotion, we decided to investigate their induction in more detail. Figure 5 shows good dose-response in the induction of dark cells in the basal layer by TPA. Normally less than 2% of the basal cells are dark cells whereas after 4 μg of TPA this increased to 35% one day after treatment. Figure 6 depicts the characteristics of the TPA induced dark cells. It is also of interest to point out that after TPA treatment approximately 40% of the dark cells incorporated thymidine. A comparison of the abilities of TPA and mezerein to induce dark basal epidermal cells was also determined. Figure 7 indicates that TPA induces 3 to 4 times more dark basal cells than mezerein. These different dark cell inducing characteristics seem to be the only major detectable difference in the early effects produced by TPA and mezerein which suggests that the induction of dark basal keratinocytes might be a critical event in tumor promotion.

Multistage Promotion

Because of the many similarities in morphological and biochemical responses induced by TPA and mezerein, we felt that mezerein, although a weak promoter, would be a good candidate as a compound to be used in the second step of a two-step promotion protocol as originally reported by Boutwell (39). His results showed that promotion could be divided into two steps, conversion and propagation (39). After DMBA initiation, the conversion step was accomplished by a limited number of croton oil treatments which, with no further treatment, only produced a few tumors and the propagation step was accomplished by repeated treatment with turpentine, a non-promoting hyperplasic agent (39). The three step protocol (initiation-conversion-propagation) produced a significant tumor response but less than that observed when croton oil was given for the complete promotion step (39). However, recent results suggest that nonpromoting hyperplastic agents such as turpentine, ethylphenylpropiolate and acetic acid when given repetitively after a few treatments with TPA were not able to complete the promotion process as reported by Boutwell (42, 70, 71). In fact, Raick reported that turpentine and ethylphenylpropiolate gave fewer tumors in a three stage system than when DMBA was only followed by the limited TPA treatment (42, 71). Similar results were reported by Slaga et al. (70) using acetic acid as a second step promoter.

Our results on the use of mezerein as a second stage promoter are shown in Table 9. As illustrated TPA is about 50 times more active as a promoter than mezerein (compare experiments 1 and 3). When 2 μg of TPA are given twice weekly for only 2 weeks after DMBA initiation, very few tumors are induced compared

to twice weekly treatments for 12 weeks. However, when mezerein is given at a dose of either 1, 2 or 4 μg twice weekly after the limited TPA treatment, it induced a significant tumor response in a dose-dependent manner (compare experiments 4, 5, 6 with 2). The ability of mezerein to act as a potent second stage promoter was repeated in three separate experiments. Also, shown in Table 9, is the ineffectiveness of ethylphenylpropiolate (EPP) as a complete promoter and as a second stage promoter. Although not shown in Table 9, we have recently found that fluocinolone acetonide can effectively inhibit both step one and two of the promotion process whereas retinoic acid specifically inhibits step two.

## CONCLUSION

An overall view of some of the important aspects of PAH tumor initiation and phorbol ester tumor promotion in mouse skin is illustrated in Figure 8. It is now quite evident that PAHs must be metabolized by the epidermal aryl hydrocarbon hydroxylase to reactive "bay region" diol-epoxides which are probably the ultimate carcinogenic forms of most PAHs. It is important to point out that the BP-diol-epoxide is a potent tumor initiator but is relatively inactive as a complete carcinogen. It is possible that some other metabolite of BP is responsbile for the promoting ability when BP is given as a complete carcinogen.

There is a good correlation between the tumor initiating activities of PAHs and their abilities to bind covalently to DNA. In addition, various inihibitors of PAH tumor initiation show a strong correlation with their abilities to inhibit the binding of the PAH to DNA and their anticarcinogenic activities. It appears that the

critical BP-DNA adduct involves the interaction of the "bay region" diol-epoxide with the exocyclic amino group of guanine and/or exocyclic amino group of adenine. Since there is a strong correlation between the carcinogenic and mutagenic activities of PAHs and since tumor initiation appears to be a irreversible step, a somatic mutation is suggested as being important in PAH tumor initiation.

The effectiveness of PAH tumor initiation appears to be related to the relative degree of activation vs. detoxification of the diol-epoxide and whether the critical adduct is repaired or not. In order to obtain a critical interaction, one can envision that either the excision repair process has to be inhibited or there has to be an increase in error prone repair by the carcinogen. In addition, a round or two of cell division appears to be necessary in order to fix the initiation event (54, 88).

Our data suggest that three and possibly four steps are important in the promotion process which can obviously be inducted by repeated TPA treatment after PAH initiation. We believe the first step involves the induction of dark basal keratinocytes. The fact that mezerein is a potent second step promoter, but only a weak complete promoter with much less ability to induce dark cells than TPA, suggests that the dark cells are important in step one of promotion. These dark cells may be primitive stem cells since they are only present in small numbers in adult skin and are more prevalent in newborn and embryonic skin (42, 43). Most of the morphological and biochemical responses caused by TPA and mezerein may be more associated with step two and three. The fact that retinoic acid is a potent inhibitor of step two and not step one in a two-step promotion process and that retinoic acid is a potent inhibitor of TPA and mezerein induced ODC suggests

that the polyamines may be important in step two. Also, since indomethacin is a potent inhibitor of TPA induced ODC activity, which can be reversed by prostaglandins (89), the prostaglandins may also be important in step two. Preliminary results suggest that nonpromoting hyperplastic agents such as EPP may be capable of inducing tumors in a three step promotion process when given after sequential treatment with tumor initiator, limited treatment of TPA and limited treatment with mezerein which do not in themselves give rise to tumors. It is possible that specific prostaglandins and polyamines given in some sequence may replace mezerein in step two. By dividing the carcinogenic process into many individual steps, we will no doubt better understand the important events in carcinogenesis which will allow us to find effective and rational ways of preventing cancer.

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TABLE 1

Correlation of Polycyclic Aromatic Hydrocarbons (PAHs) Abilities to Inhibit Epidermal DNA Synthesis and to Covalently Bind to Epidermal DNA with their Tumor Initiating Activities[a]

| PAHs | Relative ability to covalently bind to Epidermal DNA[b] | Relative ability to inhibit Epidermal DNA Synthesis[c] | Relative Tumor Initiating Activity[d] |
|---|---|---|---|
| DMBA | 10.0 | 10.0 | 10.0 |
| MC | 6.5 | 6.2 | 6.0 |
| BP | 3.3 | 3.1 | 2.0 |
| DB(a,h)A | 1.7 | 2.0 | 1.5 |
| DB(a,c)A | 0.8 | 1.1 | 0.2 |

[a] DMBA was given a value of 10 since it gave the maximum response in binding, ability to inhibit epidermal DNA synthesis and to initiate tumors in a two-stage system of tumorigenesis. All the other PAHs are expressed as values relative to DMBA's response.

[b] The relative abilities of various PAHs to covalently bind to epidermal DNA are based on dose-response binding studies. See references 6, 9 and 10 for details of actual binding levels.

[c] The relative abilities of various PAHs to inhibit epidermal DNA synthesis are based on dose-response studies using both the maximum and duration of inhibition of DNA synthesis. See references 7, 8 and 9 for details of actual response.

[d] The relative tumor initiating activities are based on dose-response studies in Charles River CD-1 mice. See references 7 and 11 for details.

TABLE 2

INHIBITORS OF TUMOR INITIATION

1. Antioxidants: butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and selenium
2. Flavones: 7,8-benzoflavone, 5,6-benzoflavone and quercetin
3. Vitamins: A, C and E
4. Certain noncarcinogenic polycyclic aromatic hydrocarbons: dibenz(a,c)anthracene, benz(a)anthracene, benzo(e)pyrene and pyrene
5. Environmental contaminants: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorobiphenyls (PCB)
6. Sulfur mustard
7. Polyriboinosinic-polyribocytidylic acid (Poly I:C)
8. Anti-inflammatory steroid

TABLE 3

Correlation of Various Compounds to Inhibit Tumor Initiation by DMBA with their Abilities to Inhibit Covalent Binding of DMBA to Epidermal DNA[a]

| Inhibitors | Relative ability to inhibit DMBA tumor initiation by at least 50% | Relative ability to inhibit DMBA binding to Epidermal DNA by at least 50% |
|---|---|---|
| TCDD | 100.0 | 100.0 |
| DB(a,c)A | 10.0 | 15.0 |
| 7,8-BF | 5.0 | 8.0 |
| B(e)P | 5.0 | 3.0 |
| BHA | 0.2 | 0.1 |
| BHT | 0.1 | 0.1 |
| Vitamin C | 0.1 | 0.1 |

[a] TCDD was given a value of 100 since it gave the greatest inhibition of tumor initiation and DMBA binding to epidermal DNA. For example, TCDD at a 1 ug dose level almost completely inhibited DMBA tumorigenesis and DMBA binding to DNA. All the other compounds are expressed as values relative to TCDD's response. For example, BHA at a 1000 ug dose level inhibited DMBA tumor initiation and binding by at least 50%. See references 9, 10, 14-16, 19-22 for details.

TABLE 4

Morphological and Biochemical Responses of Mouse Skin to Phorbol Ester Tumor Promoters

| Responses | References |
|---|---|
| Induction of inflammation and hyperplasia | 39, 40 |
| Induction of dark cells | 41-43 |
| An initial increase in keratinization followed by a decrease | 41 |
| Increase in DNA, RNA and protein synthesis | 44 |
| Increase in phospholipid synthesis | 45 |
| Increase in histone synthesis and phosphorylation | 46, 47 |
| Increase in ornithine decarboxylase activity followed by increase in polyamines | 48 |
| Decrease in histidase activity | 49 |
| Induction of embryonic proteins in adult skin | 50 |
| Increase in protease activity | 51 |
| Decrease in the isoproternol stimulation of cAMP | 52 |

TABLE 5

INHIBITORS OF PHORBOL ESTER SKIN TUMOR PROMOTION

1. Anti-inflammatory steroids: cortisol, dexamethasone and fluocinolone acetonide
2. Vitamin A derivatives
3. Combination of retinoids and anti-inflammatory agents
4. Protease inhibitors: Tosyl lysine chloromethyl ketone, (TLCK); Tosyl arginine methyl ester, (TAME); Tosyl phenylalanine chloromethyl ketone, (TPCK); antipain and leupeptin
5. Cyclic nucleotides
6. Dimethylsulfoxide (DMSO)
7. Butyrate
8. Bacillus Calmette-Guerin (BCG)
9. Polyriboinosinic: polyribocytidylic acid (Poly I:C)

TABLE 6

The Effects of Co-Treatment of Various Deterpenes, Prostaglandins and Polyamines with TPA on TPA Promotion[a]

| ← Initiation → | | ← Promotion → | Relative Tumor Response[b] |
|---|---|---|---|
| 1. DMBA | 1 wk → | TPA 2x/wk for 12 wks → | 100 |
| 2. DMBA | 1 wk → | TPA + Mezerein (5 ug) 2x/wk for 12 wks → | 36 |
| 3. DMBA | 1 wk → | TPA + Mezerein (10 ug) 2x/wk for 12 wks → | 21 |
| 4. DMBA | 1 wk → | TPA + 4-0-methyl TPA (20 ug) 2x/wk for 12 wks → | 46 |
| 5. DMBA | 1 wk → | TPA + Phorbol (20 ug) 2x/wk for 12 wks → | 95 |
| 6. DMBA | 1 wk → | TPA + Phorbol Diacetate (20 ug) 2x/wk for 12 wks → | 100 |
| 7. DMBA | 1 wk → | TPA + $PF_{2\alpha}$ (10 ug) 2x/wk for 12 wks → | 140 |
| 8. DMBA | 1 wk → | TPA + $PE_2$ (10 ug) 2x/wk for 12 wks → | 130 |
| 9. DMBA | 1 wk → | TPA + $PE_1$ (5 ug) 2x/wk for 12 wks → | 60 |
| 10. DMBA | 1 wk → | TPA + putrescine (250 ug) 2x/wk for 12 wks → | 160 |
| 11. DMBA | 1 wk → | TPA + diacetyl putrescine (250 ug) 2x/wk for 12 wks → | 150 |

[a] All the TPA-modifying compounds were applied topically either with or 5 minutes before TPA, except for putrescine and diacetyl putrescine which were given i.p. 5 minutes before TPA.

[b] The tumor response (number of papillomas per mouse) for the DMBA-TPA control experiment (# 1) was set at a value of 100 and the tumor response for all the other experiments are expressed as a value relative to the DMBA-TPA control.

TABLE 7

Time Course of Epidermal Polyamine Synthesis after TPA Treatment[a]

| Polyamine | Time after TPA Treatment (hrs) 6 | 9 | 12 | 24 | 48 | 72 |
|---|---|---|---|---|---|---|
| Putrescine | 1.55 | 2.70 | 2.40 | 1.30 | 0.75 | 1.10 |
| Spermidine | 0.95 | 1.40 | 1.60 | 2.35 | 2.20 | 1.70 |
| Spermine | 0.80 | .60 | 1.00 | 1.10 | 1.00 | 1.25 |

[a] Values given are fold change compared to control values at specified times. 0 hr control values were: Putrescine, 0.70 nmol/mg protein; spermidine, 3.5 nmol/mg protein; spermine, 2.00 nmol/mg protein. Polyamines were quantitated by HPLC separation of dansylated derivatives by use of an internal standard. The maximum percent standard deviation was 10%.

TABLE 8

Comparison of Cellular and Biochemical Responses to TPA and Mezerein

| | Relative Response | | Reference |
|---|---|---|---|
| | TPA | MEZ | |
| 1. Enhancement of neoplastic phenotype | 10 | 10 | 54 |
| 2. Promotion of neoplastic transformation (C3H-10T 1/2) | 10 | 8 | 55, 56 |
| 3. Induction of epidermal cellular proliferation | 5 | 10 | 40, 87 |
| 4. Co-mitogenesis in lymphocytes | 10 | 10 | 57 |
| 5. Inhibition of differentiation in FL cells | 10 | 10 | 58 |
| 6. Stimulation of DNA synthesis | 5 | 10 | 87 |
| 7. Stimulation of ODC activity | 8 | 10 | 87 |
| 8. Stimulation of plasminogen activator production | 2 | 10 | 54 |

TABLE 9

MULTISTAGE SKIN CARCINOGENESIS[a]

| | Initiation | | Promotion | Relative Tumor Response[b] |
|---|---|---|---|---|
| 1. | DMBA | 1 wk | TPA 2x/wk for 12 wks | 100 |
| 2. | DMBA | 1 wk | TPA 2x/wk for 2 wks acetone 2x/wk for 10 wks | 1 |
| 3. | DMBA | 1 wk | Mezerein (4 ug) 2x/wk for 12 wks | 2 |
| 4. | DMBA | 1 wk | TPA 2x/wk for 2 wks Mezerein (4 ug) 2x/wk for 10 wks | 85 |
| 5. | DMBA | 1 wk | TPA 2x/wk for 2 wks Mezerein (2 ug) 2x/wk for 10 wks | 50 |
| 6. | DMBA | 1 wk | TPA 2x/wk for 2 wks Mezerein (1 ug) 2x/wk for 10 wks | 35 |
| 7. | DMBA | 1 wk | EPP 2x/wk | 1 |
| 8. | DMBA | 1 wk | TPA 2x/wk for 2 wks EPP 2x/wk for 10 wks | 4 |

[a] The mice were initiated with 10 nmoles of DMBA and promoted with 2 ug of TPA or as shown above.

[b] The tumor response (number of papillomas per mouse) for the DMBA-TPA control (# 1) was set at a value of 100 and the tumor response for all the other experiments are expressed as a value relative to the DMBA-TPA control.

## FIGURE LEGENDS

FIG. 1. Sephadex LH-20 chromatographic elution profiles of enzyme digests from DNA isolated from mouse skin treated with (A) $^{3}$H-benzo(a)pyrene (100 nmoles/mouse) alone or (B) TCDD (1 ug) 72 hours before application of $^{3}$H-benzo(a)pyrene (100 nmoles/mouse). The DNA-digests were chromatographed on an LH-20 column (90 x 1.5 cm) and eluted with a 30 to 100% water:methanol gradient (1,000 ml). O----O U.V. was monitored continuously at 25 nm; O——O c.p.m./fraction for 1.0 ml sample of each fraction of 4.1 ml.

FIG. 2. The four possible diol-epoxides of BP from (+)- and (-)-BP-7,8-dihydrodiol. The (+)-BP 7,8-diol-9,10-epoxide-2 (or +BP-diol-epoxide anti) is the only diol-epoxide of BP which has potent tumor initiating activity.

FIG. 3. Structures of the bay region diol-epoxides of BP, BA and DMBA. The arrows depict where the bay region is located.

FIG. 4. Comparison of structures of TPA and Mezerein

FIG. 5. Percentage of dark basal epithelial cells after single applications of 1, 2, and 4 ug TPA. The percentage was derived by counting the total number of epithelial cells and the number of dark cells in the basal layer of the interfollicular epidermis. One micron thick Epon sections, stained with either toluidine-blue or Heidenhain's iron-hematoxylin were used. Total cells counted: 12,330.

FIG. 6. Hyperplasic epidermis of Sencar mouse after a single application of 4 ug TPA. A group of several dark epithelial cells are seen in the basal layer of the interfollicular epidermis (two arrows). Isolated dark cells are noted in the "intrafollicular epidermis" (single arrow). Epon section Heidenhain's iron-hematoxylin x360. Inset: normal skin, showing an extremely thin epidermis without dark basal cells. Epon-Toluidine-blue. x340. B: Basal Layer, S: Spinous Layer, G: Granular Layer.

FIG. 7. Effects of mezerein (MZ) and TPA on the percentage of dark basal epithelial cells after a single application of either chemical. Same material as described in Fig. 5. Total cells counted: 21,171.

FIG. 8. A summary diagram depicting the important events in PAH tumor initiation and phorbol ester tumor promotion.

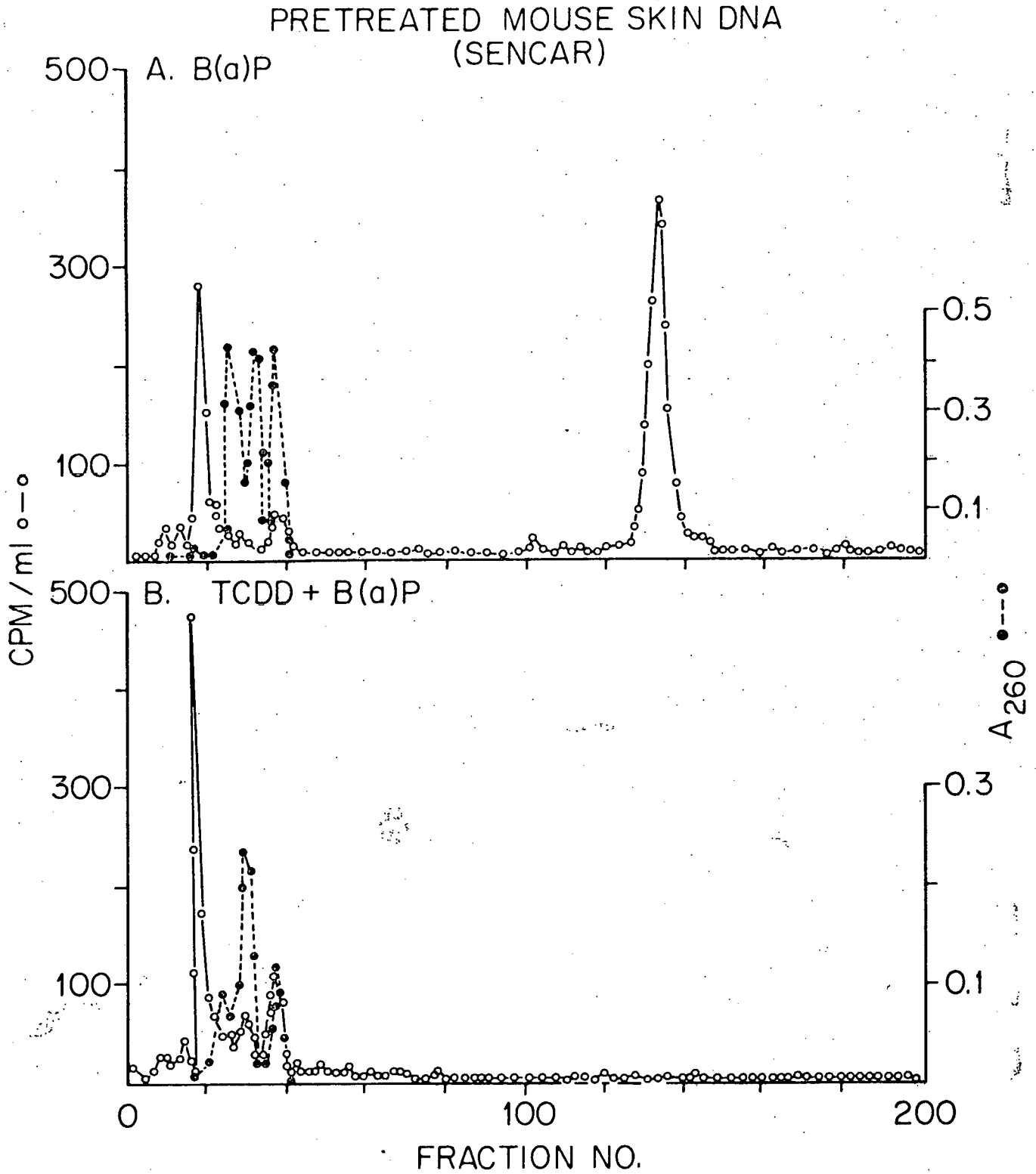
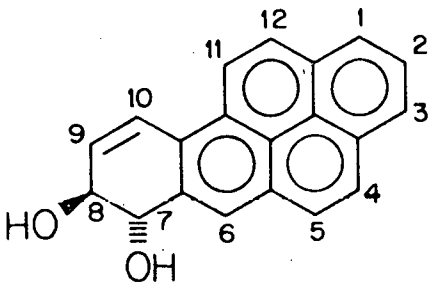
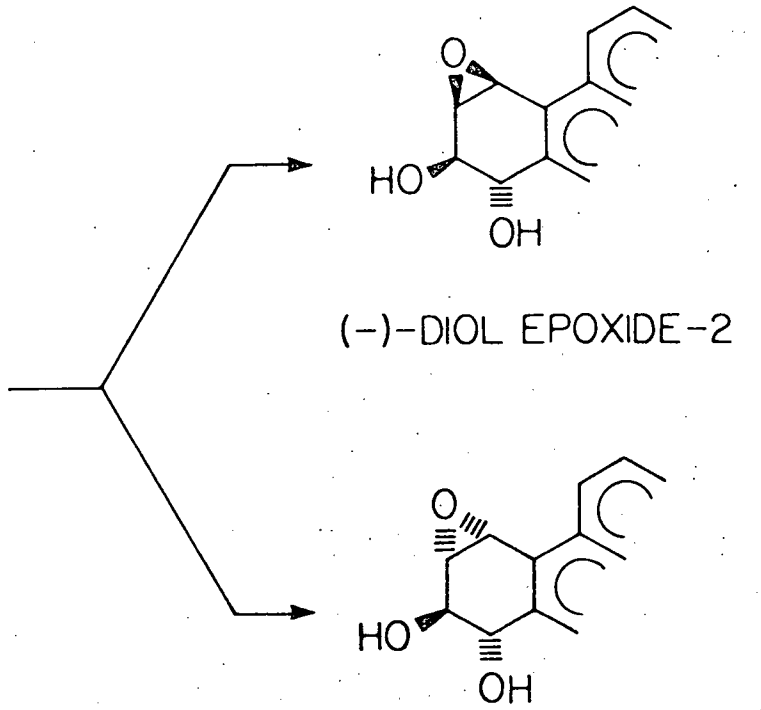

PRETREATED MOUSE SKIN DNA
(SENCAR)
A. B(a)P
B. TCDD + B(a)P
500
300
100
0.5
0.3
0.1
CPM / ml o—o
A260 •--•
0
100
200
FRACTION NO.

Fig. 1

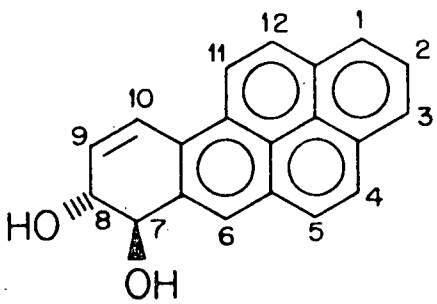



(+)-ENANTIOMER
OF BP-7,8-DIOL

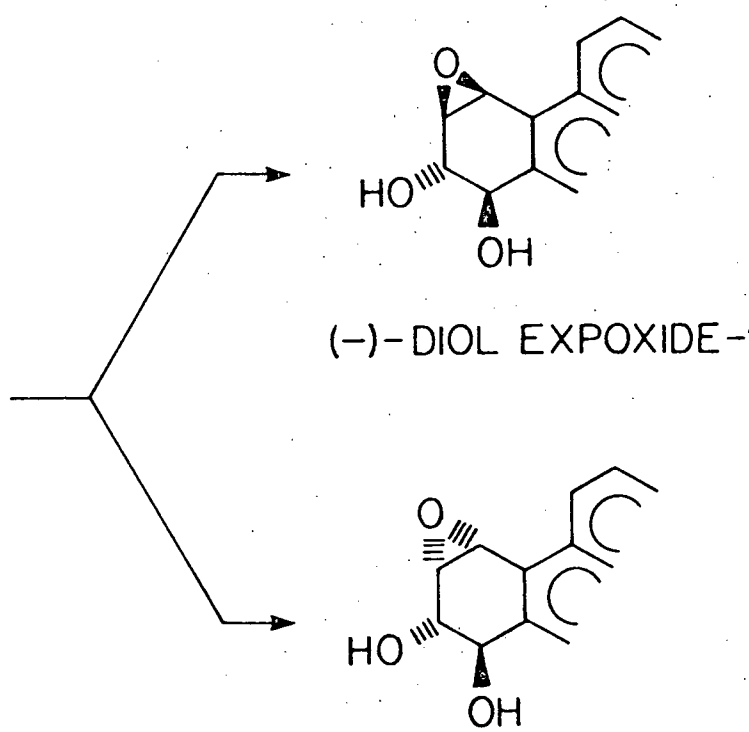



(-)-DIOL EPOXIDE-2

(+)-DIOL EXPOXIDE-1




(-)-ENANTIOMER
OF BP-7,8-DIOL



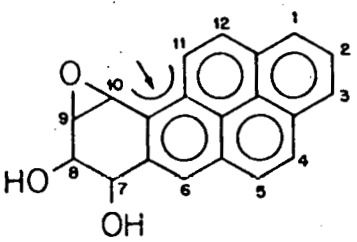

(-)-DIOL EXPOXIDE-1

(+)-DIOL EXPOXIDE-2

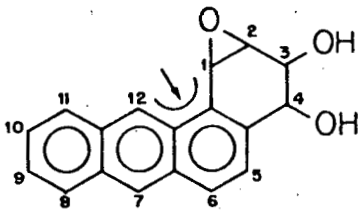
Fig. 2

BAY REGION DIOL-EPOXIDES

BP




BA




DMBA

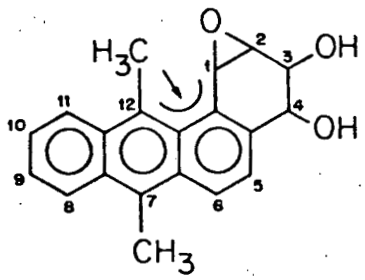
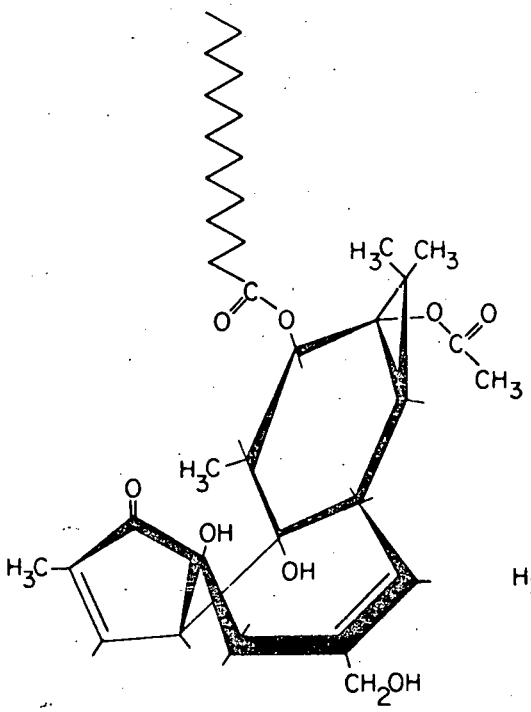
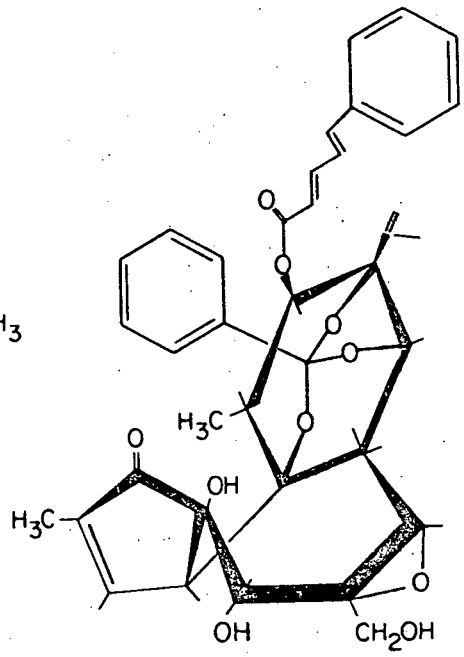



Fig. 3




TPA




MEZEREIN

Fig. 4

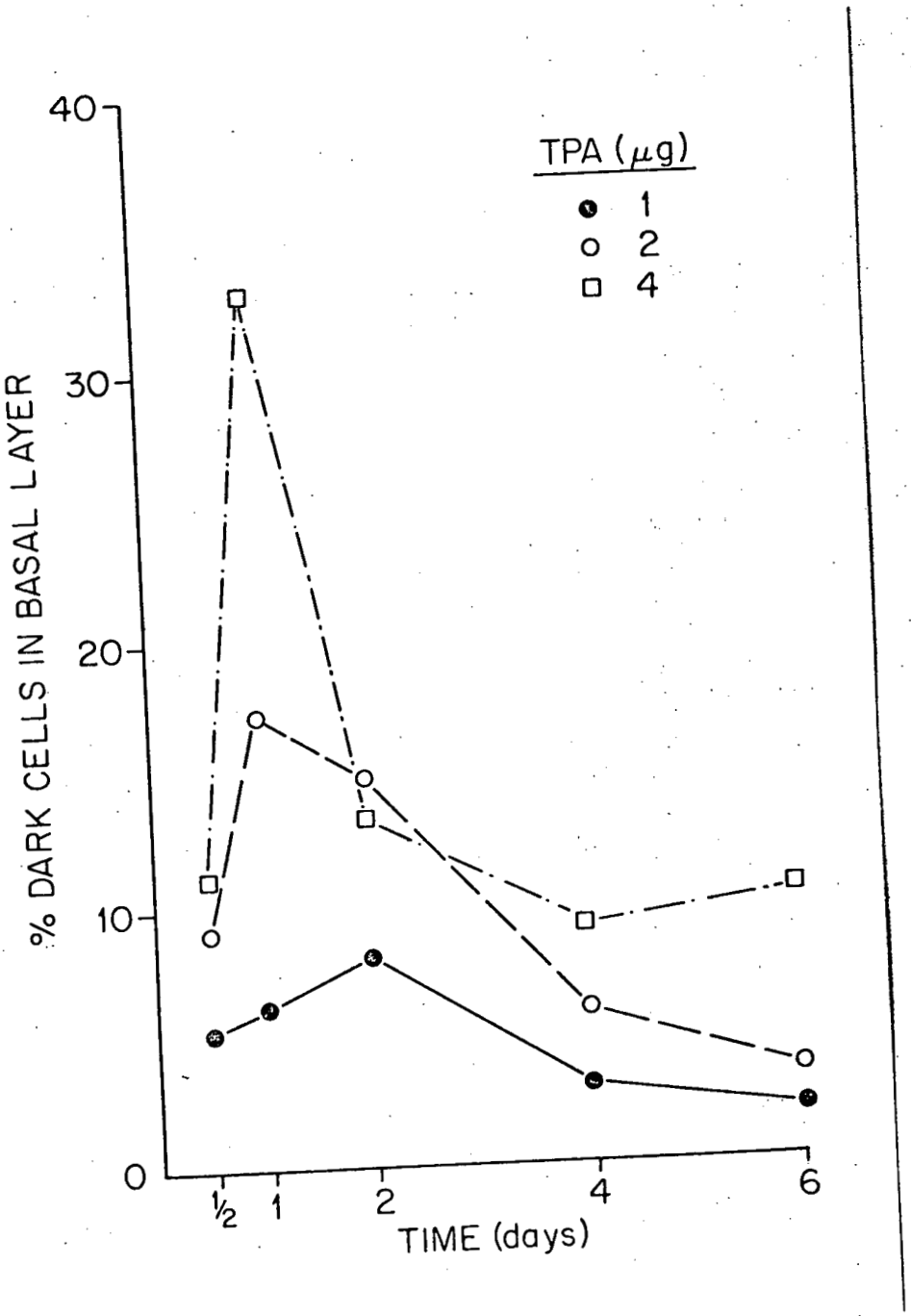

40
30
20
10
0
% DARK CELLS IN BASAL LAYER
TPA (μg)
1
2
4
½
1
2
4
6
TIME (days)

Fig. 5

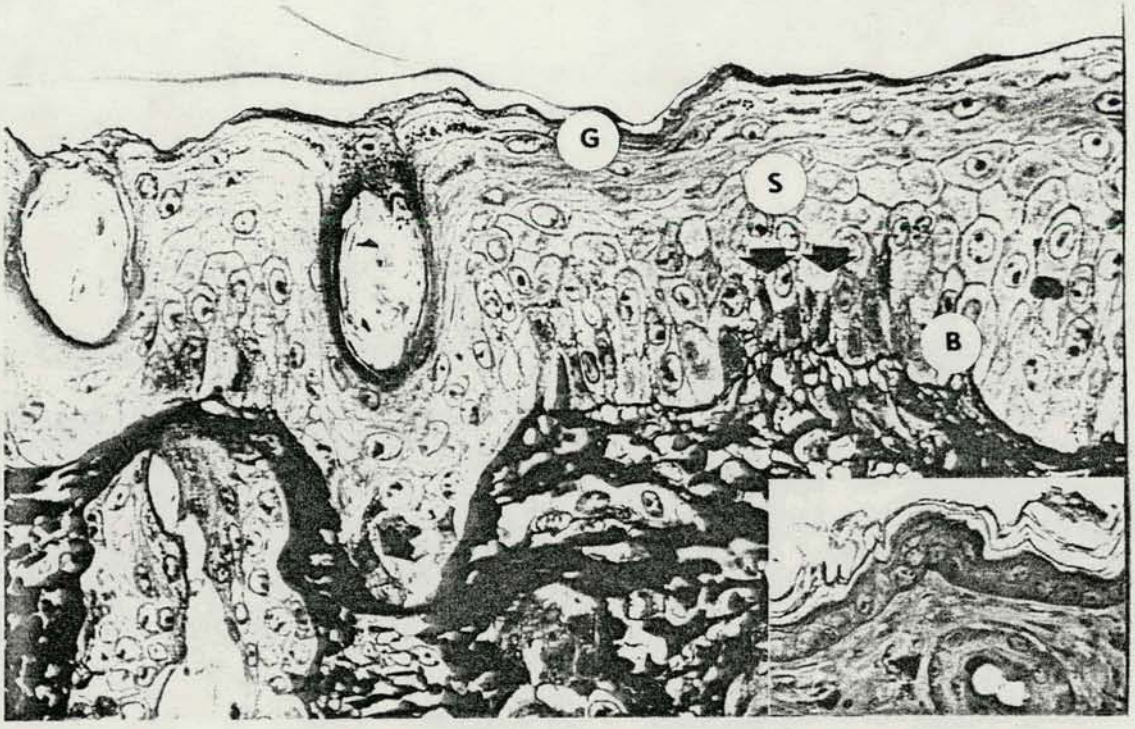



Fig. 6

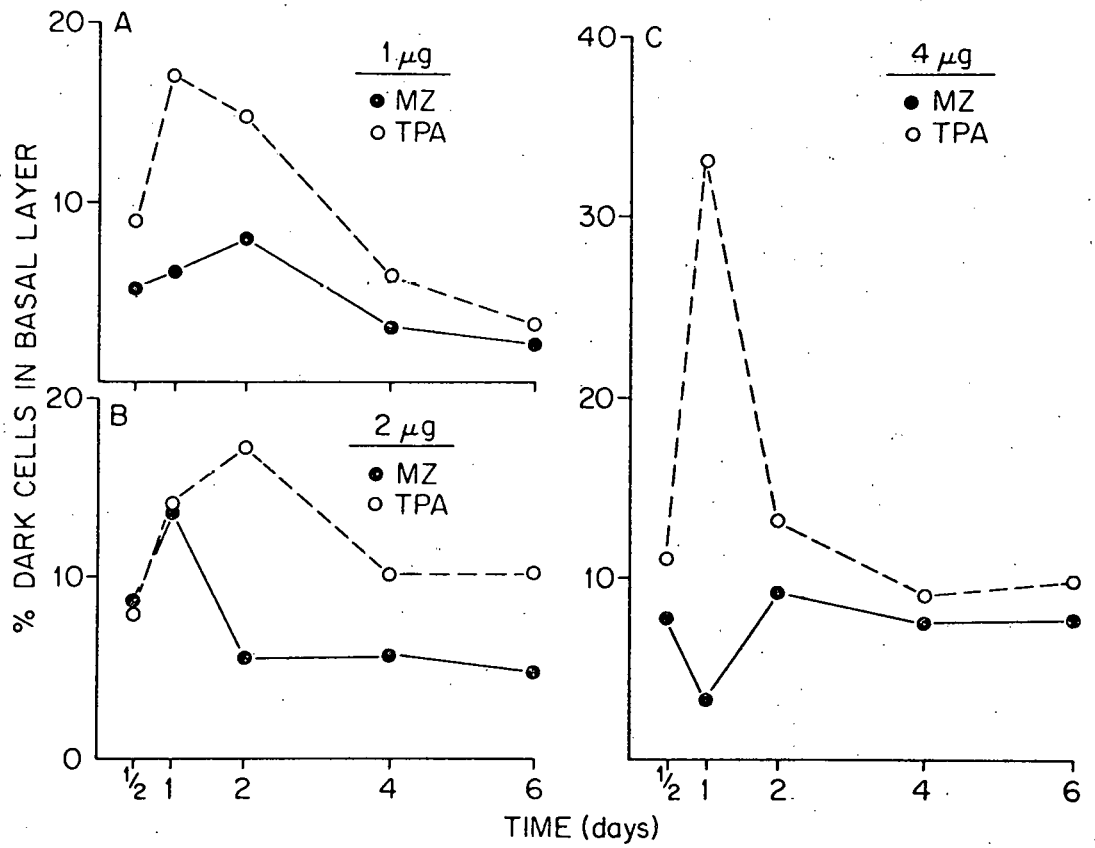

A
1 μg
MZ
TPA
B
2 μg
MZ
TPA
C
4 μg
MZ
TPA
% DARK CELLS IN BASAL LAYER
TIME (days)
20
10
0
40
30
½
1
2
4
6

Fig. 7

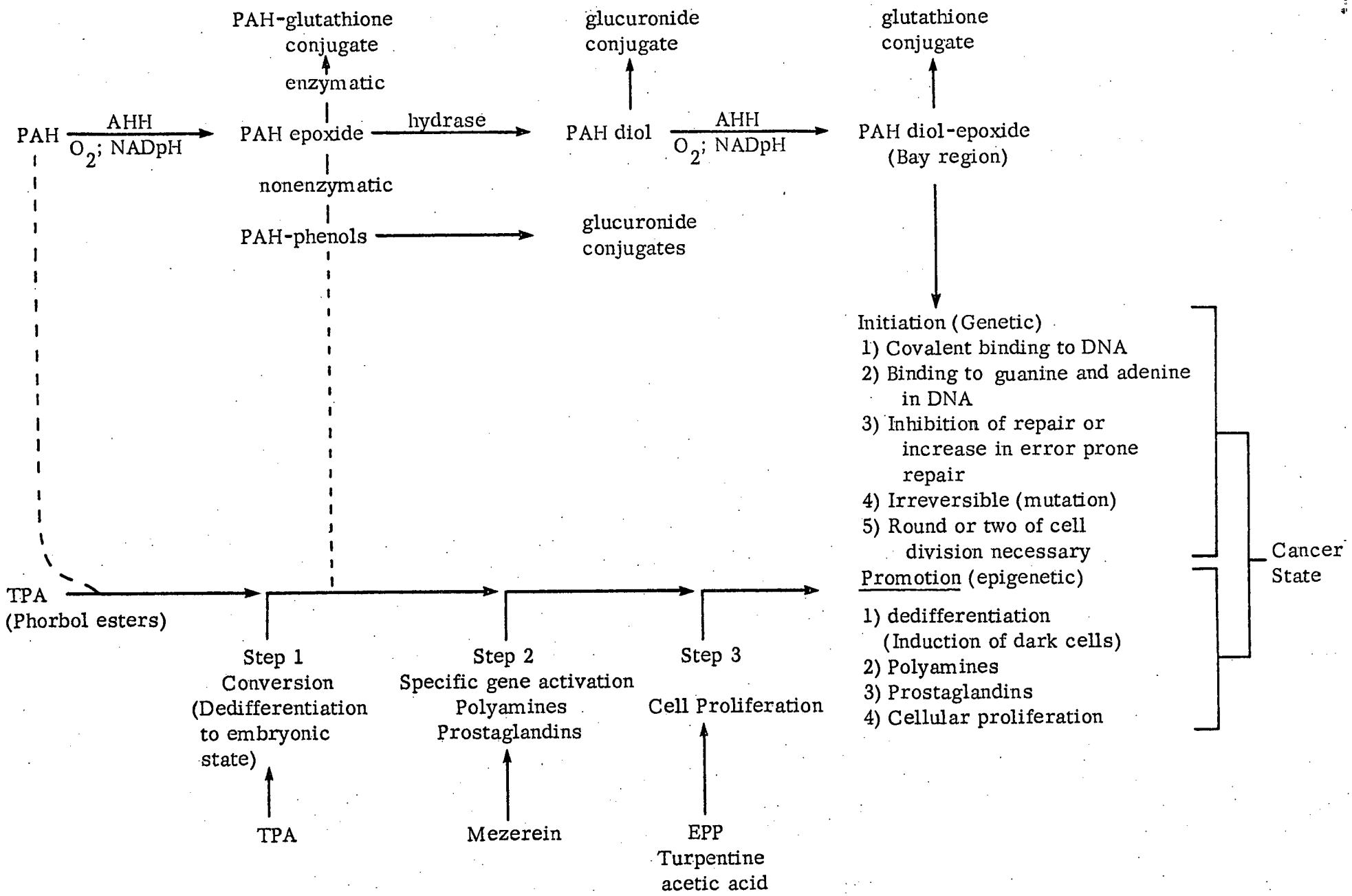

PAH-glutathione conjugate
enzymatic
glucuronide conjugate
glutathione conjugate
PAH
AHH
$O_2$; NADpH
PAH epoxide
hydrase
PAH diol
AHH
$O_2$; NADpH
PAH diol-epoxide (Bay region)
nonenzymatic
PAH-phenols
glucuronide conjugates
Initiation (Genetic)
1) Covalent binding to DNA
2) Binding to guanine and adenine in DNA
3) Inhibition of repair or increase in error prone repair
4) Irreversible (mutation)
5) Round or two of cell division necessary
Promotion (epigenetic)
1) dedifferentiation (Induction of dark cells)
2) Polyamines
3) Prostaglandins
4) Cellular proliferation
Cancer State
TPA
(Phorbol esters)
Step 1
Conversion
(Dedifferentiation to embryonic state)
Step 2
Specific gene activation
Polyamines
Prostaglandins
Step 3
Cell Proliferation
TPA
Mezerein
EPP
Turpentine
acetic acid

Fig. 8