Wide-Range Linear Dose-Response Curve for DNA Binding of Orally Administered Benzo(a)pyrene in Mice

Bruce P. Dunn

ABSTRACT

The binding of p.o. benzo(a)pyrene (BP) to the DNA of mice was investigated. With a single dose of 1 μg, levels of DNA binding were highest in the liver, followed by the intestine, colon, and stomach. In all organs, the majority of DNA-associated radioactivity was in the form of adducts which did not release ethyl acetate-soluble BP tetrols on acid hydrolysis. In both stomach and liver, the formation of acid-hydrolyzable and non-acid-hydrolyzable BP-DNA adducts was linearly related to dose, over a carcinogenic dosage range of 10^{-8} to 10^{-3} g (liver) or 10^{-7} to 10^{-3} g (stomach). Repair or removal via cell turnover of liver BP-DNA adducts over a period of 7 days proceeded with the same efficiency when the dose of the administered carcinogen was varied over a range of 100,000-fold. These results suggest that in vivo the initial interaction between DNA and ingested BP takes place in the same manner both at high doses typical of laboratory carcinogenesis experiments and at low doses typical of human exposure.

INTRODUCTION

There have been numerous reports of the presence of PAH in foods (8, 11). In general, these appear to be the result of either cooking practices which cause deposition of soot (e.g., charbroiling), preserving practices (e.g., smoking), or preexisting environmental contamination of foodstuffs. BP and other PAH, such as 7,12-dimethylbenz(e)anthracene, 3-methylcholanthrene, and dibenzo(a,h)-anthracene, have been shown to be carcinogenic when fed or administered i.g. (11). The most common tumors found include those of the lung, stomach, and mammary gland (11). However, there are only limited epidemiological data bearing on the human health risks of PAH in foods. Smoked foods have been linked with stomach cancer both in the Soviet Union (16) and in Iceland (4). These studies, although suggestive, lack epidemiological rigor.

Since foods containing PAH are often contaminated for identifiable and preventable reasons (8, 11), there is a possibility of regulating the levels of PAH in foods. For example, Germany has enacted a regulatory limit of 1 ng/g for BP in smoked foods in an attempt to reduce human consumption of carcinogens introduced by smoking as a food processing procedure (3). Such decisions on the regulation of carcinogens in foodstuffs depend critically on the perceived risk of these compounds to human health. One of the major problems in risk estimation is in determining the hazard of low doses of a carcinogen, such as are present in human foods, using animal carcinogenicity data obtained at high doses. Depending on the mathematical dose-response model used, the estimated risk of a given low dose of a carcinogen may vary by orders of magnitude (6). Models incorporating dose-response linearity at low doses give the highest estimates of risk.

The most frequently invoked mechanisms resulting in nonlinear dose-response curves for carcinogenesis involve differences in the pharmacology of the carcinogen at different doses (13). Of particular importance are the metabolic processes involved in the activation and detoxification of precarcinogens. Alterations in these metabolic steps by compounds such as antioxidants have been shown to alter the carcinogenicity of PAH (12). In cases where the DNA binding in vivo of PAH has been examined, such agents alter the degree of initial binding of carcinogens in parallel with the alteration of carcinogenicity (12). There is thus reason to believe that an examination of the dose dependency of the binding of PAH to DNA in vivo may be highly relevant to the problem of predicting the shape of the carcinogenic dose-response curve at low carcinogen doses. This communication examines the binding of p.o. BP to the DNA of target and nontarget organs of mice over a wide range of doses.

MATERIALS AND METHODS

Materials. [G-3H]BP (30 to 65 Ci/mmol) and [6-3H]BP (25 Ci/mmol) were purchased from the Amersham/Searle Corp. (Arlington Heights, Ill.). Nonradioactive BP and enzymes were from the Sigma Chemical Co., St. Louis, Mo. Radioactive and nonradioactive BP in corn oil were mixed to give the specific activity desired. All other chemicals were reagent or equivalent grade.

Animals. Mice were random-bred Swiss male mice weighing 30 to 35 g. Mice were maintained on a 12-hr light-dark cycle. For experiments, mice were starved for 6 hr before force-feeding, starting 1 hr into the light phase of their cycle.

Administration of Carcinogen. After starvation, mice were force-fed with BP in preservative-free corn oil (0.2 ml) by stomach intubation under light ether anesthesia. Animals were maintained for 1 hr without access to water or food and then maintained for another 17 hr with water but without food. Removal of food and bedding (which may be eaten in the absence of food) was done to standardize the stomach contents during the experimental period.

Isolation of DNA. Eighteen hr after force-feeding, animals were sacrificed by cervical dislocation and stomach, intestine (distal 10 cm adjacent to colon), colon, and liver samples were excised. Intestinal organs were opened and cleaned of food and fecal material by washing in ice-cold phosphate-buffered saline (140 mM NaCl and 10 mM K2HPO4, pH 7.0). Samples were immediately frozen in 5 ml of 1% sodium dodecyl sulfate/25 mM EDTA. For DNA isolation, samples in sodium dodecyl sulfate/EDTA were thawed and 100 units of RNase in 0.5 ml H2O were added. Samples were homogenized with a mechanical-sonic homogenizer (Brinkmann Polytron; Brinkmann Instruments, Inc., Rexdale, Ontario, Canada) and then incubated for 0.5 hr at 37°. Pronase (200 μg) was then added in a volume of 1 ml, and the samples were incubated...
for a further 1.5 hr. Samples were then extracted 3 times with an equal volume of phenol which had been previously equilibrated with 0.5 M Tris-HCl, pH 8.0. Supernatants were then transferred into a clean set of tubes, leaving behind any interface material, and were extracted 3 times with an equal volume of chloroform/isoamyl alcohol (24/1, v/v). Ethanol (2 volumes) containing 2% sodium acetate was added, and the DNA was precipitated at 4°C for 18 hr. The precipitate was then redissolved in 4.5 ml of 0.03 M sodium acetate in H2O, and the precipitation with ethanol was repeated. Purified DNA was dissolved in 0.03 M sodium acetate and measured spectrophotometrically at 260 nm. DNA prepared in this manner generally had a 260 nm/280 nm absorbance ratio of greater than 2.0.

Acid Hydrolysis of DNA and Ethyl Acetate Extraction. An equal volume of 1 M HCl was added to DNA samples in 0.03 M sodium acetate, and the DNA was heated at 85°C for 1 hr. These conditions have been reported to result in the hydrolysis of BP-guanine adducts and release of the BP moiety of the adducts as ethyl acetate-extractable BP tetrols (10). The acid digest was extracted twice with 1.5 volumes of ethyl acetate, and radioactivity in the ethyl acetate and acid phases was determined by scintillation counting in ACS water-miscible scintillation fluid (Amersham Corp.). Samples and backgrounds were routinely counted for 50 min in a Searle Delta 300 scintillation counter (Searle Analytic, Des Plains, Ill.). Counting efficiency was determined for each sample by the use of a 133Ba external standard. Radioactivity was expressed as mol of BP bound per g of DNA, assuming no loss of tritium label from the BP.

Enzyme Hydrolysis of DNA and Sephadex LH-20 Chromatography. In one experiment, aliquots of DNA were enzymatically hydrolyzed to deoxyribonucleosides by sequential digestion with DNase I, phosphodiesterase, and alkaline phosphatase (2). The digest was then chromatographed on a small column of Sephadex LH-20, eluted first with water to remove nucleosides and hydrophilic nucleoside-carcinogen adducts and then with methanol to remove hydrophobic nucleoside-carcinogen adducts. Aliquots of the eluates were analyzed for radioactivity by scintillation counting.

RESULTS

An experiment designed to explore the degree and nature of the covalent binding of p.o. BP to the DNA of various organs in the mouse was performed. Groups of 18 mice were fed 1 μg of either [G-3H]BP or [6-3H]BP dissolved, without the addition of nonradioactive carcinogen, in 0.2 ml of preservative-free corn oil. After 18 hr, a time shown in preliminary experiments to give near-maximal binding of BP to stomach DNA, the animals were sacrificed, and the stomach, intestine (distal 10 cm adjacent to the colon), colon, and most ventral lobe of the liver were excised. The DNA from each individual organ sample was isolated and purified separately (see "Materials and Methods"). In addition, the remaining lobes of the liver from each animal were excised and pooled in 2 groups corresponding to the 2 forms of isotopically labeled BP used. DNA from these pooled samples was isolated using a scaled-up version of the procedures described in "Materials and Methods."

After measurement of the amount of DNA by its absorbance at 260 nm, DNA from the individual samples and an aliquot of DNA from the pooled liver samples were hydrolyzed with acid, as described in "Materials and Methods." This procedure hydrolyzes the BP adducts formed by the reaction of benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide with DNA bases, releasing BP tetrols (10). BP derivatives released by this hydrolysis were extracted into ethyl acetate and determined by scintillation counting. BP-DNA adducts releasing ethyl acetate-soluble radioactivity during acid hydrolysis will be referred to in this paper as "hydrolyzable" adducts. Radioactivity remaining in the acid phase after hydrolysis and extraction with ethyl acetate was also measured and will be referred to as representing "nonhydrolyzable" adducts.

For comparison purposes, aliquots of the DNA from the pooled liver samples were analyzed by a separate procedure capable of distinguishing BP-base adducts from other forms of DNA-associated radioactivity. DNA samples were enzymatically degraded to nucleosides using the procedures of Baird and Brookes (2). Unlike acid hydrolysis, this procedure does not disrupt the covalent link between BP and DNA bases. BP nucleoside adducts are markedly more hydrophobic than the nonreacted nucleosides and were separated from them by chromatography on Sephadex LH-20 (see "Materials and Methods"). Reaction products such as that between benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide and guanosine are "hydrolyzable" adducts by the acid hydrolysis-ethyl acetate extraction procedure (above) and hydrophobic adducts by the enzyme hydrolysis-Sephadex LH-20 procedure.

Table 1 indicates the covalent binding of BP to DNA as determined by acid hydrolysis and ethyl acetate extraction. There is generally a close agreement between the amount of binding measured using [G-3H]BP and [6-3H]BP. The level of binding was lowest in the stomach and highest in the intestine and liver. In all organs, acid-hydrolyzable adducts represented only a minor fraction of the total amount of radioactivity associated with DNA, namely, 15 to 20% in liver and colon samples and 25 to 30% in stomach and intestine samples (Table 1, Columns 6 and 7). This result does not appear to arise from incomplete acid hydrolysis of the DNA samples since, in separate experiments, prolonging the time of hydrolysis did not increase the yield of ethyl acetate-extractable radioactivity. Quantitatively similar results were obtained when DNA from the pooled liver samples was analyzed by the enzyme hydrolysis-Sephadex LH-20 procedure. For the

Table 1

<table>
<thead>
<tr>
<th>Organ</th>
<th>Hydrolyzable</th>
<th>Nonhydrolyzable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1.84 ± 1.15</td>
<td>2.04 ± 2.10</td>
</tr>
<tr>
<td>Intestine</td>
<td>7.97 ± 3.17</td>
<td>7.42 ± 2.97</td>
</tr>
<tr>
<td>Colon</td>
<td>2.81 ± 1.30</td>
<td>2.30 ± 1.15</td>
</tr>
<tr>
<td>Liver, ventral lobe</td>
<td>4.96 ± 1.38</td>
<td>5.71 ± 2.14</td>
</tr>
<tr>
<td>Liver, remainder pooled</td>
<td>11.1 ± 4.44</td>
<td>10.4 ± 5.3</td>
</tr>
</tbody>
</table>

a Mean ± S.D. for groups of 18 animals.

b Single determination on a sample pooled from the nonventral lobes of the livers of 18 animals.

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DNA from animals treated with [G-3H]BP, acid hydrolysis-ethyl acetate extraction indicated 15.8% hydrolyzable adducts, while enzyme hydrolysis-Sephadex LH-20 chromatography of the same DNA indicated 14.3% hydrophobic adducts. Corresponding figures for the DNA from animals treated with [6-3H]BP were 19.4 and 19.2%, respectively. Although the relative proportions of hydrolyzable and nonhydrolyzable adducts were similar in the individual and the pooled liver samples, the total binding was substantially higher in the pooled sample. This may represent lower binding in the ventral lobe of the liver (used for individual samples), compared with the rest of the liver, which was utilized to prepare the pooled sample.

The binding of BP to DNA over a wide range of administered doses was investigated, using acid hydrolysis and ethyl acetate extraction to distinguish hydrolyzable from nonhydrolyzable BP-DNA adducts. Chart 1 shows the relationship between the administered carcinogen dose and the amount of hydrolyzable (Chart 1A) and nonhydrolyzable (Chart 1B) adducts found in stomach DNA. The amount of both hydrolyzable and nonhydrolyzable adducts formed was linearly related to dose over a range of $10^{-7}$ to $10^{-3}$ g. Over the entire dose range, the relative percentage of hydrolyzable adducts remained relatively constant, ranging from approximately 25 to 30% (Chart 1C).

Similar results were obtained from liver samples taken from the same series of experimental animals (Chart 2). In this case, because of the higher level of binding of BP to DNA and the larger amounts of DNA which could be isolated from liver relative to stomach, it was possible to extend the experiment to measure binding at an administered dose of as little as $10^{-8}$ g. Over a dose range of 100,000, the formation of hydrolyzable (Chart 2A) and nonhydrolyzable (Chart 2B) BP-DNA adducts was linearly related to the amount of ingested carcinogen. In contrast to the data from stomach DNA, the relative proportion of hydrolyzable adducts was not constant with dose, ranging from a minimum of 17% at the lowest dose to a high of 27% at $10^{-3}$ g of administered BP.

In a parallel experiment, a second group of animals was fed $10^{-8}$ to $10^{-3}$ g of BP at the same time as the animals used in the experiment shown in Charts 1 and 2. These animals were maintained for 1 week prior to sacrifice in order to examine the lifetime in vivo of the BP-DNA adducts. After 1 week of recovery time, no detectable radioactivity was found in either the hydrolyzable or nonhydrolyzable fraction of stomach DNA. Given the levels of radioactivity found in the animals at 18 hr and assuming a minimum detectable amount of radioactivity as being 10 dpm, this implies that the amount of hydrolyzable adducts had declined to less than 20% of the original value and that the amount of nonhydrolyzable adducts had declined to less than 5% of the original value. In the liver, however, significant amounts of hydrolyzable and nonhydrolyzable adducts remained after a 1-week repair period. Chart 3 shows the percentage of hydrolyzable and nonhydrolyzable adducts remaining after a 7-day repair period. Considerable variability was observed, but it is evident that, at all doses, the removal of hydrolyzable adducts was more efficient than that of nonhydrolyzable adducts. The efficiency of repair in the liver did not depend on the original dose of carcinogen over a range of 100,000-fold in dose. In comparison with the stomach, repair or removal via cell turnover of nonhydrolyzable BP-DNA adducts appeared to proceed more slowly. Thus, after 7 days, less than 5% of the nonhydrolyzable adducts present at 18 hr in stomach DNA persisted, while approximately 20 to 40% of the nonhydrolyzable adducts present in liver DNA at 18 hr persisted.

**DISCUSSION**

The use of high-specific-activity radioactively labeled carcinogens allows the binding of carcinogens to DNA to be investigated.
Chart 2. Binding of [G-3H]BP to the DNA of mouse liver. Data are the means for groups of 9 or 10 animals per point which were given either 10 ng of [G-3H]BP (dose of 10^-4 g) or 100 ng [G-3H]BP with or without the addition of nonradioactive BP to bring the total administered doses to those indicated. Bars, S.D. The apparent asymmetry of the bars is a result of the logarithmic scale used.

at very low doses. Amounts of carcinogen in the order of 10^-15 mol or less can be routinely measured in mg or larger samples of DNA. This sensitivity of 10^-12 mol of carcinogen per g of DNA (based on a 1-mg DNA sample) is appreciably better than the sensitivity of ultrasensitive enzymatic radioimmunoassay for BP adducts in DNA. The latter procedure, although it has the advantage of not needing a radioactively labeled carcinogen and of measuring one specific class of DNA adducts, is limited to a sensitivity of approximately 25 fmol of adducts in a sample of 25 μg of DNA or 10^-9 mol of carcinogen per g of DNA (9). The use of acid hydrolysis followed by ethyl acetate extraction to distinguish between hydrolyzable and nonhydrolyzable DNA adducts does not give as much information on the nature of the adducts as does the more commonly used enzyme hydrolysis-Sephadex LH-20 chromatography procedure. However, it has the advantage of speed, simplicity and, because of the lack of chromatography, sensitivity.

Reaction products between benzo(a)pyrene-diol epoxides and DNA bases have been implicated as major adducts formed when cells are exposed to BP. In the current experiments, most of the radioactivity associated with DNA in 4 different organs of mice does not appear to be in the form of such adducts, which release ethyl acetate-extractable BP tetrols on acid hydrolysis. There was insufficient radioactivity associated with DNA in the current experiments to properly explore the relationship between these "nonhydrolyzable" adducts from acid hydrolysis and the "hydrophilic" adducts from the more commonly used enzyme hydrolysis procedure. It seems likely, however, that these 2 procedures are measuring the same or closely related products. Although they are commonly ignored or eliminated by preanalysis purification procedures, hydrophilic PAH-DNA adducts are frequently reported in experiments in which DNA is degraded enzymatically. Currently, there is little or no evidence as to the nature of these adducts, although it has been speculated that they may represent noncarcinogenic lesions (5).

It seems unlikely that more than a small fraction of the DNA-associated radioactivity resistant to acid hydrolysis results simply from the exchange of tritium onto normal nucleosides. (a) There is no substantial or consistent difference in the amount or proportion of "nonhydrolyzable" adducts when a different form of tritiated BP ([6-3H]BP) is substituted for the more commonly used generally labeled material (Table 1). For exchange reactions, such differences would be expected, due to the differing degrees of lability of the tritium at different sites on the molecule. (b) The bulk of nonhydrolyzable radioactivity in liver DNA is removed during a 7-day repair period in the animal. This suggests an active repair process in living cells, which would not be expected to exist for radioactivity on normal DNA nucleosides. Finally, hydrophilic adducts have been reported from experiments utilizing [14C]BP, where there is no possibility of tritium exchange (14).

BP-DNA adducts were found not only in the stomach, a target...
organ, but also in the liver, intestine, and colon. Levels of both hydrolyzable and nonhydrolyzable adducts were higher in non-target organs than in the stomach, indicating the importance of factors other than the total amount of initial carcinogen bound to DNA in determining the susceptibility of an organ to carcinogenesis. However, it may be noted that, although the liver is not normally a target organ for BP carcinogenesis, a single treatment with BP has been reported to induce putative precancerous lesions in the livers of rats subjected to a partial hepatectomy (7).

Typical levels of BP in various foods range from 0.1 to 10 ng/g (8, 11). For a mouse consuming 5 g of food per day, this corresponds to a daily dose of 0.5 to 50 ng of this carcinogen and a total dose of PAH 1 to 2 orders of magnitude higher than these levels. Thus, the lowest doses of BP used in the current study, 10 and 100 ng, approximate levels of PAH which might be encountered as food contaminants. The highest dose of carcinogen used, 1 mg, is somewhat greater than the lowest dose which has been reported to induce stomach tumors in mice (0.2 mg) (15). The data on BP binding to DNA thus span the range from those doses typical of foods to those doses which are capable of inducing tumors in a substantial proportion of treated animals. Over this entire range of 100,000-fold (liver) or 10,000-fold (stomach), the degree of binding of p.o. administered BP is directly related to dose. There is evidence neither of a threshold effect nor of increased binding at high doses due to induction of metabolizing enzymes. In addition to the relative linearity of the dose-response curves, the relative proportion of hydrolyzable and nonhydrolyzable adducts changes either only modestly (liver) or not at all (stomach) as the dose is varied. These data are similar to those found by Pereira et al. (14), who obtained a linear dose-response relationship over a dosage range of 30,000-fold for the binding of topically applied BP to mouse epidermal DNA. Linearity for the dose-response relationship for binding of a carcinogen to DNA was also observed by Appleton et al. (1), who investigated the hepatic macromolecular binding of aflatoxin at low doses over a dosage range of 100-fold (1).

The results of this study and those of Pereira et al. indicate that the degree of initial DNA binding to BP to the DNA of target organs in vivo is directly proportional to dose, even at very low doses typical of those which might be encountered by humans as environmental contaminants. If there is a direct relation between carcinogen binding and tumor production in target organs, as is suggested by a growing body of data (12), then the carcinogenic risk of PAH is also likely to be directly proportional to dose. This in turn suggests that, in the absence of other information, linear nonthreshold dose-response models may be the most appropriate, conservative choice for estimating the risks of PAH in human foods.

REFERENCES

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