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This paper is dedicated to the memory of Professor Bronisław Filipowicz
Review

Alternative pathways of polycyclic aromatic hydrocarbons activation: The formation of polar DNA adducts[o]

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, and some are potent carcinogens in rodents. Carcinogenic PAHs are activated in the cells to metabolites that react with DNA to form covalent adducts. For most PAHs the reactive, electrophilic species which bind to DNA, are bay-region diol-epoxides. Application of ^{32}P-postlabeling to PAH-DNA adducts analysis revealed that for some PAHs the adduct profiles generated in model systems are more complex and include adducts which are more polar than those formed by classic bay-region diol-epoxides. This minireview summaries the information gained on typical representatives of polar PAH-DNA adducts. Formation of triol-epoxide-DNA adducts was proposed for chrysene and a non-alterant PAH, benzo[*b*]fluoranthene (B[b]F). 5-OH-B[b]F, the precursor of B[b]F triol-epoxide, was found to be a potent tumor initiator in mouse skin. For planar PAHs such as dibenzanthracenes the possibility of bis-diol epoxide-DNA adducts formation was suggested. The most comprehensive data were obtained for dibenz[*a,j*]anthracene (DB[a,j]A). This hydrocarbon when applied to SENCAR mouse skin forms up to 23 species of adducts, most of which are polar. Among these polar adducts seven were identified as derived from DB[*a,j*]A-3,4-10,11-bis-diol. Analysis of tumor-initiating activity showed, however, that this proximate metabolite

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Abbreviations: PAHs, polycyclic aromatic hydrocarbons; B[a]P, benzo[*a*]pyrene; DE; diol-epoxide; B[b]F, benzo[*b*]fluoranthene; DB[a,c]A, dibenz[*a,c*]anthracene; DB[a,h]A, dibenz[*a,h*]anthracene; DB[a,j]A, dibenz[*a,j*]anthracene; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

was inactive in this respect. In contrast, an excellent correlation was observed between levels of less polar DNA adducts (i.e. those derived from bay-region diol-epoxides) and skin tumor initiating activity of DB[a,j]A. Thus, while triol-epoxides seems to be involved in tumor initiating activity of the parent compound, non alternant B[b]F, the significance of bis-diol epoxide-DNA adducts, at least those derived from DB[a,j]A, is minor.

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of chemical compounds which are ubiquitously present in the environment as a result of incomplete combustion of organic matter and include some of the most powerful chemical carcinogens [1, 2]. This class of chemical carcinogens exert its effects in biological systems only after metabolic activation to reactive intermediates. The interaction between such species and the nucleophilic centers in cellular DNA can result in the formation of covalent DNA adducts, a process generally considered to lead to an initiating event in tumorigenesis [3]. Analysis and characterization of such adducts can provide information as to the nature of the activation pathways and the identity of the ultimate metabolites involved. DNA adducts are also a useful tool in biomonitoring environmental exposure to chemical carcinogens.

The most complete data on metabolism and mechanisms of activation have undoubtfully been collected for benzo[*a*]pyrene (B[a]P) [4–6]. Although its metabolism can occur *via* a variety of routes, it is clear that in almost all *in vivo* situations activation proceeds along a single pathway *via* 7,8-oxide and 7,8-dihydrodiol to 7,8-diol-9,10-epoxides, the bay-region vicinal diol-epoxides, which are generally regarded as the ultimate, carcinogenic, and DNA reactive species formed from B[a]P and most PAHs (Fig. 1).

Both the 7,8-oxide and the 7,8-dihydrodiol exist as pairs of enantiomers while 7,8-diol-9,10-oxides are formed as two diastereomers, each comprising a pair of enantiomers. Of

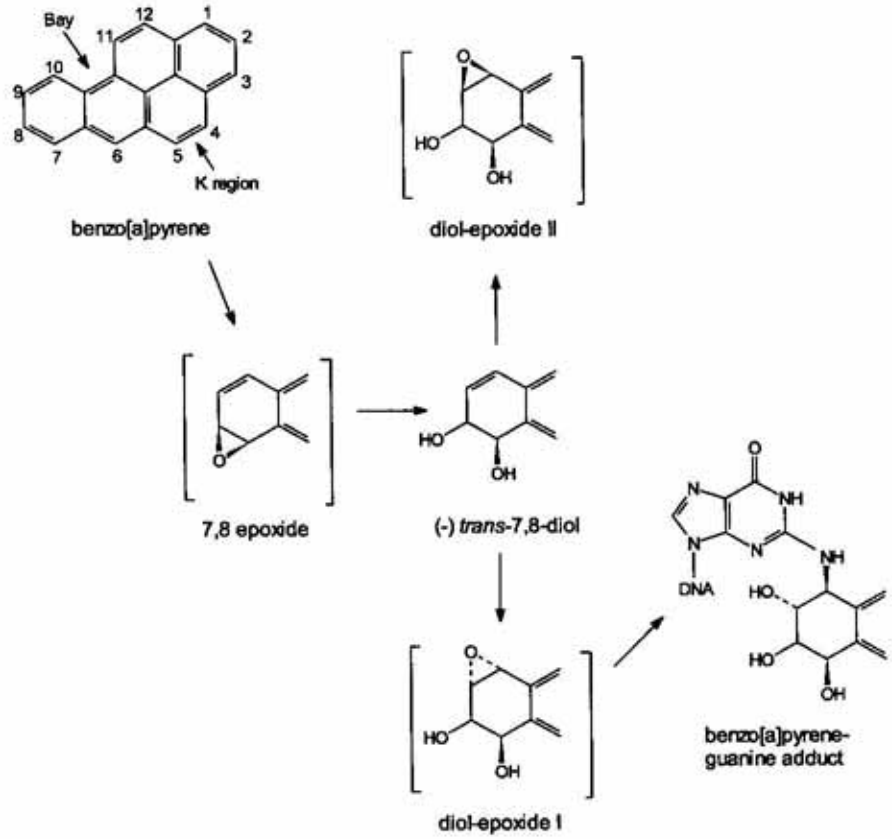

Figure 1. Metabolic activation of benzo[*a*]pyrene to diol-epoxides.

Solid triangles and dashed lines indicate that substituents are toward and away from the viewer, respectively.

these four diol-epoxides, the (+) *anti*-7,8-diol-9,10-epoxide has been found to be more biologically active than the other three in most of the systems in which they have been tested [6–8] and adducts derived from the covalent binding of *anti*-7,8-diol-9,10-epoxides to DNA have been characterized and identified as the major DNA adducts formed following the application of the hydrocarbon to cells and tissues in culture and *in vivo* in animal models such as the mouse skin [9, 10].

The available evidence also suggests that for several other PAHs, activation mechanisms closely resemble the mechanism currently accepted for benzo[*a*]pyrene, and that the major DNA-reactive species formed are also vicinal diol-epoxides of the bay-region type. For some *anti*-dihydrodiol epoxides such as that derived from the planar B[a]P, the amino group of guanine is the almost exclusive site of reaction in DNA, whereas for *anti*-dihydrodiol epoxides derived from the non-planar 7,12-dimethylbenz[*a*]anthracene or benzo[*c*]phenanthrene, comparable extents of reaction with both adenine and guanine residues in DNA were found [11–14]. However, exceptions to and modification of the general bay-region diol-epoxides activation pathway exist and are still discovered. Beside the bay-region diol-epoxides the adducts formed by K-region oxides were also reported [15]. The involvement of one-electron oxidation leading to apurinic sites in DNA was also suggested [16]. However, recent studies did not confirm this hypothesis [17]. Introducing ^{32}P-postlabeling to DNA adducts analysis enabled the identification of new adducts not described previously, very often more polar than those formed by classic bay-region diol-epoxides.

These alternative pathways of PAH activation, particularly those leading to the formation of more polar DNA adducts, will be discussed in this short review.

TRIOL-EPOXIDES

Formation of DNA adducts with the products of activation of phenolic dihydrodiols was described for two PAHs, chrysene and benzo[*b*]fluoranthene (Fig. 2).

Chrysene

The metabolism of chrysene, which is weakly carcinogenic when applied to mouse skin [18], has been extensively studied in a va-

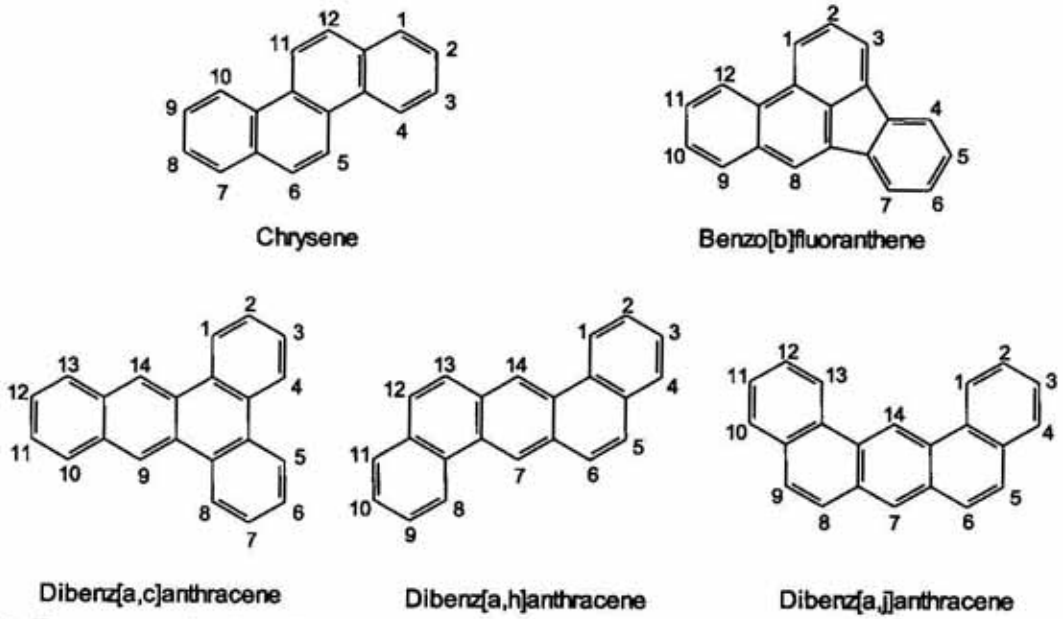

Figure 2. Structures of polycyclic aromatic hydrocarbons discussed in this review.

riety of biological systems [19–21]. Chrysene-1,2-dihydrodiol, a potent precursor of a bay-region diol epoxides is metabolized to derivatives that are mutagenic to *Salmonella typhimurium* TA100, and the (–)-*trans*-enatiomer is a tumor initiator on mouse skin [8, 22]. Although biological activities and DNA-binding properties of chrysene bay-region diol-epoxides are well documented, chrysene is also known to be activated *via* a second pathway that leads to metabolites that bind to DNA *in vivo*. This second type of ultimate carcinogen appears to be a diol-epoxide that contains a phenolic hydroxyl group situated on a ring remote from the dihydrodiol group and has been termed triol-epoxide [23]. The metabolic precursors of triol-epoxides would be expected to include phenolic dihydrodiols. Treatment of mouse skin with [^{3}H]chrysene generated two metabolites that bound to DNA. One adduct had chromatographic properties identical to those of the major adduct formed when chrysene *anti*-1,2-diol-3,4-epoxide reacts with DNA [23]. The second, more polar adduct had chromatographic properties similar to adducts arising from further metabolism of either chrysene-1,2-dihydrodiol or 3-hydroxychrysene in rat liver microsomal metabolizing system [24]. It was postulated that the second adduct was formed from a triol-epoxide that had resulted from further metabolism of 9-OH-chrysene-1,2-diol, which had been detected as a metabolite during microsomal incubations [24]. The results of incubation of rat liver microsomes with *anti*-chrysene-1,2-diol-3,4-epoxide followed by ^{32}P-postlabeling analysis suggested that further metabolism of *anti*-9-OH-chrysene-1,2-diol-3,4-epoxide to DNA binding species is also possible [25].

Benzo[*b*]fluoranthene

Benzo[*b*]fluoranthene (B[b]F) is one of the most prevalent and tumorigenic PAHs present in the environment [26, 27]. B[b]F is mutagenic and its carcinogenic activity in mice and rats is well documented [28–31]. B[b]F metabolites formed *in vitro* with rat liver homogenate and *in vivo* in mouse skin epidermis have been characterized and evaluated for mutagenic and tumorigenic activity [30, 31]. The results of initial studies indicated that none of the identified metabolites of B[b]F can account for the potent tumorigenic activity associated with B[b]F. According to the bay-region theory *anti*-B[b]F-9,10-diol-11,12-epoxide should be the ultimate carcinogenic form of B[b]F. However, the studies have not been able to implicate B[b]FDE as being primarily responsible for the genotoxicity of B[b]F [31–34]. Moreover, the analysis of DNA adducts formed *in vivo* with B[b]F demonstrated that they are more polar than those formed from B[b]F-9,10-diol [34]. In addition, metabolism studies indicate that *trans*-9,10-dihydro-5,9,10-trihydroxybenzo[*b*]fluoranthene (5-OH-B[b]F-9,10-diol) and/or *trans*-9,10-dihydro-6,9,10-trihydroxybenzo[*b*]F (6-OH-B[b]F-9,10-diol) and not B[b]F-9,10,11,12-tetraol are the principal metabolites formed *via in vitro* metabolism of B[b]F-9,10-diol [31]. These results suggest that phenolic dihydrodiols may be the key intermediates in metabolic activation of B[b]F to the ultimate carcinogen. Subsequent studies clearly demonstrated that phenolic dihydrodiols and triol-epoxides, particularly 5-OH-B[b]F-9,10-diol and 5-OH-B[b]F-9,10-diol-11,12-epoxide are involved in tumorigenesis in mouse skin [35]. Comparative studies on the tumor-initiating activity of B[b]F and its metabolites demonstrated that 5-OH-B[b]F-9,10-diol is tumorigenic in mouse skin. B[b]F was slightly more active than 5-OH-B[b]F-9,10-diol, while 6-OH-B[b]F-9,10-diol was considerably less active as a tumor initiator. Topical application of 5-OH-B[b]F-9,10-diol to mouse skin resulted in the formation of two DNA adducts as measured by ^{32}P-postalabeling technique coupled to HPLC. These adducts had identical retention times on HPLC to adducts formed from B[b]F on mouse skin *in vivo*. The similarity between the adducts formed from the reaction of 5-OH-

B[b]F-9,10-DE with dGuo and those formed *in vivo* in mouse skin with 5-OH-B[b]F-9,10-diol indicates that 5-OH-B[b]F-9,10-diol is further metabolized to the 9,10-epoxide derivative and that guanine is the principal base involved in DNA adduct formation.

Overall these data suggest that B[b]F-9,10-diol is further metabolized in mouse skin to the classical dihydrodiol-epoxide B[b]F-9,10-DE and to phenolic dihydrodiols such as 5-OH-B[b]F-9,10-diol and 6-OH-B[b]F-9,10-diol. The results of the tumor-initiating studies along with the DNA adduct data indicate that 5-OH-B[b]F-9,10-diol is the major proximate tumorigenic metabolite of B[b]F on mouse skin.

BIS-DIOL-EPOXIDES AND MULTIPLE SITES OF METABOLISM

The formation of DNA adducts with epoxides having more phenolic hydroxyl groups than triol-epoxides was described for dibenzoanthracene. This PAH forms three isomers: dibenz[*a,c*]anthracene, dibenz[*a,h*]anthracene and dibenz[*a,j*]anthracene which exhibit marked differences in mutagenic and carcinogenic potencies. The three isomers differ only in the location of the two aromatic angular rings on the anthracene "line" (Fig. 2) and all of them posses two bay regions.

Dibenz[*a,c*]anthracene

Metabolic activation of dibenz[*a,c*]anthracene (DB[a,c]A), which is a potent mutagen [36] and a weak tumor initiator [18], has been studied in a number of systems. When the three possible dihydrodiols were tested for initiating activity in mouse skin, the 1,2- and 10,11-dihydrodiols were found to be more active than the parent hydrocarbon, whereas the 3,4-dihydrodiol, a putative precursor of a bay-region diol-epoxide, was not [37]. Hydrocarbon nucleoside adducts were not detected in DNA from mouse skin treated with DB[a,c]A *in vivo*. However, when metabolized with rat liver microsomes in the presence of DNA, or when added to primary culture of hamster embryo cells, dibenz[*a,c*]anthracene did give rise to adducts, some of which had the same chromatographic characteristics as those of the adducts formed by the reaction of the *anti*-10,11-diol-12,13-epoxide with DNA [25, 38]. The 10,11-dihydrodiol has been shown to be further metabolized to this non-bay-region diol-epoxide by rat liver microsomes, but it was noted that metabolic activation of the dihydrodiol in this system gave rise to a number of additional adducts not formed by direct reaction of the diol-epoxide with DNA [38]. Thus the available evidence suggests that the 10,11-dihydrodiol is an intermediate in the metabolic activation of DB[a,c]A in some experimental systems including mouse skin, and that this dihydrodiol may be activated to a vicinal, non-bay-region diol epoxide either with or without additional metabolism elsewhere in the molecule.

Diol-epoxides formed in the 10,11,12,13-ring of DB[a,c]A would not, unlike those that could be formed from DB[a,c]A-1,2 and -3,4-diols, be vicinal diol-epoxides of the "bay-region" type. A non-bay-region diol-epoxide is known to take part in the activation of benz[*a*]anthracene [39, 40], thus the involvement of a non-bay-region diol-epoxide in the activation of DB[a,c]A, another weak carcinogen, would not be surprising. No additional analyses have been performed in order to gain further characteristics of potential polar adducts formed by this PAH.

Dibenz[*a,h*]anthracene

Dibenz[*a,h*]anthracene (DB[a,h]A) was the first pure PAH reported to be carcinogenic in experimental animals [41]. According to the bay-region theory 3,4-diol-1,2-epoxides metabolically generated from DB[a,h]A should be the ultimate carcinogenic metabolites of this PAH. However, *in vivo* [42–44] and *in vitro* [45–50] investigations on the biotransforma-

tion of DB[a,h]A have resulted in the identification of more than 20 metabolites. Of these DB[a,h]A-3,4-diol, a precursor of the bay region diol-epoxides, is formed extensively [47, 48] and is a potent initiator of skin tumors in mice [51]. However, work by Platt & Reischmann [48] provided no evidence for the conversion of DB[a,h]A-3,4-diol into vicinal diol-epoxides when this diol was incubated with liver microsomes prepared from rats pretreated with Aroclor 1254. Studies of microsomal metabolism of DB[a,h]A showed, however, that both the hydrocarbon and the related 3,4-diol could be converted to a range of polyhydroxylated products that included bis-dihydrodiols. It was therefore suggested that the ultimate reactive metabolite formed from DB[a,h]A-3,4-diol might be an epoxide formed through further metabolism of a bis-diol. In a subsequent work of Lecoq *et al.* [52] ^{32}P-postlabeling coupled with HPLC was used for the analysis of DNA adducts formed after the application to mouse skin of DB[a,h]A-3,4,-5,6-diols, and *anti-* or *syn*-DB[a,h]-diol-epoxides. The results of these studies showed that DB[a,h]A is activated to a minor extent *via* a bay-region diol-epoxide, *anti*-3,4-diol-1,2-epoxide, in mouse skin treated *in vivo* with the parent compound or 3,4-diol. The HPLC elution profile provided also evidence for the existence of a second activation pathway which leads to more polar adducts than those formed when *anti-* or *syn*-3,4-diol-1,2-epoxides reacted with DNA or were applied to skin. Further studies designed to examine the nature of these adducts revealed that treatment of mouse skin only with DB[a,h]A-3,4,10,11-bis-diol produced a major DNA adduct. The major adduct formed from DB[a,h]A-3,4,10,11-bis-diol cochromatographed on TLC and HPLC with the major adducts formed following treatment of mouse skin with DB[a,h]A or DB[a,h]A-3,4-diol [53]. Hence it was suggested that this particular symmetrical bis-diol may represent the penultimate metabolite in a pathway by which a bis-diol-epoxide of the bay region type is formed as the ultimate DNA binding species. This route, however, was not exclusive, since a minor adduct derived from the binding of DB[a,h]A-3,4-diol-1,2-epoxide was also identified [52–55]. No further characterization of bis-diol-epoxides or their adducts and tumorigenic activity of bis-diol was performed.

Dibenz[*a,j*]anthracene

Untill recently dibenz[*a,j*]anthracene (DB[a,j]A) had not been extensively studied in terms of its metabolism and metabolic activation compared to other isomers, although its mutagenic and carcinogenic activity was demonstrated relatively early [56–58]. Currently the most comprehensive data on the metabolism and activation pathways of this compound are available thanks to the works performed by the group of DiGiovanni [58, 60, 62, 63, 66–67].

According to the bay-region theory [6], 3,4-diol-1,2-epoxides metabolically generated from DB[a,j]A *via* the *trans*-3,4-dihydrodiol should be ultimate carcinogenic metabolites. Studies performed by Sawyer *et al.* [58] demonstrated that (±)*anti*-DB[a,j]A-diol-epoxide was significantly more active as a skin tumor-initiator than the parent compound, suggesting that metabolic formation of this diol epoxide and its covalent modification of DNA may be involved in the process of tumor initiation by DB[a,j]A. Structural characterization of DNA adducts derived from the reaction of (±)*anti* DB[a,j]A-DE and pure enantiomers of both the *syn* and *anti* diol epoxides of DB[a,j]A with calf thymus DNA showed that these compounds bound extensively to both deoxyguanosine (dGuo) and deoxyadenosine (dAdo) residues [60, 61]. Examination of the covalent DNA adducts formed in cultured mouse keratinocytes exposed to ^{3}H DB[a,j]A revealed the formation of a variety of bay-region diol epoxide DNA adducts including both dGuo and dAdo adducts [62]. In addition, much of the radioactivity was observed in the more polar part of the HPLC chroma-

togram. Analysis of DNA adducts formed in mouse epidermis, 24 h after topical treatment with ^{3}H DB[a,j]A, revealed 11 radioactive peaks consistently observed in HPLC chromatograms [63]. Their identity, as in the above studies, was established based on cochromatography with marker DNA adducts. Beside the early eluting polar portion of chromatogram, the principal DNA adducts were formed from (+)*anti*-DB[a,j]A-DE. The major DNA adduct formed from (+)*anti*-DB[a,j]A-DE in mouse epidermis following topical application of ^{3}H DB[a,j]A was tentatively identified as (+)*anti*-DB[a,j]A-DE-*trans*-N^2-dGuo. This isomer has the (4*R*,3*S*)-diol-(2*S*,1*R*)-epoxide absolute configuration [60, 61]. Diol epoxides with such configuration have been shown to be the most tumorigenic of the four corresponding configurational isomers of bay-region diol epoxides derived from B[a]P, chrysene, benzo[*a*]anthracene, and benzo[*c*]phenanthrene [7, 64]. In addition, after topical application of ^{3}H DB[a,j]A to mouse epidermis, two dAdo adducts were tentatively identified as arising from the (+)*anti*-diol epoxide (both the *cis* and *trans* addition products). The relative proportions of dGuo *vs* dAdo adducts formed *in vivo* was 3.9 and was very similar to that observed for *in vitro* reactions. It is also interesting that several DNA adduct peaks were tentatively identified as arising from the *syn*-diol epoxide of DB[a,j]A. In addition the late eluting peak was identified as dAdo adduct formed with K-region 5,6-oxide. These studies showed also the presence of material not retained on reversed-phase columns when conventional methodology was used (i.e. separation of hydrocarbon-deoxyribonucleoside adducts). Actually it represented the major portion of radioactivity eluted from the column. The consistent presence of this early eluting radioactive material as well as the large fraction of DNA-associated radioactivity eluting in the water phases from the initial cleanup step raised the question about the nature of this material. One possibility is that the early eluting material was composed of highly polar DNA adducts. This suggestion was further explored by ^{32}P-postlabeling analysis. The HPLC profiles of 3',5'-diphosphodeoxyribonucleoside adducts obtained from epidermal DNA isolated from mice treated with the parent compound DB[a,j]A or its *trans*-3,4-diol revealed 12 and 11 adduct peaks, respectively. Notably, in each case approximately half of these peaks eluted prior to standard *anti*- and *syn*-diol epoxide adduct peaks. At least some of the polar DB[a,j]A-DNA adducts formed in mouse skin had retention times similar to those of the early eluting adducts formed after topical application of DB[a,j]A-3,4-diol. This observation suggested indirectly that DB[a,j]A-bis-diol-epoxide metabolites may account for at least some of the polar DB[a,j]A-DNA adducts formed in mouse epidermis. This possibility was further developed by the application to the mouse skin of a series of potential precursors of bis-diol-epoxides, bis-dihydrodiols and phenolic dihydrodiols of DB[a,j]A and subsequent analysis of epidermal DNA adducts. For the analysis of DNA adducts, ^{32}P-postlabeling coupled with TLC was used [65]. The major findings of this study were as follows: i/ three of the highly polar DNA adducts produced following topical application of DB[a,j]A were tentatively identified as arising from the 3,4-10,11-bis-diol; ii/ topical application of the 5,6-diol of DB[a,j]A produced two major highly polar DNA adduct spots in TLC chromatograms that did not correspond to any of the highly polar DNA adducts derived from the parent compound; iii/ DB[a,j]A-3,4-8,9-bis-diol and the 10-OH and 11-OH-diols produced only low levels of DNA adducts when topically applied at doses equivalent to DB[a,j]A. Analysis of the tumor-initiating activity showed, however, that DB[a,j]A-3,4-10,11-bis-diol did not posses skin tumor initiating activity at a dose of 400 nmole in SENCAR mice. In contrast, an excellent correlation was observed between levels of less polar DNA adducts (i.e. those derived from bay-region diol-epoxides) and skin tumor initiat-

ing activity of DB[a,j]A, DB[a,j]A-3,4-diol, DB[a,j]A-3,4-10,11-bis-diol, and (±)*anti*-DB[a,j]ADE.

Thus, the above results indicate that several routes of activation to DNA binding intermediates are possible for DB[a,j]A. One pathway involves the formation of simple epoxides of the K-region type. A second pathway involves the bay-region diol epoxides, and another one involves the formation of more polar metabolites derived from bis-dihydrodiol epoxides. The most important, however, seems to be the pathway which leads to bay-region diol-epoxides, which should be considered as the ultimate carcinogenic metabolites of DB[a,j]A This statement is further supported by the results of the analysis of c-Ha-*ras* mutations in skin papillomas initiated by DB[a,j]A. The data obtained in this experiments showed exclusively the A^{182}→T mutation [66] which additionally implicated the role of dAdo adducts in the tumor-initiating activity of DB[a,j]A. Finally, in studies in which the mutagenicity of site-specific DNA adducts was examined, the (+)*anti*-DB[a,j]A-DE-*trans*-N^6-dAdo adduct produced exclusively A→T transversions [67]. Thus, while some of the polar DNA adducts detected in mouse skin arise from DB[a,j]A-3,4-10,11-bis-diol, the contribution of these adducts to the tumor-initiating activity of DB[a,j]A may be relatively small.

CONCLUSIONS

In the years following the establishing of the concept of "bay-region diol-epoxide" as the ultimate carcinogenic form of PAH, new mechanisms of activation of this structurally diverse class of carcinogenic chemicals to DNA-binding species were proposed. The examination of DNA adducts have benefited greatly from the application of ^{32}P-postlabeling technique, whereby a radiolabeled test compound is not required. The analysis of some PAH-DNA adducts performed in this way showed the possibility that these compounds may undergo additional metabolism at other sites of the molecule, not only in the bay or K-region. The significance of these alternative activation pathways is not clear yet. Certainly, on the basis of the evidence accumulated so far, the larger symmetrical PAHs, such as the dibenzanthracenes, appear to be likely to undergo activation in more than one aromatic ring. The resulting polar adducts, although predominant in the adduct profiles of such PAHs as DB[a,j]A, seem to play a minor role in their tumorigenic activity. More data are necessary in order to evaluate the significance of these alternative activation pathways of PAHs.

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