ABSTRACT

Previous studies have shown that the carcinogenic metabolite of benzo(a)pyrene [B(a)P], B(a)P-7,8-diol-9,10-epoxide (BPDE), is transported in serum after B(a)P injection in mice. It is possible that serum transport is an important source of carcinogenic metabolite and results in DNA adduction formation in tissues. This possibility was studied by comparing the time course for BPDE appearance in serum with that for BPDE/DNA adduct formation in tissues. BPDE serum levels and DNA adduct levels were measured by 32P-postlabeling analysis. Results indicate that, after a 200-mg B(a)P/kg i.p. injection, BPDE/DNA adduct levels rose sharply in liver, lung, kidney, stomach, and spleen through 5 h and then more gradually through 24 h. Adduct levels were similar in all tissues at 24 h. BPDE levels in serum reached a plateau within 2.5 h and remained constant thereafter (10 to 11 nM). B(a)P levels in serum fell steadily from 1980 nmol at 1 h to 350 nmol by 24 h. Levels of serum BPDE and DNA adducts showed a similar dose dependency at 10- and 100-fold lower B(a)P i.p. doses. After BPDE i.v. injection, BPDE levels in serum decreased to 0.16% of the initial level within 5 min. By this time, BPDE/DNA adducts were at peak levels in all tissues assayed. Lung adduct levels were 10 to 100 times greater than those in the other tissues. These results support a role for serum transport of BPDE in the production of DNA adducts after B(a)P since BPDE was available in serum throughout the time course for DNA adduction formation. Further, injected BPDE rapidly formed DNA adducts and this occurred primarily in the lung, which had the greatest access to the transported carcinogen.

INTRODUCTION

The PAH, B(a)P, is a potent mutagen and carcinogen in animals and is widely distributed in the human environment (1–3). Its carcinogenic effects depend upon host metabolic activation to produce electrophilic metabolites capable of forming DNA adducts (4–6). The major adducting metabolite formed in a variety of animal test systems is BPDE (4–6). DNA adducts resulting from BPDE and/or related metabolites have also been detected in the peripheral blood lymphocytes of workers occupationally exposed to PAH (7–10). Since there are competing pathways for the activation and detoxification of B(a)P, it is generally viewed that the cellular balance between these pathways determines the extent of genetic damage (4–6, 11). However, tissues deficient in B(a)P activation (e.g., brain, muscle, spleen) contain levels of BPDE/DNA adducts that are similar to those in metabolically competent tissues (e.g., liver) (11–14). One explanation is that cells which activate B(a)P can secrete BPDE into blood where it is stabilized and transported to distal tissues. Thus, cells which are unable to form BPDE might still be at risk due to exposure via the systemic circulation. This hypothesis is supported by evidence that in vitro, serum components can sequester BPDE, prevent its hydrolysis, and transfer BPDE to target cells in which it forms DNA adducts (12, 15, 16). Further, BPDE can be transported out of the cells in which it is formed to adduct DNA in neighboring cells (17) or purified DNA in the medium (18).

We have previously shown that BPDE is detectable in serum 4 h after an i.p. dose of B(a)P in mice (12). Detection was based upon BPDE trapping with spDNA to form DNA adducts followed by 32P-postlabeling quantitation of the adducts. The amount of BPDE in serum was greater than the total bound to lung DNA, suggesting that serum might be an important source of adducting metabolite. However, the ability of cells in vivo to take up BPDE from serum and form DNA adducts is unknown. The present studies were designed to further evaluate the importance of serum transport of BPDE in the production of DNA adducts. We gave mice injections of BPDE i.v. to assess the uptake of BPDE into cellular DNA. Additionally, we injected B(a)P i.p. to compare the time course for BPDE appearance in serum with that for BPDE/DNA adduct formation. Results indicate that, after B(a)P injection, BPDE reaches peak levels in serum prior to the peak in DNA adduct formation. After BPDE injection, BPDE removal from serum and uptake into tissue DNA were very rapid. These findings provide additional evidence that circulating BPDE may be an important source of adducting metabolite after B(a)P treatment.

MATERIALS AND METHODS

Animal Treatments. Female C57BL/6 × C3H F1 mice, (hereafter called B6C3F1), mice, 3 to 4 mo of age (22 to 28 g), were used throughout these studies. Groups of mice received a single i.p. injection of B(a)P (Sigma) at a dose of 2, 20, or 200 mg/kg or vehicle (corn oil) in a volume of 10 ml/kg. Other mice received a single i.v. (tail vein) injection of ±-anti-BPDE (NCI Chemical Carcinogen Repository) diluted in B6C3F1 mouse serum. BPDE was dissolved in absolute ethanol and stored at −20°C for up to 2 mo before use. The integrity of BPDE stock solutions was assessed by the alkaline β-mercaptoethanol/high-performance liquid chromatography technique of Michaud et al. (19). For BPDE administration to mice, 5 μl of the BPDE ethanol stock were added to 150 μl of mouse serum (on ice) to deliver 8.25 nmol of BPDE per mouse. The dose was used immediately after preparation. At selected times after injection, mice were bled by retroorbital puncture under ether anesthesia. Livers, lungs, kidneys, spleens, and, in certain experiments, stomachs were excised, rinsed, and stored frozen (−20°C) until DNA extraction.

BPDE Levels in Serum. Serum levels of BPDE were measured as described previously (12) by trapping the reactive metabolite with spDNA, followed by 32P-postlabeling analysis of the resulting DNA adducts. Immediately after mice were killed, blood was centrifuged for 2 min at 16,000 × g in a microfuge (Eppendorf), the serum was collected, and 0.2 ml were added in duplicate to 100 μg of spDNA.
SERUM BPDE AND TISSUE DNA ADDUCTS

A standard curve was assayed in parallel by adding 1 to 10 ng of BPDE (5 µl of ethanol stocks) to 0.2 ml of control mouse serum, followed by the addition of 100 ng of spDNA (12). The DNA was repurified after incubation for 2 h at 37°C on a shaking platform.

DNA Isolation. DNA was isolated from tissues, and spDNA was repurified after incubation with serum as described previously (12, 20) except that samples were incubated with RNase A (0.15 mg/ml; 37°C for 1 h) immediately after protease K treatment (0.25 mg/ml; 55°C for 16 h), and all solvent extractions followed the enzyme treatments.

32P-Postlabeling Analysis. DNA (5 µg) digestion to 3'-monophosphate nucleotides, adduct enrichment by butyl alcohol extraction, PEI thin-layer chromatography of postlabeled adducts, and quantitation of adduct levels were adapted from Gupta (21) as previously described (12). The only exception was that, after D1 development onto a wick (overnight), the origin area was contact transferred to a new PEI sheet (5 cm x 4 cm) according to Yu et al. (14). This avoided flaking of the PEI from its plastic backing which otherwise occurred with these thin-layer plates (Merck) after sequential development in D1 and then D2 buffers. Background radioactivity was consistently seen near the center of autoradiograms (e.g., Fig. 1b) and usually appeared flattened, in contrast to adduct spots which were nearly round. This region of background radioactivity was well separated from the BPDE adduct (Fig. 1A, Spot 3), but did overlap with adduct Spot 2 (e.g., Fig. 1c).

Since this background region was absent when mock digests (no DNA) were postlabeled, it is possible that it represents incompletely digested oligonucleotides. Other background spots (e.g., Fig. 1g, bottom) were present infrequently and did not interfere with adduct analyses. To test whether the chromatographic properties of adducts found in different DNA preparations were the same, 2.5 µg of the test DNAs were mixed, digested, postlabeled, and cochromatographed. Adduct separation utilized the standard solvent system, or one in which the D1 buffer was replaced by 2-propanol/4 N NH₄OH (14, 22).

B(a)P Serum Levels. B(a)P was recovered from 175 µl of serum by extraction with ethyl acetate (0.5 ml, 3 extractions). After evaporation of ethyl acetate, the residue was dissolved in 150 µl of methanol for high-performance liquid chromatography analysis (Vydac C₁₈, 5-µm diameter, 4.6 mm x 25 cm). B(a)P was eluted in 100% methanol with a retention time of 8.5 to 9.5 min as measured by UV (254 nm) detection.

Statistical Analysis. To compare BPDE/DNA adduct levels among tissues (Tables 1 and 2), adduct levels were subjected to one-way analysis of variance. Where F values were significant (P < 0.05), the Studentized Range test was used for specific comparisons (23).

RESULTS

32P-Postlabeling analysis was used to measure B(a)P metabolite/DNA adduct levels in liver, lung, spleen, and stomach and to measure BPDE levels in serum (Fig. 1). Consistent with our previous results (12), B(a)P i.p. injection (200 mg/kg) resulted in three B(a)P metabolite/DNA adducts in mouse tissues. The adducts, labeled J, 2, and 3 in Fig. 1c, were not seen in the tissues of vehicle-dosed mice (e.g., Fig. 1b). Cochromatography studies (data not shown) demonstrated that adduct Spot 3 cochromatographed with the adduct produced by the in vitro reaction of (±)-anti-BPDE with calf thymus DNA (Fig. 1a). The Cherenkov counts of this adduct spot were used to quantify the level of BPDE/DNA adducts. Adduct Spots 1 and 2 are presently unidentified but appear to result from B(a)P metabolites other than BPDE. Adduct Spot 2 was difficult to quantify because of background radioactivity in the zone to which it migrated. However, a crude estimate of its levels after subtraction of background from control autoradiograms indicated that it was present at approximately one-fifth the level of Adduct 3, similar to our previous report. This ratio did not appear to change over time. Adduct 1 was present in all tissues assayed except for the spleen, and appeared to be present at slightly lower levels than Adduct 2.

The tissue distribution of BPDE/DNA adducts 1 to 24 h after i.p. injection of 200 mg of B(a)P/kg is shown in Fig. 2. Adduct levels rose sharply in all tissues through 5 h, followed by a more gradual increase through 24 h. Between 1 and 5 h, levels in the kidney and stomach were one-third to one-half the levels in the spleen, liver, and lung. In a previous study a similar adduct distribution was seen 4 h after B(a)P with levels in the stomach and kidney significantly below those in liver (12). As seen in Fig. 2, this difference in adduct levels was no longer apparent at 24 h, which is again consistent with our previous results (24).

DNA-adducting metabolites of B(a)P present in serum were quantitated by 32P-postlabeling analysis after trapping the metabolites with spDNA. After B(a)P injection, two adding metabolites were detected in mouse serum (Fig. 1d). The adducts formed from these metabolites cochromatographed with adduct Spots 2 and 3 from tissue DNA (e.g., Fig. 1c). The major adding metabolite in serum appears to be BPDE based upon the chromatographic behavior of the resulting DNA adduct (Spot 3). Fig. 3 compares BPDE and B(a)P levels in serum at various times after injection of 200 mg of B(a)P/kg. BPDE levels reached a plateau within 2.5 h and then remained constant through 24 h (10 to 11 nm). In contrast, B(a)P serum levels declined from 1980 nm at 1 h to approximately one-sixth this level by 24 h.

The dose response for BPDE appearance in serum and DNA adduct formation after B(a)P i.p. injection are shown in Table 1. A decrease in B(a)P dose from 200 mg/kg (Figs. 1 and 2) to 20 mg/kg (Table 1) resulted in an approximately 10-fold reduction in both the BPDE level in serum and BPDE/DNA adduct levels in tissues at 24 h. An exception to this was the adduct level in the spleen, which declined by a factor of only 3 compared to the level after 200 mg/kg. At both 2 and 20 mg/kg, the 24-h splenic DNA adduct levels were significantly above those in liver and kidney, and at 2 mg/kg, the splenic adduct level was also above that in the lung. The decrease in dose from 20 to 2 mg/kg (Table 1) resulted in similar declines in DNA adduct and serum BPDE levels (4- to 8-fold). The time course for these effects was consistent at the three BaP doses tested. Between 2.5 and 24 h adduct levels rose 5- to 10-fold in most tissues while serum levels of BPDE remained constant (2 and 200 mg/kg) or increased 2-fold (20 mg/kg).

The above studies demonstrated that BPDE is present in serum throughout the time course for DNA adduction at all doses tested. To determine whether circulating BPDE can actually enter tissues to form DNA adducts, BPDE was injected i.v., and the levels of BPDE in serum and of BPDE/DNA adducts in tissues were determined. Based upon the amount of BPDE injected and an approximate blood volume of 1.5 ml (25), the theoretical time zero serum concentration of BPDE was 5.5 µM. Table 2 shows that, within 5 min of injection, the BPDE serum level was approximately 3 orders of magnitude below this theoretical initial value. At 30 min, BPDE levels in serum were near the limit of detection of the assay system (≥0.2 nm), but BPDE was still detectable in serum at 60 min. Representative autoradiograms (Fig. 1, g and h) show the decline in serum levels of adducting metabolite over time. DNA adduct levels peaked within 5 min of the i.v. injection (Table 2), and the levels in each tissue were constant between 5 and 60 min. Lung DNA adduct levels were 10 to 100 times greater than those in liver, kidney, or spleen (P < 0.05). This difference is exemplified in Fig. 1, e and f.
DISCUSSION

Previous studies from this laboratory (12) as well as others (16, 26, 27) have demonstrated that BPDE can enter cultured cells to form DNA adducts and that this process is facilitated by serum components (12, 16). These findings take on added importance in light of the current evidence that BPDE is present in serum for extended periods after B(a)P injection (Table 1; Fig. 3). Thus, it is possible that circulating BPDE is a major source of the DNA-adducting metabolite, especially in tissues such as kidney and spleen which do not appear to be competent in B(a)P activation (24, 28). The importance of BPDE uptake from serum into tissue DNA is supported by the evidence (Table 2) that, when injected i.v., BPDE is removed from serum and forms DNA adducts extremely rapidly. Within 5 min of the i.v. injection, only 0.16% of the injected BPDE remained in serum. This is faster than the rate of B(a)P removal from serum after i.v. injection, although the rate for B(a)P is also quite rapid (29, 30). The speed with which circulating BPDE adds cellular DNA is demonstrated by the fact that peak adduct levels occurred within 5 min of the BPDE i.v. dose (Table 2). The percentage of BPDE injected that was recovered as DNA adducts was small (approximately 0.02%). However, the rapid rate of DNA adduction suggests that after B(a)P dosing, a fraction of the circulating BPDE may continuously adduct DNA leading to an accumulation of adducts over time. The fate of the BPDE in serum which did not form DNA adducts is unknown, but previous in vitro experiments suggest that hydrolysis to tetrads would only be a minor pathway (12). BPDE
may also be removed through covalent binding to proteins in blood (7) or tissues (31), or via enzymatic detoxification systems (32).

In establishing the BPDE i.v. model to study the fate of circulating BPDE, we assumed that once injected, this metabolite will behave similarly to that formed endogenously from B(a)P. This assumption is based upon our previous studies (12) which showed that, when BPDE is added to B6C3F1 mouse serum at a high concentration (66 μM), the BPDE is completely sequestered by serum components. In this form BPDE is stabilized, but can be released from serum components in the presence of cells or spDNA to form DNA adducts. Most importantly, BPDE added to control serum exhibited a similar ability to adduct both spDNA and cellular DNA as did BPDE present in serum as a result of in vivo metabolism of B(a)P. This was true even though the BPDE level was 50-fold lower in the serum from B(a)P-dosed mice than in the serum that was spiked in vitro. Thus, it is reasonable to assume that the i.v. administration of BPDE (preloaded into serum) resulted in its partitioning into serum components and subsequent distribution into tissues, in a manner similar to that for BPDE formed endogenously. However, the actual rate and efficiency of DNA adduct formation from circulating BPDE probably differed between the two protocols. This is because the i.v. studies used a large bolus dose, whereas metabolism of B(a)P after i.p. injection leads to lower, but much more prolonged steady-state levels of BPDE.

The B(a)P i.p. studies also support the hypothesis that serum is an important source of adducting metabolite. After injection of 200 mg of B(a)P/kg, BPDE rapidly attained peak levels in serum (Fig. 3, 2.5 h), and this occurred during the phase in which BPDE/DNA adduct levels were sharply rising (Fig. 2, 1 to 5 h). BPDE levels in serum were constant beyond 2.5 h, which corresponded to a continued, but more gradual rise in BPDE/DNA adduct formation (5 to 24 h). Thus, BPDE was available in serum throughout the time course for DNA adduct formation and was at peak levels prior to the peak in adduct formation. These kinetics appear to hold true for 10- and 100-fold lower doses of B(a)P (Table 1), although additional data are needed to determine the time at which DNA adduct levels plateau at the lower doses. The apparent plateau in adduct levels after 200 mg/kg in spite of continued availability of BPDE in serum, suggests that DNA repair may have offset continued adduction. Mammalian cells contain inducible DNA repair systems to remove alkylation damage (33) and possibly also bulky adducts (34). The much faster removal of aflatoxin B1 or B(a)P adducts at high compared to low adduct levels suggests that an inducible repair system is activated at high levels of bulky adduct (11, 34–38). Since DNA adduction was extensive by 5 h after the 200-mg/kg injection (Fig. 2), it is feasible that an inducible DNA repair system was activated which placed a limit on adduct levels. However, other factors, such as a limitation in the number of available sites in DNA or an induction of BPDE detoxification systems, may account for the plateau in adduct levels.

The data in Fig. 3 demonstrate that BPDE remains at peak levels in serum through 24 h following the 200-mg B(a)P/kg dose, in spite of its rapid removal from serum after BPDE i.v. injection (Table 2). The steady-state in serum after B(a)P is probably maintained by continued metabolic conversion of B(a)P to BPDE within tissues. This view is supported by pharmacokinetic studies showing that tissue levels of B(a)P are still considerable 24 or more hours after injection (39, 40). As suggested elsewhere (41), the continuous metabolic activation of B(a)P over a prolonged period makes an accurate assessment of DNA repair impossible. The rate of adduct removal would be underestimated due to continued adduct formation. Thus, BPDE serum levels need to be monitored for extended periods after a single B(a)P dose in order to determine the time at which DNA repair can be accurately measured. However, injec-

![Graph](image)

**Fig. 3.** Serum concentrations of B(a)P and BPDE over time after i.p. injection of B(a)P. Groups of 2 or 4 mice were dosed with 200 mg of B(a)P/kg and killed at the indicated times. BPDE levels in serum were measured by incubating serum with spDNA and quantitating the resulting BPDE/DNA adducts by 32P-postlabeling analysis. Adduct levels were converted to serum concentration by a standard curve of BPDE-spiked serum incubated with spDNA. B(a)P levels in serum were measured by high-performance liquid chromatography analysis. BPDE and B(a)P assays were performed on serum from different mice, and each serum sample was assayed in duplicate. Points, mean; bars, SE (n = 4). Where no error bars appear, n = 2.

<table>
<thead>
<tr>
<th>B(a)P (mg/kg)</th>
<th>No. of h after injection</th>
<th>BPDE (nmol/liter of serum)</th>
<th>DNA adducts (fmol/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0.32 ± 0.13</td>
<td>17.1 ± 5.3</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.34 ± 0.11</td>
<td>43.0 ± 3.2</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
<td>0.67 ± 0.16</td>
<td>71.6 ± 19</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>1.26 ± 0.07</td>
<td>363 ± 65</td>
</tr>
</tbody>
</table>

| a | Mean ± SEM (n = 3 to 5 mice/group). |
| b | Spleen and lung adduct levels are significantly greater than those in liver at P < 0.05. |
| c | Spleen adduct level is significantly greater than that in lung and kidney at P < 0.05. |
| d | Spleen adduct level is significantly greater than that in liver and kidney (P < 0.05) but not lung. Other comparisons against levels in liver are not statistically significant. |
The preferential formation of BPDE/DNA adducts in the lung Twenty-four h after i.p. injection of the high dose of B(a)P, activate B(a)P compared to microsomes from liver or lung (24). The tissue distribution of BPDE/DNA adducts varied in mouse tissues. At 200 mg of B(a)P/kg, detoxification systems in a more uniform adduct distribution. However, since the relative capability of these tissues to detoxify BPDE is unknown, other intertissue differences (e.g., DNA repair, BPDE transport of BPDE in the production of DNA adducts after B(a)P injection. This is based upon the availability of BPDE in serum at both early and extended periods after a wide range of B(a)P doses. Further, BPDE injection resulted in the rapid formation of DNA adducts, primarily in the lung, which had the greatest access to the transported carcinogen. Although our studies show that circulating BPDE can enter cells to adduct DNA, additional research is needed to clarify the relative importance of BPDE uptake versus in situ formation from B(a)P within target tissues.

### Table 2 DNA adduct levels and BPDE serum concentrations after i.v. injection of BPDE

<table>
<thead>
<tr>
<th>Time after injection (min)</th>
<th>BPDE (nmol/liter of serum)</th>
<th>DNA adducts (fmol/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Lung</td>
<td>Kidney</td>
</tr>
<tr>
<td>5</td>
<td>8.60 ± 1.92*</td>
<td>161 ± 33</td>
</tr>
<tr>
<td>30</td>
<td>0.47 ± 0.10</td>
<td>135 ± 19</td>
</tr>
<tr>
<td>60</td>
<td>0.28 ± 0.11</td>
<td>129 ± 31</td>
</tr>
</tbody>
</table>

* Mean ± SEM (n = 4 to 6 mice/group).

*Lung adduct levels are significantly greater than those in liver at P < 0.05. Other comparisons against levels in the liver are not statistically significant.

### REFERENCES

27. Pal, K. The relationship between the levels of DNA-hydrocarbon adducts and the formation of sister-chromatid exchanges in Chinese hamster ovary


37. Rojas, M., and Alexandrov, K. In vitro formation and persistence of DNA adducts in mouse and rat skin exposed to (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene and (±)-7,8,9,10-tetrahydrobenzo(a)pyrene. Carcinogenesis (Lond.), 7: 1553–1560, 1986.


DNA Adduct Formation in Mouse Tissues in Relation to Serum Levels of Benzo(a)pyrene-diol-epoxide after Injection of Benzo(a)pyrene or the Diol-epoxide

Gary L. Ginsberg and Thomas B. Atherholt