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*Advances in Brief*

# Conjugation of Benzo(*a*)pyrene 7,8-dihydrodiol-9,10-epoxide in Infant Swiss-Webster Mice[1]

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## Abstract

**Benzo(*a*)pyrene 7,8-dihydrodiol-9,10-epoxide (BPDE), accepted as the ultimate carcinogen of benzo(*a*)pyrene, has a very short half-life in aqueous solutions yet induces lung tumors when injected into infant mice. To evaluate the possibility that metabolites of BPDE, principally in the form of stable conjugates, contribute to binding to DNA in peripheral tissues, infant mice were injected i.p. with 39 nmol (±) *anti*-BPDE. One h after injection, 5% of the dose was recovered in serum and appeared mostly as conjugated metabolites (54% as glucuronides and 16% as glutathione conjugates). Amounts of direct acting electrophiles in serum estimated by trapping with DNA comprised less than 0.02% of the injected dose. No more than 10% of the radioactivity in extracts of liver, lung, and kidney was recovered as BPDE. Glutathione conjugates predominated in the liver and lung, whereas glucuronides were the major metabolites in kidney. Radioactivity bound to DNA in liver, lung, and kidney was 21.5, 42.7, and 7.8 pmol/mg, respectively. Despite the rapid conversion of BPDE to stable conjugates, $^{32}$P-postlabeling profiles of DNA adducts in lung closely resembled that noted after addition of BPDE directly to lung homogenate. Thus, the reactive intermediate as well as stable conjugates of BPDE may be transported to target tissues where they initiate tumors.**

## Introduction

It is well established that the environmental carcinogen benzo(*a*)pyrene is converted to the (+)*anti* isomer of benzo(*a*)pyrene 7,8-dihydrodiol-9,10-epoxide, which is highly mutagenic (1) and causes lung tumors in newborn mice (2). Despite the long awareness of this phenomenon, the metabolic fate of BPDE[3] *in vivo* has not been studied. In the classic study by Buening *et al.* (2), 14 nmol of (+)*anti*-BPDE administered i.p. to infant mice produced tumors in 100% of the animals after 38 weeks. While it was assumed that BPDE was the agent responsible for tumors, it is not known whether BPDE administered i.p. per se or a metabolite of this compound caused lung tumors. One problem is that BPDE is extremely unstable in aqueous environments and reacts rapidly with nucleophilic sites on macromolecules. There is some evidence that BPDE can reach extrahepatic tissues via the circulation protected from hydrolysis by serum proteins or lipids (3, 4); however, it has not been demonstrated that this occurs under conditions that induce lung tumors. Alternatively, BPDE administered i.p. may be converted into stable conjugates in the liver that are exported to susceptible tissues as precursors of the ultimate carcinogen. In accord with this possibility, Wall *et al.* (5) showed recently that conjugated metabolites of benzo(*a*)pyrene are released from orthotopically transplanted livers in rats and are transported via the blood to susceptible tissues such as lung where they bind to DNA.

Several examples support the hypothesis that conjugated metabolites mediate carcinogenicity *in vivo*. The sulfate ester of 6-hydroxymethylbenzo(*a*)pyrene is highly mutagenic in the Ames assay and induces liver tumors in mice (6). The hepatocarcinogenicity of this metabolite exceeds the activity of a comparable dose of benzo(*a*)pyrene and 6-hydroxymethylbenzo(*a*)pyrene by at least 10-fold. Furthermore, the selective nephrotoxicity and carcinogenicity of several halogenated alkanes and alkenes have been attributed to bioactivation involving glutathione conjugate formation (7, 8) and translocation from the liver to the kidney (9). Thus, it is reasonable to propose that stable conjugates of benzo(*a*)pyrene can function as carriers of carcinogenic precursors to target organs. The purpose of the present study was to characterize the fraction of BPDE converted to various conjugates compared to the fraction remaining as a direct-acting electrophile in blood when administered to infant mice under conditions known to induce lung tumors.

## Materials and Methods

**Reagents.** [$^{14}$C] (±) *anti*-BPDE was purchased from the NCI Chemical Carcinogen Standard Repository (specific activity, 55.8 mCi/mmol). Just before use, the solvent (tetrahydrofuran:triethylamine, 95:5) was removed under nitrogen and the compound was dissolved in dimethyl sulfoxide (high-performance liquid chromatography grade; Aldrich Chemicals, Milwaukee, WI). Glucuronide and sulfate conjugates of benzo(*a*)pyrene were also obtained from the repository. All other solvents were high-performance liquid chromatography grade from Fisher Scientific (Springfield, NJ), and biochemicals were the highest grade available from Sigma Chemical Co. (St. Louis, MO). Preparative thin-layer chromatography plates (type PLK5F) were the products of Whatman, Inc. (Clifton, NJ).

Reagents used for $^{32}$P-postlabeling of DNA adducts including nuclease P1, micrococcal nuclease, and deoxyadenosine 3′-monophosphate were purchased from Sigma Chemical Co. Calf spleen phosphodiesterase and T4 polynucleotide kinase were products of Boehringer Mannheim (Indianapolis, IN) and U.S. Biochemicals (Cleveland, OH), respectively. [$\gamma$-$^{32}$P]ATP (specific activity, 7000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Polyethyleneimine cellulose thin-layer chromatography plates were prepared according to methods described by Randerath and Randerath (10).

**Administration of BPDE.** Newborn mice of the Swiss-Webster strain were obtained from Harlan Sprague-Dawley and housed with their mothers. At 15 days of age, pups weighing an average of 10 g were given a single i.p. injection of [$^{14}$C] (±) *anti*-BPDE (39 nmol, 2.2 $\mu$Ci in 10 $\mu$l dimethyl sulfoxide) as described by Buening *et al.* (2). This dose of the racemic mixture contains 1.4 times the amount of (+) *anti*-BPDE used by these investigators to induce lung tumors. Tissues were

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[3] The abbreviations used are: BPDE, benzo(*a*)pyrene 7,8-dihydrodiol-9,10-epoxide; [$^{14}$C] (±) *anti*-BPDE, (±) *r*-7, *t*-8-dihydroxy-*t*-9, 10-epoxy-7,8,9,10-tetrahydro-[7-$^{14}$C]benzo(*a*)pyrene.

removed 1 h after administration, since initial experiments indicated that radioactivity in blood was maximal at 1 h after i.p. injection of [$^{14}C$]BPDE.

**Determination of BPDE Conjugates.** To quantitate the fraction of administered dose in various organs, tissues were removed, minced, and digested in 1 ml tissue solubilizer (NCS; Beckman, Fullerton, CA), and total radioactivity was determined in the digest. In other experiments, metabolites were determined in serum or tissues homogenized in 0.15 M potassium phosphate buffer, pH 7.4. An aliquot of the homogenate (1 ml) was extracted with an equal volume of ice-cold acetone and centrifuged to remove insoluble material. The supernatant was concentrated under vacuum using a Speedvac concentrator (Savant Instruments, Farmingdale, NY). Conjugates of BPDE in the supernatant concentrate were separated by thin-layer chromatography according to the method of Zaleski *et al.* (11). Glutathione, glucuronide, and sulfate conjugates were visualized under long-wavelength UV light and scraped from the plates. Hydrofluoric acid (1 ml of 24%) was added to the samples, and radioactivity was quantitated by scintillation spectroscopy. BPDE metabolites in serum from blood obtained by cardiac puncture were analyzed as described for tissue homogenates, except that serum was applied directly onto thin layer chromatographic plates.

**Estimation of Reactive Material.** Electrophilic metabolite(s) were estimated in serum collected from mice 1 h after administration of [$^{14}C$] (±) *anti*-BPDE by measuring radioactivity bound to salmon sperm DNA (12). Specifically, serum (200 µl) was added to 400 µg salmon sperm DNA in phosphate-buffered saline, the mixture (total volume 300 µl) was incubated at 37°C for 2 h, and DNA was isolated as described above. Results are expressed as pmol of electrophile bound/mg of DNA in the assay. As a positive control, 2 nmol of [$^{14}C$] (±) *anti*-BPDE were added directly to control serum, and binding to DNA was determined.

**DNA Isolation and $^{32}P$-Postlabeling of DNA Adducts.** DNA was isolated from tissue homogenates by phenol:chloroform:isoamylalcohol extraction following Marmur's procedure (13), and radioactivity bound to DNA was determined. $^{32}P$-Postlabeling was carried out according to Reddy and Randerath (14) with minor modifications. Briefly, DNA was digested to deoxyribonucleotide 3'-monophosphates. Nonadducted nucleotides were dephosphorylated by incubation with nuclease P1 (14). Adducted nucleotides were labelled with 100 µCi [$^{32}P$]ATP in the presence of 4.8 units T4 polynucleotide kinase, 10 mM $MgCl_2$, 10 mM dithiothreitol, 20 mM sodium glycine (pH 9.6), and 1 mM spermidine for 30 min at 37°C. Labeled nucleotides were separated by spotting the equivalent of 7.5 µg DNA on polyethyleneimine-cellulose plates and developing these plates in two dimensions (14). Dried plates were exposed to Kodak XAR-5 or CRONEX-4 X-ray film for 8 h in a cassette containing intensifying screens.

## Results and Discussion

Distribution of radioactivity derived from [$^{14}C$] (±)*anti*-BPDE in various organs 1 h after i.p. injection is presented in Table 1. The greatest fraction of administered material was found in the intestines, followed by liver, kidney, and lung. Total radioactivity recovered in serum was approximately 6 nmol/ml, which corresponded to about 5.3% of the administered dose, assuming 3.5 ml serum/100 g body weight.

BPDE administered i.p. to infant mice was converted rapidly to stable conjugates (Fig. 1), in all likelihood by first-pass metabolism in the liver. Glucuronides represented the highest fraction of benzo(*a*)pyrenyl metabolites in serum and in kidney (55% and 80%, respectively) (Table 2). This fraction may also contain the cysteinyl glycine conjugate of BPDE (Rf, 0.45) if further metabolism of the glutathione conjugate occurred *in vivo*. In contrast, conjugates with glutathione were found in highest concentration in liver and in lung and accounted for 55% and 39% of radioactivity in tissue extracts, respectively. These data support the idea that the lung is very efficient in extracting BPDE conjugates as well as BPDE (4) and benzo(*a*)pyrene (15) from the circulation. Large amounts of glutathione conjugates found in lung may reflect uptake of the glutathione conjugate from blood as well as the specificity of isoforms of glutathione *S*-transferases that are highly efficient in conjugating BPDE (16).

Approximately 10% of radioactivity found in tissue extracts and in serum migrated with standard BPDE by thin-layer chromatography. A fraction of the radioactivity migrating with

Table 1 *Distribution of radioactivity derived from [$^{14}C$] (±) anti-benzo(a)pyrene 7,8-dihydrodiol-9,10-epoxide in serum and tissues of infant mice*

Serum and organs of 15-day old mice were removed 1 h after an i.p. injection of 39 nmol [$^{14}C$] (±) *anti*-BPDE. Tissues were homogenized in 0.15 M phosphate buffer, and total radioactivity was determined in the homogenates as described in "Materials and Methods." Results are means ± SEM of serum from four mice and various tissues from six mice.

| Tissue | Amount in tissues (nmol/g) | % of injected dose |
|---|---|---|
| Serum | 5.97 ± 0.47 | 5.25 ± 0.42 |
| Liver | 9.48 ± 0.83 | 7.88 ± 0.69 |
| Lung | 12.5 ± 2.48 | 3.46 ± 0.68 |
| Kidney | 12.2 ± 1.42 | 4.30 ± 0.50 |
| Heart | ND[a] | 0.59 ± 0.07 |
| Spleen | ND | 0.29 ± 0.02 |
| Stomach | ND | 1.14 ± 0.16 |
| Intestines | ND | 10.6 ± 1.10 |

[a] ND, not determined.

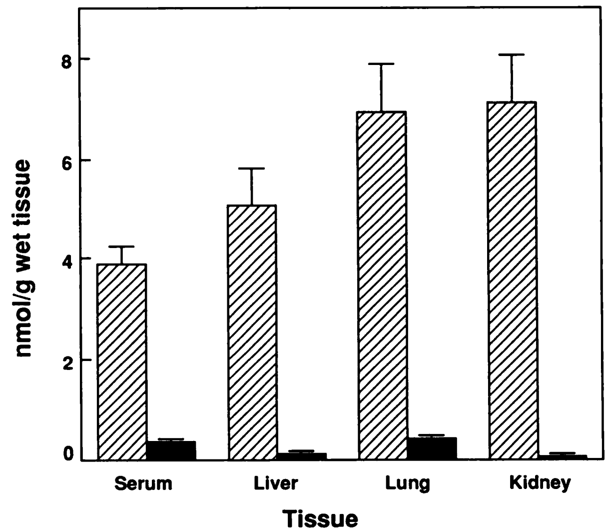


Fig. 1. Distribution of radiolabel from [$^{14}C$] (±) *anti*-BPDE as metabolites and parent compound in mouse tissues. The distribution of BPDE (■) and metabolites (▨) was determined in serum and in tissues of infant mice receiving injections of 39 nmol [$^{14}C$] (±) *anti*-BPDE. Metabolites were measured after separation on thin-layer plates as described in "Materials and Methods" and represent the sum of glutathione, glucuronide, sulfate conjugates and tetrols. Results are means ± SEM of serum from three mice and tissues from five mice.

Table 2 *BPDE and conjugated metabolites in serum and tissues of infant mice*

BPDE and conjugated metabolites were determined in serum and in tissue extracts 1 h after injection of 39 nmol [$^{14}C$] (±) *anti*-BPDE. Metabolites in tissues were extracted into water:acetone and separated by thin-layer chromatography as described in "Materials and Methods." Serum was spotted directly onto thin-layer plates. Results are means ± SEM of samples obtained from the number of animals shown in parentheses.

| Tissue (*n*) | Conjugates: Glutathione (nmol/ml serum or nmol/g tissue) | Glucuronide | Sulfate/tetrols | BPDE |
|---|---|---|---|---|
| Serum (3) | 0.67 ± 0.47 | 2.29 ± 0.24 | 0.87 ± 0.11 | 0.37 ± 0.07 |
| Liver (5) | 2.80 ± 0.62 | 1.12 ± 0.11 | 1.08 ± 0.21 | 0.14 ± 0.03 |
| Lung (5) | 2.80 ± 0.49 | 0.86 ± 0.26 | 3.17 ± 0.59 | 0.39 ± 0.07 |
| Kidney (5) | 0.63 ± 0.12 | 5.71 ± 0.82 | 0.72 ± 0.07 | 0.08 ± 0.01 |

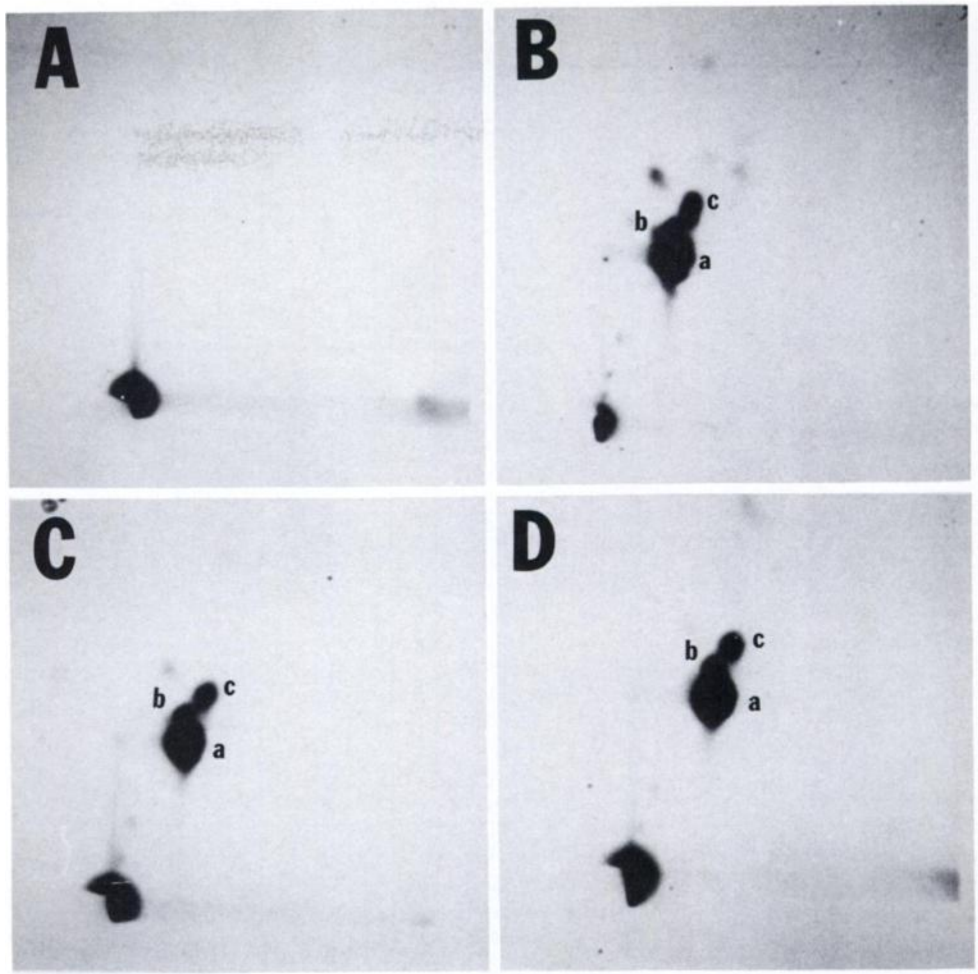


Fig. 2. $^{32}$P-Postlabeling analysis of DNA nucleotides isolated from lungs of mice receiving injections of (±) *anti*-BPDE. Autoradiograms were obtained after development of the plates at −80°C for 8 h. The origin is in the lower left corner. *a–c*, adducts. The samples are lung DNA digests from vehicle-treated control mouse (*A*), BPDE added directly to lung homogenate (*B*), mouse treated with 39 nmol (±) *anti*-BPDE 1 h after i.p. injection (*C*), and mouse treated with BPDE, 24 after i.p. administration (*D*).

sulfate conjugates were benzo(*a*)pyrenyl tetrols which arose from spontaneous hydrolysis of the diolepoxide. Although the major fraction of administered BPDE was recovered as stable conjugates, a small amount of injected BPDE remained in the blood as such and may be extracted from the circulation by peripheral tissues. Recent work (4) indicating that BPDE injected i.v. into adult mice is stabilized by serum proteins and forms maximal amounts of DNA adducts within 5 min after administration is in accord with this idea.

$^{32}$P-Postlabeling profiles of nucleotides from DNA extracted from lungs of infant mice indicated that the major adduct (Fig. 2, adduct a, *C* and *D*) at either 1, 6, 12 or 24 h after injection corresponded to the same adduct generated when BPDE was added directly to a lung homogenate (Fig. 2*B*). These data support the idea that BPDE per se is transported to and taken up by the lung after i.p. injection. In addition to the major BPDE adduct, a number of other adducts were detected by the $^{32}$P-postlabeling technique. In general, the distribution and intensity of labeling noted with these adducts in lungs of animals given BPDE also corresponded with those noted in the homogenate to which BPDE had been added directly.

The possibility that BPDE is protected from hydrolysis in serum is suggested by adduction of DNA added to serum. Direct-acting electrophiles in serum were trapped by binding to salmon sperm DNA. The amount of radioactivity in 200 μl serum that could be trapped under these experimental conditions was 15.4 pmol/mg DNA. Assuming 3.5 ml serum/100 g body weight, total amounts estimated to be direct-acting electrophile(s) represented only 0.02% of the administered dose 1 h following injection of BPDE. When BPDE was added directly to mouse serum, only 1% of the added radioactivity was trapped by DNA (19.7 pmol of 1990 pmol added). This low recovery may be explained by hydrolysis of the diolepoxide to tetrols. Alternatively, a large fraction of BPDE may bind to serum proteins, or react inefficiently with DNA used as the trapping agent in these experiments. Binding to DNA did not occur when BPDE was added to phosphate-buffered saline containing DNA. Thus, some degree of protection of BPDE from hydrolysis was clearly apparent in mouse serum. This protection appears sufficient to allow BPDE to be transported to lung, as suggested by the $^{32}$P-postlabeling patterns noted above.

In view of the large fraction of BPDE converted to conjugates in this study, the possibility that these compounds serve as precursors of carcinogenic metabolites in susceptible tissues cannot be ruled out. Conjugation reactions are generally associated with detoxification of xenobiotics (17); however, several studies indicate that conjugated metabolites of a variety of chemicals (7, 18), including benzo(*a*)pyrene (6), are actually bioactivation products that are highly mutagenic and carcinogenic. Early studies by Kinoshita and Gelboin (19) had shown that hydrolysis of benzo(*a*)pyrene 3-glucuronide by β-glucuronidase generated a product that bound to DNA to a greater extent than 3-hydroxybenzo(*a*)pyrene (19). Recent studies in which rat lung slices were incubated with glutathione, glucuronide, and sulfate conjugates generated by hepatocytes demonstrated these metabolites were taken up by lung slices and converted to protein-binding derivatives (20). The nature of electrophiles generated from the conjugates is not known; however, the binding is dependent on the hydrolysis of the glucuronide and sulfate conjugates by β-glucuronidase and arylsulfatase, respectively. Taken together, results presented in this study indicate that small amounts of injected BPDE, despite its instability, may travel to target organs unchanged. In addition, conjugated metabolites of BPDE must also be considered in the production of tumors in lungs from animals injected with BPDE. Identification of the chemical nature of DNA adducts under these experimental conditions will not only provide clues to the validity of this hypothesis but will also provide information on metabolic pathways unique to the target tissue and may lead to strategies to inhibit this process.

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