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Genetics

Chronic, topical exposure to benzo[*a*]pyrene induces relatively high steady-state levels of DNA adducts in target tissues and alters kinetics of adduct loss

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ABSTRACT **Carcinogen–DNA adduct measurements may become useful biomarkers of effective dose and/or early effect. However, validation of this biomarker is required at several levels to ensure that human exposure and response are accurately reflected. Important in this regard is an understanding of the relative biomarker levels in target and nontarget organs and the response of the biomarker under the chronic, low-dose conditions to which humans are exposed. We studied the differences between single and chronic topical application of benzo[*a*]pyrene (BAP) on the accumulation and removal of BAP–DNA adducts in skin, lung, and liver. Animals were treated with BAP at 10, 25, or 50 nMol topically once or twice per week for as long as 15 weeks. Animals were sacrificed either at 24, 48, or 72 hr after the last dose at 1 and 30 treatments, and after 24 hr for all other treatment groups. Adduct levels increased with increasing dose, but the slope of the dose-response was different in each organ. At low doses, accumulation was linear in skin and lung, but at high doses the adduct levels in the lung increased dramatically at the same time when the levels in the skin reached apparent steady state. In the liver adduct, levels were lower than in target tissues and apparent steady-state adduct levels were reached rapidly, the maxima being independent of dose, suggesting that activating metabolism was saturated in this organ. Removal of adducts from skin, the target organ, was more rapid following single treatment than with chronic exposure. This finding is consistent with earlier data, indicating that some areas of the genome are more resistant to repair. Thus, repeated exposure and repair cycles would be more likely to cause an increase in the proportion of carcinogen–DNA adducts in repair-resistant areas of the genome. These findings indicate that single-dose experiments may underestimate the potential for carcinogenicity for compounds that follow this pattern.**

Benzo[*a*]pyrene (BAP) is a ubiquitous environmental carcinogen found within complex mixtures, which often contain many polycyclic aromatic hydrocarbons (PAH). BAP is often used to model exposure and effects for the family of PAH. These compounds have relatively low vapor pressures, but can be vaporized when heated and form condensation nuclei when the vapors cool (1, 2). In addition, PAH have been shown to condense onto soot, silica, and other particles and enter various regions of the respiratory tract as appropriate for the size of the particles (3–5). Persons employed in aluminum reduction plants, coke ovens, and foundries may be exposed to BAP and PAH via inhalation. PAH are also well-absorbed through the skin (6). For occupations such as road pavers, roofers, and automobile mechanics, the transdermal route may be the major route of entry (7, 8). Although the carcinogenicity of BAP in the animal skin model has been well-documented, particularly regarding initiation and promotion, less is known about chronic exposure and pre-malignant genetic effects in local and distal target tissues.

Increased levels of BAP–DNA adducts have also been described in target tissues for exposed animals. BAP has been studied widely with regard to its ability to bind to DNA and produce genotoxic effects (1, 9, 10). BAP has been shown to be both a systemic and local animal carcinogen by a number of routes of administration such as topical, oral, intraperitoneal, intratracheal, and subcutaneous. In particular, chronic exposure to BAP in animal studies has been shown to cause cancer in skin, lung, and stomach (1, 11). BAP is a suspect human carcinogen based on the fact that the compound is a potent carcinogen after chronic multiple-dose or single high-dose exposure and the BAP–DNA covalent products in animals are very similar to those found in human cells *in vitro* (1, 9, 10).

BAP must first be metabolized to a reactive metabolite(s) that can bind to DNA and subsequently exert its mutagenic and/or carcinogenic effect in target tissue (12–14). Sensitive assays have been developed to assess exposure to BAP by measuring DNA adducts in human blood lymphocytes that have included immunoassays, synchronous fluorescence spectroscopy, fluorescence–HPLC, and [^{32}P]-postlabeling (15–21). The bay-region diol epoxide of BAP has been shown to bind to guanine at the N2 position in native DNA and also forms lesser amounts of adducts with deoxyadenosine (9). The anti-diol epoxide of BAP has been shown to induce mutations in the HPRT gene at both GC and AT base pairs depending on the dose of metabolite used (22). Consistent with these data, the majority of the K-*ras* mutations found in BAP-induced lung tumors in strain A/J mice were GC to AT transitions at the first base of the 12th codon with the rest of the mutations occurring either as GC to AT transitions or GC to TA transversions at the second base of codon 12. The mutations involving a guanine base were consistent with the major DNA adduct formed with the bay-region diol epoxide of BAP (23). Lastly, BAP or its metabolites have caused sister chromatid exchanges and micronuclei in human lymphocytes *in vitro* and *in vivo* and in animal cells (24–29). Studies have shown a correlation between the sister chromatid exchange and DNA adduction for BAP exposure (26).

The development of carcinogen biomarkers to monitor human exposure and predict effects requires an understanding of the basic kinetics of response in a model that mimics the human exposure situation. Beland, Poirier and coworkers have studied the relationship between chronic carcinogen treatment and target organ carcinogen–DNA adduct levels in some detail (30, 31). They have reported that in a chronic feeding study with high levels of acetylaminofluorene, hepatic carcinogen–DNA adduct levels appear to peak in about 20 days. These authors report that there appeared to be two compartments of adducts in the liver. Adducts were formed twice as fast and

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Abbreviations: BAP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbons; RAL, relative adduct labeling.

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removed 10 times as fast in one compartment relative to the other.

The importance of measuring the levels of specific adducts with chronic exposure has been shown in several studies. Boucheron *et al.* (32) reported that O^4-ethylthymidine, a minor adduct with acute exposures, became the major adduct when dimethylnitrosamine was applied chronically. In addition, Beland *et al.* (30) reported that with chronic exposure to acetylaminofluorene, the relative importance of the *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene DNA adducts was greatly decreased compared with *N*-(deoxyguanosin-8-yl)-2-aminofluorene. Fedtke *et al.* (33) showed that 7-(2′-oxoethyl)guanine was removed from DNA with a half-life of 62 hr, whereas N^2,3-ethenoguanine, a relatively minor adduct was removed with a half-life of greater than 30 days. The relative levels of the two adducts changed from 1:100 immediately after exposure to 1:14 at the end of 1 week.

One potential and often imputed problem with carcinogenicity studies is that physiological effects may be elicited at high doses that do not reflect the normal occurrences at lower doses that occur in the environment. Sensitive techniques are now available to evaluate the effects of chronic exposures at and below the levels that are neoplastic in animal studies. This study uses these techniques to determine the effects of low-level chronic exposure by a route common in occupations and the general environment. Only the highest dose reported in this work was used in a chronic topical application study and was found to be carcinogenic in the skin (11).

MATERIALS AND METHODS

Animals and Treatments. Female Hsd (ICR) Br mice were purchased from Harlan–Sprague–Dawley at 5–7 weeks of age and allowed to acclimatize for 2 weeks prior to treatment. Animals were housed individually in plastic cages in conditions of controlled temperature, humidity, and day/night cycle. Animal chow and water were given ad libitum. The backs of the animals were shaved with electric clippers 48 hr prior to treatments with BAP. The backs of animals were treated either once or twice per week, for as many as 15 weeks, with 10, 25, or 50 nMol of BAP (Aldrich; 99% pure-purification described elsewhere, ref. 11) in 50 μl of acetone (HPLC grade, Fischer Scientific) or acetone alone. Animals were sacrificed by i.p. overdose of pentobarbital (100 mg/kg). Tissues of interest (skin, liver, lung, and urinary bladder) were rapidly removed and immediately frozen in liquid nitrogen. Blood was processed to separate nucleated fractions for carcinogen–DNA adduct analysis. Tissues were stored at −80°C until analyzed. In single acute treatment studies animals were treated with either 10 or 50 nMol BAP in 50 μl of acetone and then sacrificed at either 24, 48, or 72 hr after the treatment. In the long-term studies, groups of animals were treated twice per week with either 10, 25, or 50 nMol of BAP. Groups were sacrificed 24 hr following treatment after 3, 6, 12, and 15 weeks of treatment. Groups of animals treated for 15 weeks were also sacrificed at 48 and 168 hr following the last dose. There were 3–5 animals in each group except for the 10 and 25 nMol treatment at 12 weeks when two animals each were sacrificed.

[^{32}P]-Postlabeling. DNA from the various tissues was obtained using a technique that employs hydrolytic enzymes to degrade RNA and protein, followed by solvent extraction to remove the latter, and ice-cold ethanol addition to precipitate polymerized nucleotides (34).

The [^{32}P]-postlabeling assay was run for all samples essentially as described (35–37). [^{32}P]ATP was synthesized in the laboratory according to the procedures of Johnson and Walseth (38). Multiple determinations in the lab indicated that the specific activity of the [^{32}P]ATP is approximately 3000 Ci/mM (range 2500–3300; 1 Ci = 37 GBq); this value is used in all calculations. A stratified block randomization scheme was used to minimize the effect of run-to-run variability common with [^{32}P]-postlabeling. This scheme requires that each [^{32}P]-postlabeling run contain animals from many treatment and sacrifice interval groups. DNA was hydrolyzed by incubation for 3 hr at 37°C in the presence of 2.5 units of micrococcal endonuclease and 0.25 μg of calf spleen phosphodiesterase. We have shown that these conditions result in efficient labeling of BAP adducts (39). DNA digests were then further incubated with 0.75 unit of nuclease P_1 for 40 min at 37°C. This treatment has been shown to increase sensitivity of [^{32}P]-postlabeling for BAP–DNA adducts. On the other hand, the recovery of the various BAP–DNA adducts following nuclease P_1 treatment has not been determined and absolute quantitation of the adducts is, therefore, not possible. Because of the possibility of digestion by nuclease P_1, the values reported likely represent minimums. Adduct levels are given as relative adduct labeling (RAL) × 10^9 to reflect this fact. Assuming complete recovery, RAL × 10^9 and adducts per 10^9 nucleotides are equivalent. However, because labeling conditions were controlled within the study and the stratified block sampling method was employed, these results will be internally consistent. All analyses were run at least in duplicate.

RESULTS

Fig. 1 gives representative chromatograms of [^{32}P]-postlabeled DNA from skin, liver, and lung of animals treated with BAP. Adduct 1, which cochromatographed with a standard of (+)-*trans*-anti-7R,8S,9S-trihydroxy-10R-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene, obtained from the National Cancer Institute chemical repository, is the major adduct in all cases. Adduct 2, which cochromatographed with (−)-anti-7S-8R,9R-trihydroxy-10S-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene, and obtained from L. Marnett (Vanderbilt University) was seen in almost all samples from each tissue. Adducts 3 and 4, the identity of which have not yet been determined, were more variable. Under different dosing or treatment and chromatographic conditions, Ross *et al.* (40), show several adducts that were not seen in the current study. Although the labeling conditions are not necessarily identical in each case, the autoradiograms do suggest that the relative DNA binding in these tissues is skin ≫ lung > liver. Figs. 2–4, which show the time course of total BAP–DNA adduct accumulation with an increasing number of doses, corroborate the tissue distribution of these adducts. Following a single dose, the levels of BAP–DNA adducts were 125 adducts per 10^9 nucleotides in the skin.

The data given in these figures are for total adducts. Adduct 1 accounted for the majority of the total in every case. When individual adducts were analyzed, no significant differences were detected in the time-course of adduct loss (data not shown).

The rate of adduct accumulation with chronic topical exposure was tissue- and dose-specific. In the skin-a target tissue total BAP–DNA adducts appeared to increase linearly at the 10 and 25 nMol doses, whereas the accumulation of adducts at the highest dose appeared to plateau after an initial early increase. The maximum total BAP–DNA adduct level in the skin was approximately 200 adducts per 10^9 nucleotides, a level that was seen between 12 and 15 weeks of twice per week treatments. At lower-dose levels, adduct levels increased throughout the treatment period reaching respective zeniths of 61 and 144 adducts per 10^9 nucleotides for the 10 and 25 nMol per application treatments, respectively. When the adduct levels in skin at 15 weeks of treatment were normalized by dose, the efficacy of adduct formation decreased with increasing dose from 0.61 to 0.58 and 0.38 adduct per nMol for the 10, 25, and 50 nMol doses, respectively.

The increase in BAP–DNA adduct level in the lung also appeared to increase linearly at low doses, whereas at the high

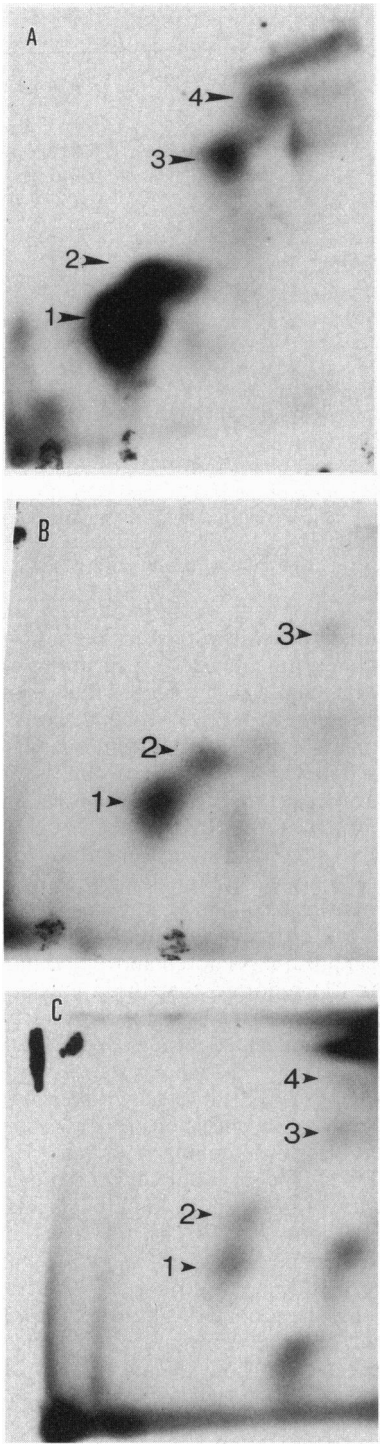

FIG. 1. Autoradiograms of [^{32}P]-postlabeled DNA from (*A*) skin, (*B*) lung, and (*C*) liver of animals treated topically with BAP. DNA was isolated, hydrolyzed, and [^{32}P]-postlabeled as described.

dose the adduct levels more rapidly increased with the increasing number of treatments. The maximal total BAP–DNA adduct levels in the lung were at about 20 adducts per 10^9 nucleotides at the 50 nMol treatment at 15 weeks. There was no clear dose-response in total BAP–DNA adduct levels because the levels at the 10 and 25 nMol doses were essentially the same (Fig. 3). At these lower levels of adducts it would be difficult to discern differences. There was a 3-fold increase in total BAP–DNA adduct levels between the 10 and 50 nMol doses, however, although the difference was not statistically significant.

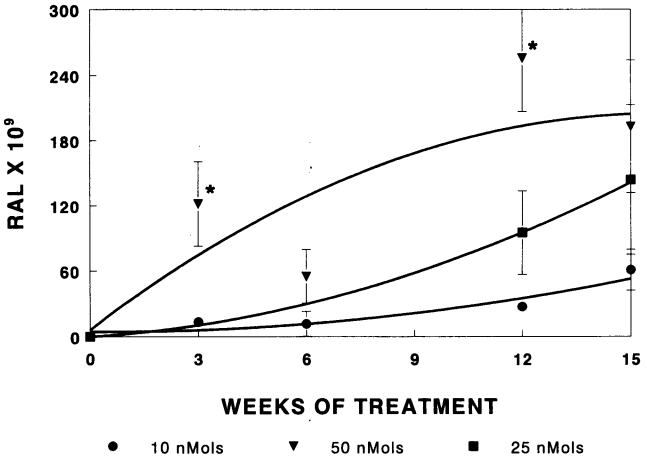

FIG. 2. Time course of total BAP–DNA adduct accumulation in the skin of mice treated topically twice weekly for as many as 15 weeks with either 10, 25, or 50 nMol of BAP in 50 μl of acetone. Animals were sacrificed 24 hr after the last treatment. Error bars indicate standard error of the mean. The curves were generated by using computer-generated second order polynomials. Asterisks indicate where there were statistically significant differences between the 50 nMol and 10 and 25 nMol doses.

The liver is not a target tissue for BAP carcinogenicity. As seen in Fig. 4, total BAP–DNA adduct levels in the liver were much lower in this tissue than in the skin and about one-half that of the lung. There was also no clear dose-response with adducts appearing to reach a maximum in the 25-nMol dose after 15 weeks, although the differences were not statistically significant due to the variability in these data and the low levels of adducts seen.

Because groups of animals were also sacrificed at intervals following the last dose after 1 and 30 treatments, the kinetics of adduct loss could be after acute and chronic exposure. Fig. 5 shows that adducts were rapidly lost following one treatment, whereas Fig. 6 indicates that the rate of adduct loss was much slower after 30 treatments.

DISCUSSION

The effects of chronic, low-level exposure of a common environmental carcinogen have been studied to determine the dose- response at different exposure levels, the effect on target organs under these exposure conditions, and the time course of adduct removal. These are questions basic to the application

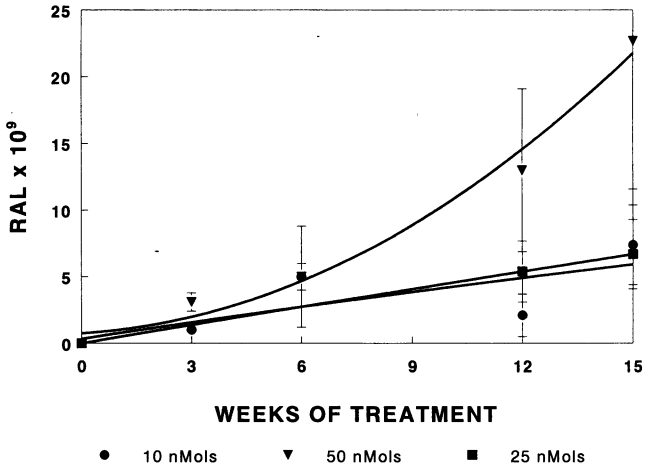

FIG. 3. Time course of total BAP–DNA adduct accumulation in the lungs of mice treated with BAP; details are a given in Fig. 2.

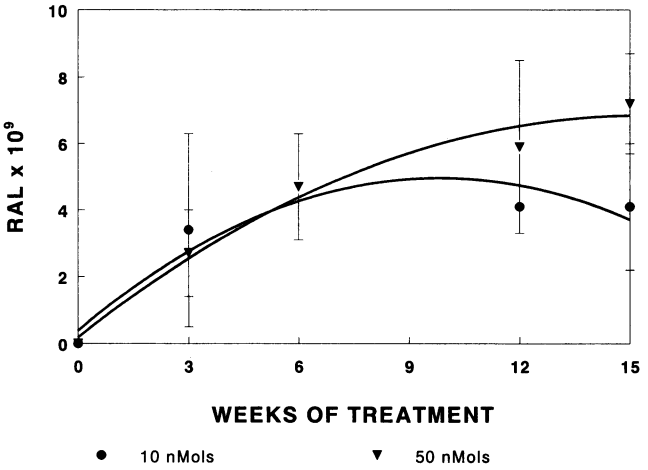

FIG. 4. Time course of total BAP–DNA adduct accumulation in the livers of mice treated with BAP; details are as given in Fig. 2.

of carcinogen–DNA adduct analysis to occupational and environmental exposures and to the understanding of carcinogenic initiation in exposed humans.

We report that BAP–DNA adducts are 10-fold higher in the primary target tissue, the skin, than in an equivocal target tissue, the lung, and one-half that of the lung in a nontarget tissue, the liver. Although adduct levels are lower in nontarget tissues, adducts are still present, so that with the appropriate stimulus, i.e., increased cell proliferation caused by toxic or physical injury, the potential exists for these adducts to be converted to mutations and initiate tumors (41). We did not examine nontarget tissues, such as heart and brain, following the chronic study. Although others have seen high levels of DNA damage in several of these tissues, heart in particular, they are in a nonproliferative state and thus not usually subject to mutation (42).

We report that the maximal level of adducts was only about twice as high following 30 twice weekly treatments with sacrifice 24 hr after the last treatment as when only one topical application was given with the same and the animals also sacrificed after 24 hr. There have been few studies comparing carcinogen–DNA adducts in acute and chronic exposure studies. However, we have also shown that in the skin following exposure to 4-aminobiphenyl or BAP, adduct levels were not directly proportionate with the increased numbers of treatments (43, 44).

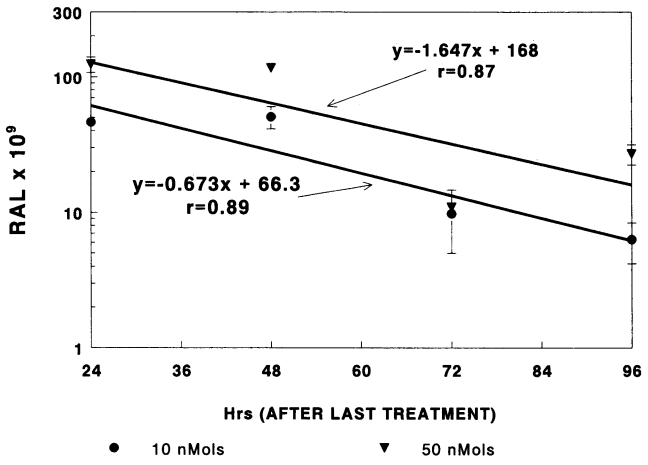

FIG. 5. Time course of the loss of total BAP–DNA adducts in the skin following one topical treatment with the indicated amount of BAP. The first animals were sacrificed 24 hr after the treatment. The equations for the slope of adduct loss are given for each treatment.

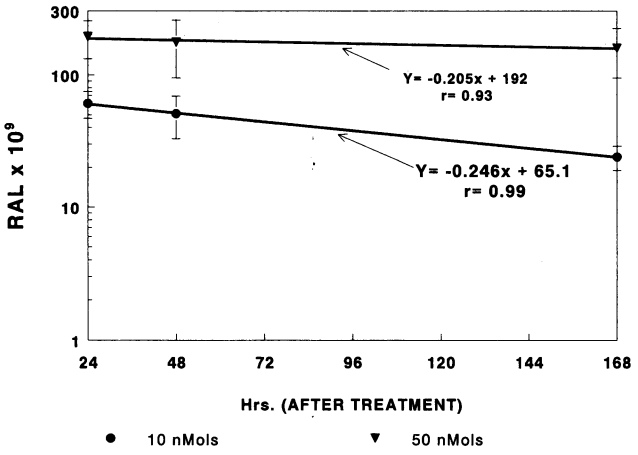

FIG. 6. Time course of the loss of total BAP–DNA adducts in the skin following 30 topical treatments (2 treatments per week) with the indicated amounts of BAP. The first animals were sacrificed 24 hr after the last treatment. The equations for the slope of adduct loss are given for each treatment.

Previous work by other investigators has indicated that there are different compartments of DNA regarding adduct formation and removal. Gupta (34) reported that certain restriction enzyme fragments of repetitive sequences on DNA were as much as 13 times more likely to form acetylaminofluorene adducts as was bulk DNA. They found that this was in spite of a slightly lower proportion of the target base, guanine, in the fragments as in bulk DNA. In fact, there was no correlation between percent guanine in any fragment (or total DNA) and the level of DNA adducts (34). The rate of adduct loss from these fragments was also significantly faster than from bulk DNA because the difference in adduct levels disappeared 9 days after the exposure. As noted above, Beland, Poirier, and coworkers (30, 31) reported that there appeared to be two compartments of DNA in the liver; in one adducts were formed twice as fast and removed 13 times as fast as in the other. Preliminary data that was generated using (±)-anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, and therefore controlling for metabolism of the compound, suggests that there are three compartments for BAP–DNA adducts in skin DNA. The slopes of adduct removal seen for the three phases are −640, −13.1, and −3.6 adducts per 10^9 nucleotides per hour, for 1–3 hr, 4–12 hr, and 12–36 hr, respectively (K. Dixon, K. Vasunia, and G.T., unpublished).

In the critical study reported here, comparable data were available for only the second and third phases. However, the slopes of adduct removal were similar: −7.6 and −1.3 adducts per 10^9 nucleotides per hour, 12–24 hr and 24–96 hr after treatment. The slope of the last phase is consistent with removal of adducts based on a cell half-life of about 20 days. These data indicate that there is a compartmentalization of damage and repair following exposure and that with chronic exposure an accumulation of adducts occurs in a repair resistant compartment. Thus, although we found that the maximal adduct levels in skin DNA following chronic exposure were only about twice as high as with acute exposure, the removal of the damage was much slower, suggesting that much more of the damage was resided in repair resistant compartments. Whether adducts in repair-resistant compartments are more or less important for carcinogenesis is still subject of debate (45). However, a recent study involving the formation of adenomas induced by PAHs in strain A/J mouse lung, as a function of time integrated DNA adduct levels, suggested that the formation and persistence of DNA adducts determined the carcinogenic potencies of PAHs (40). It seems plain that the interaction between DNA damage and cell proliferation is

critical for fixation of mutations in any tissue and the fact that a tissue such as skin or lung may be a target because critical damage is not repaired prior to cell replication. In the current study we report that the rate of adduct removal is 2.5–8 times faster following a single dose than when animals are exposed for 15 weeks. Our data indicate that chronic exposure produces peak adduct levels only twice as high as acute exposure. However, these adducts appear to be much more persistent, so that the integral of peak levels and adduct persistence would appear to be extremely important in making an accurate assessment of risk.

We thank Drs. Kathleen Dixon and Kersi Vasunia for use of unpublished data and helpful discussions. (−)-Anti-7S-8R,9R-trihydroxy-10S-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene adduct standard was kindly provided by Dr. Lawrence Marnett. This research was supported by U.S. Environmental Protection Agency Grant R 81-8088 to D.W. and Grant 1 P30 ES06096–04 from the National Institutes for Environmental Health Sciences.

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