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COMMENTARY

THE PRICE OF PROGRESS OR PANDORA'S PURSE

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One of the major advances in unifying the field of chemical carcinogenesis came with the hypothesis by the Millers that *all* carcinogens exert their effects after their transformation into electrophiles (see Ref. 1 for review). For many of these carcinogens, the biotransformation was accomplished enzymatically within the endoplasmic reticulum (reviewed in Ref. 2) and the nucleus (reviewed in Ref. 3) of mammalian cells. Specifically, the polycyclic aromatic hydrocarbons are metabolically activated to their purported ultimate carcinogenic forms, the diol epoxides, through the concerted action of a cytochrome P-450-dependent monooxygenase and epoxide hydrolase [4–6]. The diol epoxides spontaneously rearrange to electrophiles that interact with nucleophilic centers of macromolecular components such as DNA.

In a similar sense, agents such as ethyl- and methylnitrosoureas are activated to electrophilic species (see Ref. 1); the nitrosoureas spontaneously give rise to ethyl or methyl electrophiles while the nitrosamines are metabolically activated to these electrophiles. The methyl or ethyl electrophile will alkylate specific portions of the DNA, i.e. at various positions of the different bases.

The exact nature of the nucleophilic target responsible for the transformation of a normal to a neoplastic cell after exposure to a polycyclic aromatic hydrocarbon is not established at this time. Most investigators would believe that DNA is the target nucleophilic acceptor and that its alkylation contributes in a substantive manner to neoplastic transformation, i.e. somatic mutation. To the contrary, Cairns [7] has provided an interesting argument *against* this hypothesis and has suggested an alternative acceptor. However, in this commentary, I will consider an alkylation of DNA as the focal point within the cell from which a series of events ensue, culminating in neoplasia. The major points to be discussed will include (a) whether metabolism to electrophilic species represents the rate-limiting component in chemical carcinogenesis, (b) whether susceptibility or resistance to a particular chemical-induced carcinogenic event is a reflection of the formation of different adducts in DNA, i.e. different alkylated deoxyribonucleosides, (c) whether various tissues are able to repair different adducts at different rates, if at all, and, furthermore, whether adducts result in specific biological lesions, and (d) whether repair of DNA adducts can be affected by cocarcinogenic agents and, specifically, mineral particulates. I will mainly use polycyclic aromatic hydrocarbons and methylating agents to demonstrate the various points.

Activation of carcinogens. The activation of polycyclic aromatic hydrocarbons may proceed through two mechanisms: (a) cytochrome P-450 and epoxide hydrolase systems, and (b) arachidonic acid-dependent cooxygenation. The mechanism of activation of benzo[a]pyrene through pathway "a" has been fully discussed by Gelboin in a recent review [6]. The purported ultimate carcinogenic forms of benzo[a]pyrene are believed to be 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene (diol epoxide I or *anti* isomer) and 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (diol epoxide II or *syn* isomer). Benzo[a]pyrene is epoxidated by the cytochrome P-450-dependent monooxygenase system to the 7,8-epoxy derivative which subsequently is hydrated through the catalytic action of epoxide hydrolase to the stereospecific (−)-*trans*-7,8-diol derivative. The latter undergoes a further oxidation as catalyzed by the cytochrome P-450 system to yield predominantly the *anti* diol epoxide, although the ratio of *syn* to *anti* derivatives depends upon tissue and species [6]. The *anti* diol epoxide appears much more carcinogenic than the corresponding *syn* derivative [8, 9]. Pathway "b" consists of several steps involving fatty acid cyclooxygenase, which catalyzes the bisdioxygenation of arachidonic acid to prostaglandin G_2, and prostaglandin hydroperoxidase, which catalyzes the reduction of prostaglandin G_2 to the alcohol, prostaglandin H_2. This system cooxidizes the proximate carcinogen, *trans*-7,8-dihydroxy 7,8-dihydrobenzo[a]pyrene, i.e. *trans*-7,8-diol, to electrophilic forms that covalently bind to macromolecules [10, 11]. These electrophiles are also mutagenic and, as shown by the laboratories of Marnett [12] and Eling [13], arise from diol epoxides of benzo[a]pyrene.

In regard to the methylating or ethylating agents, as mentioned previously, they are either directly alkylating, i.e. spontaneously forming electrophiles, or they are metabolically activated. The latter requires a cytochrome P-450-dependent monooxygenase as presented in Ref. 1.

Adduct formation. A number of investigators have studied the nature of polycyclic aromatic hydrocarbon adducts formed with DNA of various cells and tissues under *in vitro* and *in vivo* conditions. Furthermore, several have compared, both qualitatively and quantitatively, DNA adducts formed from polycyclic aromatic hydrocarbons in cells that are susceptible, i.e. can be neoplastically

transformed, and resistant to these agents. In this presentation, I will limit the polycyclic aromatic hydrocarbon under discussion to benzo[a]pyrene.

That the diol epoxides of benzo[a]pyrene can form adducts with DNA using a number of cell cultures, tissue explants, or perfused organ systems has been demonstrated by a number of investigators [14–32]. Diol epoxides of benzo[a]pyrene are also produced by isolated nuclei prepared from rodents that had been pretreated with either vehicle alone or with an inducing agent, e.g. 3-methylcholanthrene [33–36]. Furthermore, adduct formation has also been reported after *in vivo* exposure of animal systems to benzo[a]pyrene; some of these adducts arose through the intermediate diol epoxides [32, 37–39].

Studies on the nature of the nucleophile present in DNA with which the electrophilic rearrangement products of benzo[a]pyrene diol epoxides interact, have been reviewed by Weinstein *et al.* [40]. Covalent interactions are produced mainly with guanine and to a lesser extent with cytosine and adenine. The major adduct was derived from the *anti* diol epoxide isomer by the *trans* addition of the N2-amino group of guanine to the 10-position of the benzo[a]pyrene moiety. The corresponding adduct formed from the *syn* isomer has also been observed. Other *trans* additions to the 2-amino of cytosine and the 6-amino of adenine occurred even less frequently. Eastman *et al.* [41] have reported the occurrence of benzo[a]pyrene diol epoxide-adenine adducts in a hamster tracheal epithelial cell, a cell that is sensitive to the carcinogenic action of this polycyclic aromatic hydrocarbon. Lo and Kakunaga [42] have looked at adduct formation in a cell that is highly transformable by benzo[a]pyrene and have reported only the *anti* diol epoxide guanine derivative. Furthermore, they have suggested the involvement of only this adduct in the transformation process, an extrapolation which appears rather simplistic. The formation of benzo[a]pyrene metabolite adducts with DNA has been studied in intact animal systems by Anderson and colleagues. Under *in vivo* conditions, adducts produced in rat lung and liver resulted from the further metabolism of benzo[a]pyrene phenols and, furthermore, diol epoxide adducts were not detected in liver; only 3% of the amount of benzo[a]pyrene phenol generated adducts was of the diol epoxide variety in lung [37]. This was unlike that reported by Eastman *et al.* [32] with the A/J mouse which is sensitive to the carcinogenic action of benzo[a]pyrene (unlike the rat). In the latter report, the major adduct in lung was of the diol epoxide type. More recently, Ioannou *et al.* [38] have reported the formation of diol epoxide-guanine adducts in the DNA from lung, liver and forestomach from A/HeJ mice that were treated with benzo[a]pyrene. All three mouse tissues are target organs for the polycyclic aromatic hydrocarbons.

Benzo[a]pyrene metabolite-DNA adduct formation has been studied in human explants of a variety of tissues including bronchus, esophagus, colon and duodenum by Harris and his colleagues [24, 43, 44]. Although large interindividual variation was observed, the major DNA adduct was that formed by the *trans* addition of the *anti* diol epoxide of benzo[a]pyrene to the 2-amino of guanine. The same adduct was reported by Theall *et al.* [45] after incubation of human epidermal cells with benzo[a]pyrene and by Stampfer *et al.* [46] with human mammary epithethial cells. In the latter study, some *syn* products with guanine as well as *anti* diol epoxide adducts with cytosine were reported. The formation of benzo[a]pyrene metabolite-adducts has been studied in human alveolar tumor A549 cells by Cerutti *et al.* They have noticed the presence of two major adducts, one formed from the *syn* diol epoxide with the 2-amino of guanine and the other from the *anti* derivative.

The benzo[a]pyrene metabolite which ultimately serves to contribute to the formation of the DNA adduct is not restricted to the diol epoxides I and II. In fact, Ashurst and Cohen [14] as well as Boroujerdi *et al.* [37] have reported the diol epoxides I and II to be relatively insignificant in this regard, while benzo[a]pyrene phenols proved to be major contributors. Jernstrom *et al.* [18] similarly have reported that the major adduct seen in hepatocytes obtained from 3-methylcholanthrene-treated rats was generated from the further metabolism of 9-hydroxy benzo[a]pyrene. Other adducts formed from the further metabolism of the 4,5-oxide of benzo[a]pyrene have been reported in isolated liver preparations after treatment of rats with β-naphthoflavone [27]. King *et al.* [48] have also demonstrated the involvement of a metabolite of 9-hydroxy benzo[a]pyrene in the interaction with DNA; the latter metabolite may have been the 4,5-epoxy 9-hydroxy derivative. The data of Guenthner and Oesch [49] offer a partial explanation for the formation of different adducts. They have reported that high levels of epoxide hydrolase favor the formation of the 7,8-diol of benzo[a]pyrene and hence the subsequent formation of diol epoxides I and II. On the other hand, low levels of this hydrolase will cause preferential formation of 9-hydroxy benzo[a]pyrene. In addition, altered rates of detoxification of the various epoxide hydrolase products, i.e. 4,5-, 7,8-, or 9,10-diols of benzo[a]pyrene, by glucuronidation, sulfation, or glutathione conjugation, may also contribute to the different DNA adducts which are formed.

Finally, some recent work in our own laboratory is germane to this discussion of DNA adducts. Ireland *et al.* [50] have found that benzo[a]pyrene treatment of a sensitive hamster tracheal epithelial cell line is followed by the formation of polycyclic aromatic hydrocarbon DNA-protein crosslinks. These DNA-protein crosslinks may, in fact, be responsible for the "early peaks" seen during Sephadex LH20 chromatography or high performance liquid chromatography (HPLC) as reported previously [32, 37]. The establishment of a covalent bridge between the DNA and the attendant nuclear proteins may play an important role in the carcinogenic process.

In summary, a number of different benzo[a]pyrene metabolite-DNA adducts have been reported in a variety of cells and tissue systems in different species (including the human). Which one (or ones), if any, is (or are) important to the process of neoplastic transformation still remains to be established. Furthermore, the recent observations of

benzo[a]pyrene metabolite-induced DNA-protein crosslinking may also provide a clue to the alterations observed during neoplasia development.

Biological effects of adduct formation. The benzo[a]pyrene diol epoxides I and II have been tested for mutagenicity in several systems including both bacterial and mammalian. In the Ames assay, the *anti* and *syn* isomers were both very potent mutagens although the latter was slightly more effective [51]. Malaveille *et al.* [52] have also demonstrated the potent mutagenic properties of the *anti* isomer in the Ames assay. A number of investigators have also established the mutagenic potency of the diol epoxides using a variety of marker systems in mammalian cells [52–56]. In an interesting study by Wood *et al.* [57], the four optical enantiomers of the diol epoxides were compared as to their mutagenic efficacy in V79 cells; the (+)-*anti* isomer was the most active.

The tumorigenicity of the diol epoxides as carcinogens has been tested by the "Hoffmann–LaRoche–NIH axis" [58]. Of the four stereoisomers, the (+)-*anti* isomer proved extremely active as a lung carcinogen upon injection into newborn mice. The other three compounds were not very active. The diol epoxides of benzo[a]pyrene have also been tested on mouse skin [59, 60]. Both *syn* and *anti* diol epoxides exhibited a potent hyperplasiogenic effect on mouse skin after topical administration [59]. As in the case of pulmonary tumorigenesis cited above, the (+)-*anti* isomer was the most effective as both a complete mouse skin carcinogen and initiator [60].

Several investigators have attempted to establish the effects of interaction of the diol epoxides with DNA upon some biological parameter. Thus, Mizusawa and Kakefuda [61] have interacted the *anti* isomer with the bacterial plasmid pBR322 and observed that the subsequent replication of the resultant plasmid DNA was inhibited by only a small number of adducts. Similar results have been obtained with ØX174 [62] and pKo482 [63] plasmid DNAs.

The effect of alkylation of SV-40 DNA upon its efficiency of transfection of mammalian cells has also been investigated by several groups. Viral infectivity was reduced significantly after alkylation with the *anti* diol epoxide [64–66]. Alkylation of SV-40 DNA was accompanied by a transitory decrease in viral DNA synthesis which was partially restored concomitant with the appearance of single-stranded regions. These data suggested that the reduced transfection efficiency may be the result of either a block in transcription at the remaining adducts or a high error rate in a repair process.

Finally, the effects of adduction of DNA by the *anti* diol epoxide of benzo[a]pyrene have been studied using the lacI gene of uvrB$^-$ *Escherichia coli* as the target site [67]. Adduction in this model leads to the production of nonsense mutations in approximately 10% of the instances. The diol epoxide causes rather specific transversions by inducing GC → TA and, to a lesser extent, AT → TA. The former could be the result of the rotation of modified guanine around the glycosylic bond, changing the *anti* conformation to a *syn*.

In summary, alkylation of DNA by a benzo[a]pyrene metabolite(s) exerts potent biological effects, including mutagenesis in bacterial and mammalian systems, and neoplastic transformation *in vitro* and *in vivo*. At least some of these effects may result from specific transversions.

Persistence of adducts as related to biological effects. Prior to discussing the persistence of polycyclic aromatic hydrocarbon lesions in target cell DNA, I'll digress for a few moments to consider ethylation and methylation of DNA bases and the repair thereof. This digression is germane to the question of repair. One of the first suggestions of the relationship between persistence of DNA adducts and the occurrence of neoplasia came from the observations of Goth and Rajewsky [68]. They reported that neonatal rat brain was more sensitive to ethylnitrosourea carcinogenicity because of a longer persistence of O^6-ethylguanine. Non-target tissues such as liver, whose DNA also became alkylated, possessed a mechanism for the rapid removal of the lesions. The importance of this observation is that neoplastic transformation is *not* simply the result of interaction of DNA with oncogenic electrophiles but depends upon (a) the rate of proliferation of the target cell, and (b) persistence of specific alkylated groups. The concept of alkylation at specific sites has been very adequately addressed by Magee [69] and Singer [70].

Although liver is relatively resistant to methylating agents, it is possible to obtain angiosarcomas and some hepatocellular carcinogens after administration of 1,2-dimethylhydrazine (see, for example, Ref. 71) and dimethylnitrosamine (see, for example, Ref. 72). In the former [71], the greater incidence of angiosarcomas over carcinomas was related to the greater accumulation and persistence of O^6-methylguanine in non-parenchymal cells. It was Pegg (reviewed in Ref. 73) who first showed that one could oversaturate the capacity to remove the methylated lesion in liver and hence could neoplastically transform this tissue. Subsequent work in my laboratory [74] defined the methylation repair process in liver as a transfer of the methyl group from O^6-methylguanine of the DNA to a sulfhydryl group on an acceptor protein [74]. With this information as background, we will now return to the question of persistence of polycyclic aromatic hydrocarbon moieties in the DNA of target cells.

As indicated above, differential repair processes appear to render some tissues more susceptible to neoplastic transformation. The difference in rates of repair of polycyclic aromatic hydrocarbon-DNA adducts has been reported in cultured cells by several investigators. Dipple and Hayes [75] reported the efficient removal of 3-methylcholanthrene metabolite adducts from mouse embryo cell cultures while 7-bromomethylbenz[a]anthracene metabolite-DNA adducts were eliminated with considerably greater difficulty. Benzopyrene *anti* diol epoxide moieties are removed from DNA of the human lung tumor cell with some rapidity although incompletely [47]. In the latter report, DNA strand breakage was seen by 3 hr, and 30–53% of the adducts still remained by 30 hr after exposure of the cells to *anti* benzo[a]pyrene diol epoxide. It is apparent from several studies that the ease of excision of the DNA adducts depends upon the type of adduct. Thus,

Dipple and Roberts [76] reported the preferential excision of modified adenine moieties after exposure of mouse embryo cells to 7-bromomethylbenz[a]anthracene, and Eastman *et al.* [41] have communicated the complete removal of benzo[a]pyrene metabolite-adenine adducts from the DNA of a hamster tracheal epithelial cell; guanine adducts were very resistant to excision. The latter observation is in accordance with incomplete removal of these lesions from baby hamster kidney and secondary C57BL mouse embryo cells [20]. The slow excision rate may be caused by the location of the benzo[a]pyrene derivative in the minor groove of DNA, a position which would not substantially distort its double helical nature [20]. Only minimal distortion of hamster tracheal cell DNA, as determined by its susceptibility to alkaline elution, has been reported [41] at doses of benzo[a]pyrene which were toxic to the cells.

Several groups of investigators have also studied the persistence of polycyclic aromatic hydrocarbon DNA adducts under *in vivo* conditions. Based upon the known fact that 7,12-dimethylbenz[a]anthracene (DMBA) causes a greater incidence of hepatic cancer in partially hepatectomized rodents than in intact animals, Tomsak and Cook [77] examined the retention of labeled DMBA in rat livers. Two weeks after partial hepatectomy, considerably more radioactivity was associated with the regenerating than the intact liver.

It struck us that mice of various strains would provide a well characterized system for initially assessing the relationship between persistence and neoplasia. We employed a strain of mouse, the A/J, which was highly susceptible to 3-methylcholanthrene-induced pulmonary neoplasia, the C3H which was moderately susceptible, and the DBA and C57BL which were quite resistant [78]. Adult liver in all four strains was remarkably resistant to polycyclic aromatic hydrocarbon-induced carcinogenesis. We administered [^{3}H]3-methylcholanthrene, i.v., to all the mice and periodically assessed the specific activities of the liver and lung DNA [78]. Expressed as amount of deoxyribonucleoside adduct/mg DNA, the liver radioactivity disappeared rapidly in all four strains so that by 28 days later the specific activity was close to 0. In contrast, the lung specific activity of deoxyribonucleoside adduct/mg DNA reflected the relative susceptibility to 3-methylcholanthrene-induced neoplasia. The lungs from the susceptible strains, C3H and A/J, did not clear the 3-methylcholanthrene metabolite from its DNA as rapidly as did the tissue from the resistant DBA and C57BL. Consequently, persistence was correlated with susceptibility.

Modulation of kinetics of disappearance of benzo[a]pyrene from tracheal DNA by asbestos. Asbestos represents a group of silicate minerals that have broad commercial utility. Recently, we have come to recognize the occupational exposure to asbestos as a major health hazard. Thus, fibrotic lung disease and a rare neoplasia, mesothelioma, have increased incidence in asbestos miners [79]. Furthermore, among asbestos workers who smoke, an increased frequency of bronchigenic carcinoma of 80 to 92-fold is noted over the non-smoking general population [80]. Cocarcinogenesis between asbestos and normal components of tobacco smoke is believed responsible for this increased incidence. We have tested the effects of combined benzo[a]pyrene and asbestos upon the hamster tracheal epithelial cell line [81]. The benzo[a]-pyrene coated onto asbestos fibers was more rapidly taken up by these cells than uncoated polycyclic aromatic hydrocarbon. Furthermore, the benzo[a]pyrene remained for longer periods of time within the epithelial cells. By 4 days after a single administration of the coated asbestos or benzo[a]pyrene alone to the cells, considerably higher levels of the polycyclic aromatic hydrocarbon under the former conditions were associated with tracheal DNA. This effect was not observed if the asbestos was added to the cells 1 hr before benzo[a]pyrene. Consequently, the enhanced uptake of the polycyclic aromatic hydrocarbon contributed by the coated asbestos markedly influences the kinetics of disappearance of benzo[a]pyrene from the tracheal cell DNA and, therefore, may significantly increase the incidence of neoplastic transformation.

I can summarize the nature of this commentary as follows. First, the metabolic activation of benzo[a]pyrene has been discussed, and it appears unlikely to this author that activation will prove a major rate-limiting component in the carcinogenic process. Virtually every cell that has been tested has the capacity to form activated species from polycyclic aromatic hydrocarbons. Second, the activated metabolites interact with DNA, contributing to a number of DNA adducts only some of which have been identified. The tendency in most research of this type is to cast the blame upon the adduct which is present in greatest amount, i.e. the so-called "looking for the key under the streetlight syndrome". In this case, the blame has fallen upon the shoulders of the *anti* diol epoxide-guanine adduct. However, it is to be emphasized that whether or not this is indeed the major cause of the neoplastic transformation is still very problematical. Third, the rate of repair of the DNA adducts varies depending upon the nature of the adduct and upon the cell in which the alkylation has occurred. Unfortunately, we currently have no knowledge of the mechanism responsible for the removal of polycyclic aromatic hydrocarbon adducts, an aspect of research that must be thoroughly investigated. Fourth, it is not established whether *persistence* of the adduct in DNA or an error-prone repair system, or both, contributes to the problem of neoplasia. Whether in fact an error-prone DNA repair system (in regard to removal of polycyclic aromatic hydrocarbon-DNA adducts) exists or not in mammalian cells is certainly not established. Finally, the cocarcinogenic role of asbestos and polycyclic aromatic hydrocarbons may be caused by enhanced uptake of the latter by susceptible cells and the consequent apparent persistence of adducts in DNA. This aspect must also be pursued and perhaps extended to include mixtures of polycyclic aromatic hydrocarbons, as they occur "naturally" in incomplete combustion smokes.

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