The Role of Acetylation in Benzidine Metabolism and DNA Adduct Formation in Dog and Rat Liver

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To determine whether benzidine is acetylated in dog, like rat, the metabolism of benzidine was assessed with dog and rat liver slices. Slices were incubated with 0.05 mM [^{3}H]benzidine for 4 h. Media and cellular DNA were analyzed for acetylated benzidine metabolites and adducts. In rat, benzidine was rapidly converted to acetylated metabolites. At 1 h, benzidine, *N*-acetylbenzidine, and *N,N'*-diacetylbenzidine represented 5%, 23%, and 54%, respectively, of the total radioactivity in media. Within 2 h, 75% of the radioactivity was *N,N'*-diacetylbenzidine. In dog, 45% of the radioactivity was present in metabolites more polar than benzidine by 4 h. No N-acetylated metabolites were observed in dog liver slice media. To identify acetylated benzidine DNA adducts, *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine was prepared and identified by FAB MS. This nucleoside adduct was used to synthesize *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine and *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine. Nucleoside adducts from slices incubated with [^{3}H]benzidine were analyzed by HPLC. With this method of analysis, the ^{3}H-material did not correlate with the synthetic adduct standards. To improve sensitivity and identify liver adducts, a ^{32}P-postlabeling method was developed. 2'-Deoxyguanosine 3'-monophosphate adduct standards of acetylated benzidine were prepared. ^{32}P-Postlabeling analysis demonstrated that rat liver contained only *N'*-(3'-monophosphodeoxyguanosine-8-yl)-*N*-acetylbenzidine after a 1- or 4-h exposure to benzidine. In contrast, no acetylated adducts were detected in dog. Results indicate that dog is a nonacetylator with respect to benzidine. The availability of acetylated benzidine nucleotide standards allowed unambiguous identification of *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine as the adduct present in rat liver slices. These nucleotide adduct standards will be useful in subsequent studies in animals and human.

Introduction

Benzidine, an aromatic diamine, induces bladder tumors in man and dog, and predominately liver tumors in rat (*1–4*). One distinguishing characteristic between rat and dog arylamine metabolism is that the former has been considered an acetylator, and the latter a nonacetylator (*5, 6*). In rats, benzidine is rapidly acetylated to *N*-acetylbenzidine and *N,N'*-diacetylbenzidine (*7*). Following administration of benzidine to mice and rats and *N*-acetylbenzidine to hamsters, the major DNA adduct observed was *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (*8, 9*). This adduct is thought to initiate the carcinogenic process. A recent study examining liver suggested dog may be an acetylator rather than a nonacetylator (*10*). Thus, tumor formation in dog, like rat, may be initiated by the formation of acetylated benzidine DNA adducts. This possibility was tested in the following study. Rat was used as a positive control for comparison to dog. Liver slices from rat and dog were incubated with [^{3}H]-benzidine. Media were analyzed for benzidine, *N*-acetylbenzidine, and *N,N'*-diacetylbenzidine, and slices for DNA adducts. To identify adducts, synthetic acetylated benzidine deoxyguanosine nucleoside and nucleotide standards were prepared. The availability of acetylated benzidine nucleotide standards allowed unambiguous identification of adducts by ^{32}P-postlabeling. Under identical incubations, rats produced acetylated benzidine metabolites and an adduct, but not dog. The results suggest that dog is a nonacetylator with respect to benzidine.

Materials and Methods

Materials. Caution: *The following chemicals are hazardous and should be handled carefully: benzidine, N-acetylbenzidine, N'-hydroxy-N-acetylbenzidine, N-hydroxy-N-acetylbenzidine, and N-hydroxy-N,N'-diacetylbenzidine.* [^{3}H]Benzidine (180 mCi/mmol) and carrier-free [γ-^{32}P]ATP (7000 Ci/mmol) were purchased from Chemsyn (Lenexa, KS) and ICN (Irvine, CA), respectively. *N*-Acetylbenzidine was synthesized by acetylation of benzidine in glacial acetic acid, and *N,N'*-diacetylbenzidine was synthesized by acetylation of benzidine with acetic anhydride as previously described (*11*). The purity of these labeled and unlabeled *N*-acetyl derivatives of benzidine was greater than 99%. Scintiverse was purchased from Fisher Chemical Co. (St. Louis, MO). Benzidine (free base and hydrochloride salt), 2'-deoxyguanosine, 2'-deoxyguanosine 3'-monophosphate, EDTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic

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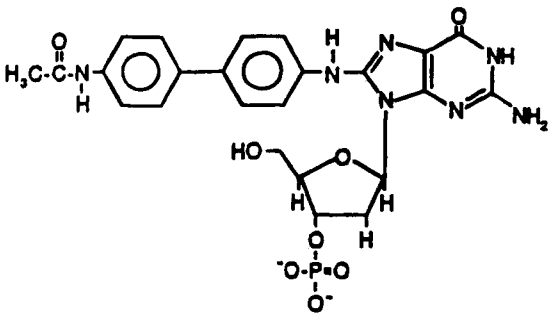

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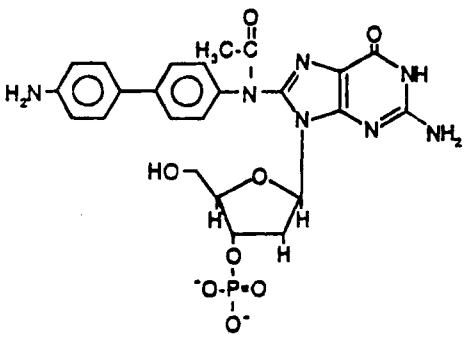

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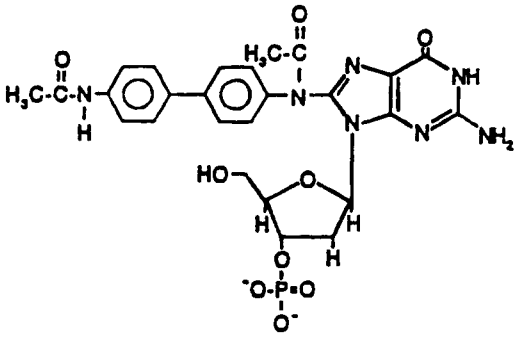

N-(3'-monophospho-deoxyguanosin-8-yl)-N,N'-Diacetylbenzidine

Figure 1. Structures of synthetic nucleotide adduct standards used for ^{32}P-postlabeling.

acid (EGTA),[1] phenylmethanesulfonyl fluoride, deoxyribonuclease I (DN-25 from bovine pancreas; EC 3.1.21.1), phosphodiesterase I (Type VII from *Crotalus atrox* venum; EC 3.1.4.1), alkaline phosphatase (Type III-S from *Escherichia coli*; EC 3.1.3.1), porcine liver esterase (EC 3.1.1.1), and rabbit liver esterase (EC 3.1.1.1) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 (EC 3.1.30.1) was from Boehringer Mannheim (Indianapolis, IN). The following were purchased for ^{32}P-postlabeling: micrococcal endonuclease (Sigma Chemical Co; grade VI; EC 3.1.31.1), spleen exonuclease (Boehringer-Mannheim Corp.; EC 3.1.16.1), T4 polynucleotide kinase (PL-Biochemicals; EC 2.7.1.78), and potato apyrase (Sigma Chemical Co.; grade I; EC 3.6.1.5). Male Fischer 344 rats (150–200 g) were purchased from Harlan Industries (Indianapolis, IN). N-Acetylated and nonacetylated benzidine adduct standards were synthesized using either 2'-deoxyguanosine or 2'-deoxyguanosine 3'-monophosphate by a modification of previous procedures (*8*). Preparation of different nucleoside and nucleotide adduct standards is described below. The nucleoside standards and starting compound (*N*-acetoxy-*N*,*N*'-diacetylbenzidine) are identified by a letter designation which correlates to their order of elution from a C-18 HPLC column (Figures 5 and 8). The nucleotide standards are identified by their chemical name and are illustrated in Figure 1.

Compound E: *N*-Acetoxy-*N*,*N*'-diacetylbenzidine. To 4-acetamido-4'-nitrophenyl (*11*) (40 mg, 160 μmol) in 4 mL of dimethylformamide at 5 °C was added 2 mL of 20% aqueous ammonium sulfide, dropwise with stirring. After 18 h at 5 °C, the solution was diluted with 20 mL of cold water (argon degassed) and the resulting solids were isolated by centrifugation. The precipitate was washed sequentially, with ice-cold 20 mL portions of argon degassed water, and twice with argon degassed 20 mM ascorbic acid, and the resulting white solids were lyophilized to dryness. The crude hydroxylamine was dissolved in 16 mL of ethyl acetate, cooled to 5 °C, and treated with 4 mL of acetic anhydride/pyridine (9:1), dropwise with stirring. After 18 h, the reaction mixture was evaporated in vacuo and washed with ice-cold water, and the resulting precipitate lyophilized to dryness and stored at −70 °C. HPLC analysis (Program 1 or 2, described below) readily separated reaction components. HPLC analysis (Program 1) indicated that the product was stable upon storage under argon at −70 °C for several months and contained 5–10% *N*,*N*'-diacetylbenzidine.

Compound B: *N*-(Deoxyguanosin-8-yl)-*N*,*N*'-diacetylbenzidine. Under an argon atmosphere at 37 °C, *N*-acetoxy-*N*,*N*'-diacetylbenzidine (**E**) (12 mg, 46 μmol) in 2 mL of acetonitrile was added dropwise with stirring to 2'-deoxyguanosine (30 mg, 105 μmol) in 14 mL of 30 mM sodium chloride/3 mM sodium citrate (pH 7.0). After stirring 6 h, the solution was extracted four times with 10 mL portions of diethyl ether. The resulting aqueous phase was reduced to $^2/_3$ volume under a stream of argon at room temperature and then applied to LH20 Sephadex (5 × 150 mm column bed). The column was eluted sequentially with 8 mL aliquots of distilled water, methanol/water (at 10%, 25%, 50%), and finally 100% methanol. After HPLC analysis (Program 1) of the fractions, those containing the product (primarily the 25% and 50% methanol fractions) were pooled, concentrated, and purified by HPLC (Program 2) to yield 4.0 mg, 24% yield, 99+% pure **B** (HPLC analysis, Program 1). The amount of **B** and **E** in the reaction mixture before purification was approximately 3:1. Compound **B** was used to synthesize the other benzidine nucleoside adducts described below using modifications of published procedures by Martin et al. (*8*).

Compound D: *N*'-(Deoxyguanosin-8-yl)-*N*-acetylbenzidine. To *N*-(deoxyguanosin-8-yl)-*N*,*N*'-diacetylbenzidine (**B**) (4 mg, 7 μmol) in 8 mL of methanol was added potassium carbonate (75 mg, 542 μmol), and the mixture was stirred at room temperature for 2 h. After cooling on ice, 0.058 mL of glacial acetic acid was added and volatiles were removed in a stream of argon at room temperature. The sample was stored overnight at −70 °C. The reaction was 80–90% complete by HPLC (Program 1), and the product was isolated by repeated HPLC

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; NBA, *m*-nitrobenzyl alcohol.

(Program 2). Pooled fractions were reduced in volume by $^1/_3$ in a nitrogen stream and lyophilized to dryness. Purity was >99% by HPLC analysis (Program 1). FAB MS showed $[MH]^+$ at *m/z* 492 and BH_2^+ at *m/z* 376. High resolution MS of MH^+ corresponds to $C_{24}H_{26}O_5N_7$ (calculated: 492.1995; measured: 492.2001).

Compound A: *N*-(Deoxyguanosin-8-yl)-*N*-acetylbenzidine. *N*-(Deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**) (2 mg, 3.5 μmol) in 0.1 mL of dimethyl sulfoxide was added dropwise with stirring to 10 mL of 50 mM sodium phosphate buffer (pH 7.7) at 37 °C containing 90 units each of pig and rabbit esterase. After 48–72 h, the reaction was terminated by centrifugation at 3500 rpm for 10 min at 4 °C. The supernatant was lyophilized to dryness, reconstituted in 30% methanol/70% 20 mM ammonium acetate buffer (pH 6.7), and purified by HPLC (Program 2). The purity of pooled fractions was determined by HPLC to be >99%. FAB MS showed $[MH]^+$ ion at *m/z* 492 and BH_2^+ at *m/z* 376.

Compound C: *N*-(Deoxyguanosin-8-yl)benzidine. Treatment of **D** with porcine and rabbit esterase, as described above, afforded *N*-(deoxyguanosin-8-yl)benzidine (**C**) which is identical to the product of potassium carbonate/methanol hydrolysis of **A**. **C** is identical to the main adduct from the reaction of benzidinediimine with DNA (*12*, *13*).

Synthesis of Nucleotide Adduct Standards. Nucleotide adducts were made by minor modifications of the above procedures described for making the nucleoside adducts. *N*-Acetoxy-*N,N'*-diacetylbenzidine (**E**) was added to 2'-deoxyguanosine 3'-monophosphate to make the initial product, the *N*-(3'-monophosphodeoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (Figure 1). Treatment with base (potassium carbonate) or enzymes (esterases) resulted in the formation of the different acetylated adducts. Structures of the nucleotide adducts were confirmed by conversion to their corresponding nucleosides by alkaline phosphatase treatment and identified by HPLC.

Metabolism of Benzidine by Liver Slices. Livers were removed from anesthesized rats and dogs and immediately placed in a room temperature saline solution containing 1 mM phenylmethanesulfonyl fluoride, 1 mM EGTA, and 1 mM EDTA. Tissue was quickly washed three times with this solution and placed in ice-cold saline. Tissue was sliced with a Stadie-Riggs microtome. Slices (6 g) were placed in 125 mL flasks containing 25 mL of Krebs–Ringer's bicarbonate or α-modified Eagle's medium with 0.05 mM [^{3}H]benzidine (4 μCi/mL) at 37 °C with 5% CO_2/95% O_2. Every hour, 0.5 mL of media was removed and the sample regassed with 5% CO_2/95% O_2. After a 4-h incubation, media were removed by filtration and slices frozen at −70 °C. Slices were analyzed for DNA adducts described below and media for acetylated products of benzidine metabolism.

To media was added ice-cold methanol (2 volumes), the samples were centrifuged, and the supernatant was frozen at −70 °C. Metabolism was assessed using HPLC Program 1, except that 0.02 mM sodium phosphate buffer (pH 6.8) was used. Benzidine, *N*-acetylbenzidine, and *N,N'*-diacetylbenzidine eluted from the HPLC at 10, 16, and 21, min, respectively. Radioactivity in the eluent was measured with a FLO-ONE radioanalytical detector (Packard Instrument Co., Meriden, CT). Data are expressed as a percentage of the total amount of radioactivity recovery by HPLC.

DNA Preparation and Hydrolysis. DNA was prepared and hydrolyzed using standard methods. Homogenates were treated with RNase followed by sodium dodecyl sulfate and proteinase K treatment and extracted with phenol (*11*) and chloroform/isoamyl alcohol (24:1) (*14*). Recovery of DNA ranged from 2 to 4 mg of DNA/g wet liver weight. Purity and quantitation of DNA were determined by absorbance at 260 and 280 nm. A ratio of A_{260}/A_{280} of approximately 1.7 was achieved for each sample. DNA was hydrolyzed to nucleosides for HPLC analysis (*13*) and to nucleotides for ^{32}P-postlabeling analysis (*15*).

HPLC Analysis of Adducts. 1-Butanol-extracted nucleoside samples were analyzed on C-18 Ultrasphere 5 μm Beckman columns employing methanol/0.02 mM ammonium acetate buffer (pH 6.7). Standards were coinjected in sufficient quantity to overcome residual UV absorption in hydrolyzed samples. UV spectra (280 nm) were taken during elution with a Beckman Model 166 detector, and radioactive measurements were made using a FLO-ONE radioanalytical detector. The solvent system for Program 1 consisted of the following (4.6 × 250 mm column at 1 mL/min flow rate): 0–2 min, 35% methanol; 2–15 min, 35–50% methanol; 17–23 min, 50–80% methanol; 25–30 min, 80–35% methanol. Program 2 consisted of the following (10 × 250 mm column at 2 mL/min flow rate): 0–10 min, 40% methanol; 10–40 min, 40–60% methanol; 45–50 min, 60–40% methanol.

^{32}P-Postlabeling Analysis of Adducts. Samples enriched by 1-butanol extraction (*16*) or nuclease P_1 treatment (*17*) were ^{32}P-postlabeled (*15*) to access adduct formation. Labeled adducts were separated on PEI–cellulose sheets using two different multicomponent solvent systems. Solvent system 1 contained D-1 = 1.7 M sodium phosphate (pH 5.5); D-3 = 2.25 M lithium formate, 3.5 M urea (pH 3.5); D-4 = 2-propanol/4 M ammonium hydroxide (1:1). Solvent system 2 contained D-1 = 1.7 M sodium phosphate (pH 5.5); D-3 = 4.5 M lithium formate/7 M urea (pH 3.5); D-4 = 0.8 M lithium chloride/0.5 M Tris-HCl/7 M urea (pH 8.0). ^{32}P-Labeled adducts were observed by autoradiography using Kodak X-Omat AR or XAR-5 films. DNA adduct content was calculated by determining the amount of radioactivity contained in the TLC spot. Adduct content was based on recovery of radioactivity and the specific activity of ATP and was expressed as amol/μg of DNA. Cochromatography of adduct standards with corresponding tissue adducts was accomplished by two independent methods. In the first method, adducts extracted from plates with 6 M ammonium hydroxide/2-propanol (1:1) were applied to a plate and developed in a different solvent system. For example, with the rat liver adduct, D-4 in solvent system 2 was replaced with 2-propanol/4M ammonium hydroxide (1:1). The appearance of a single autoradiographic spot suggested that the standard and tissue adduct were identical. Radiochemical analysis indicated that >90% of the material applied was recovered. The second method relied on cochromatography of standards with nonpurified tissue samples. Adduct standard was applied along with the tissue sample such that similar amounts of radiochemical material was present for the standard and corresponding unknown tissue adduct. Following thin-layer chromatographic development, autoradiography indicated that a single spot was obtained, and radiochemical analysis indicated that the total amount of material recovered in this spot represented the sum of the standard and corresponding unknown tissue adduct applied.

Mass Spectrometry. Both fast atom bombardment (FAB) mass spectra and accurate mass measurement of the MH^+ ion by peak matching were conducted by a VG ZAB-SE (VG Analytical, Cheshire, U.K.) double-focusing mass spectrometer equipped with a VG 11-250 J data system. FAB ionization was performed with an Ion Tech (Middlesex, U.K.) gun using xenon atoms accelerated to 8 kV (1 mA). *m*-Nitrobenzyl alcohol (NBA) was used as the matrix. For the hydrogen–deuterium exchange experiment, CH_3OD was added to NBA and dried under vacuum (Spec-Vac) several times until NBA gave a FAB mass spectrum indicating deuterium exchange had been completed. Compounds analyzed were dissolved in CH_3OD and mixed with the above deuterated NBA matrix and subjected to FAB source for mass spectra.

Results

Rat liver slices were incubated for 4 h with 0.05 mM [^{3}H]benzidine. Figure 2 illustrates the metabolism of benzidine to *N*-acetylbenzidine and *N,N'*-diacetylbenzidine. Within 1 h, benzidine, *N*-acetylbenzidine, and *N,N'*-diacetylbenzidine represent 5%, 23%, and 54%, respectively, of the total radioactivity in media. By 2 h, *N,N'*-diacetylbenzidine represents 75% of the total radioactivity with benzidine and *N*-acetylbenzidine, each less than 3%.

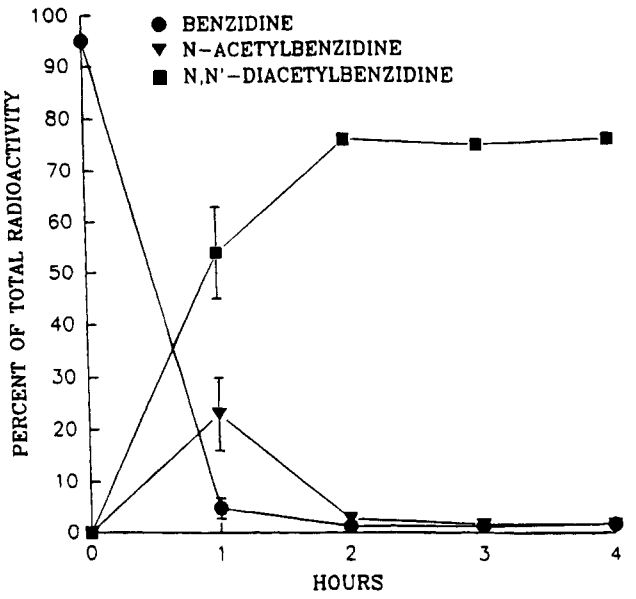

Figure 2. Metabolism of benzidine by rat liver slices. Slices were incubated for 4 h (0.05 mM [^{3}H]benzidine) and the media sampled each hour for acetylated benzidine products. Values represent the percent of total radioactivity recovered from media following HPLC analysis (mean ± SE; n = 4).

Thus, benzidine is rapidly converted to its acetylated products by rat liver slices.

In contrast to rat, benzidine does not appear to be converted to its acetylated products in dog. Dog liver slices were incubated in a similar manner as the rat slices described above. As illustrated in Figure 3, neither *N*-acetylbenzidine nor *N,N'*-diacetylbenzidine was detected in media after a 4-h incubation. Identical results were observed at earlier incubation times. Considerable metabolism of benzidine was observed with 45% of the total radioactivity in media represented as polar metabolites in the early eluting peak. Recent studies indicate that a majority of this radioactivity is benzidine *N*-glucuronide (*18*).

A previous protocol used for identifying benzidine nucleoside adducts in rat liver was modified for this study (*8*). Using the synthetic scheme described in the Materials and Methods section, *N*-acetoxy-*N,N'*-diacetylbenzidine (**E**) was synthesized from 4-acetamido-4'-nitrobiphenyl. The N-acetoxy compound was incubated with 2'-deoxyguanosine to produce *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**). Illustrated in Figure 4A is the FAB mass spectrum of the deoxyguanosine adduct, which shows MH^+ ion at *m/z* 534. Loss of deoxyribose from MH^+ gives protonated nucleobase ion (BH_2^+) (*19–22*), as observed at *m/z* 418. Loss of CH_2=CO and CH_3CO from BH_2^+ gives *m/z* 376 and 375, respectively, and supports the presence of acetyl groups. Additional support for the assigned structure is provided by hydrogen–deuterium exchange experiments (*23*), in which each of the aforementioned ions were replaced by the ions resulting from exchange of the appropriate number of active hydrogens (Figure 4B). The accurate mass measurement of the MH^+ ion gives a composition of $C_{26}H_{28}N_7O_6$ (calculated: 534.2101; measured: 534.2119), which also supports the structural assignment. This adduct was used as starting material to prepare *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (**A**) and *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (**D**).

HPLC analysis of rat liver nucleosides is illustrated in Figure 5. A minor radioactive peak corresponding to *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**) was

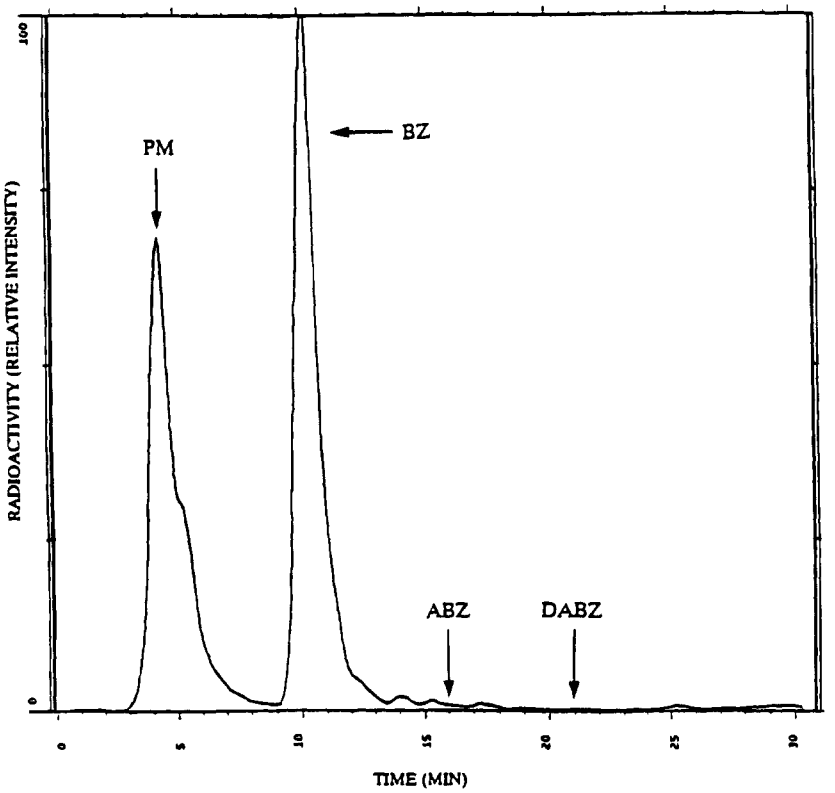

Figure 3. Metabolism of benzidine by dog liver slices. Slices were incubated for 4 h with 0.05 mM ^{3}H-benzidine. Media samples were removed each hour to assess the appearance of *N*-acetylbenzidine and N,N'-diacetylbenzidine along with disappearance of benzidine. Only the 4-h time point is illustrated. Values represent the percent of total radioactivity recovered from media following HPLC analysis.

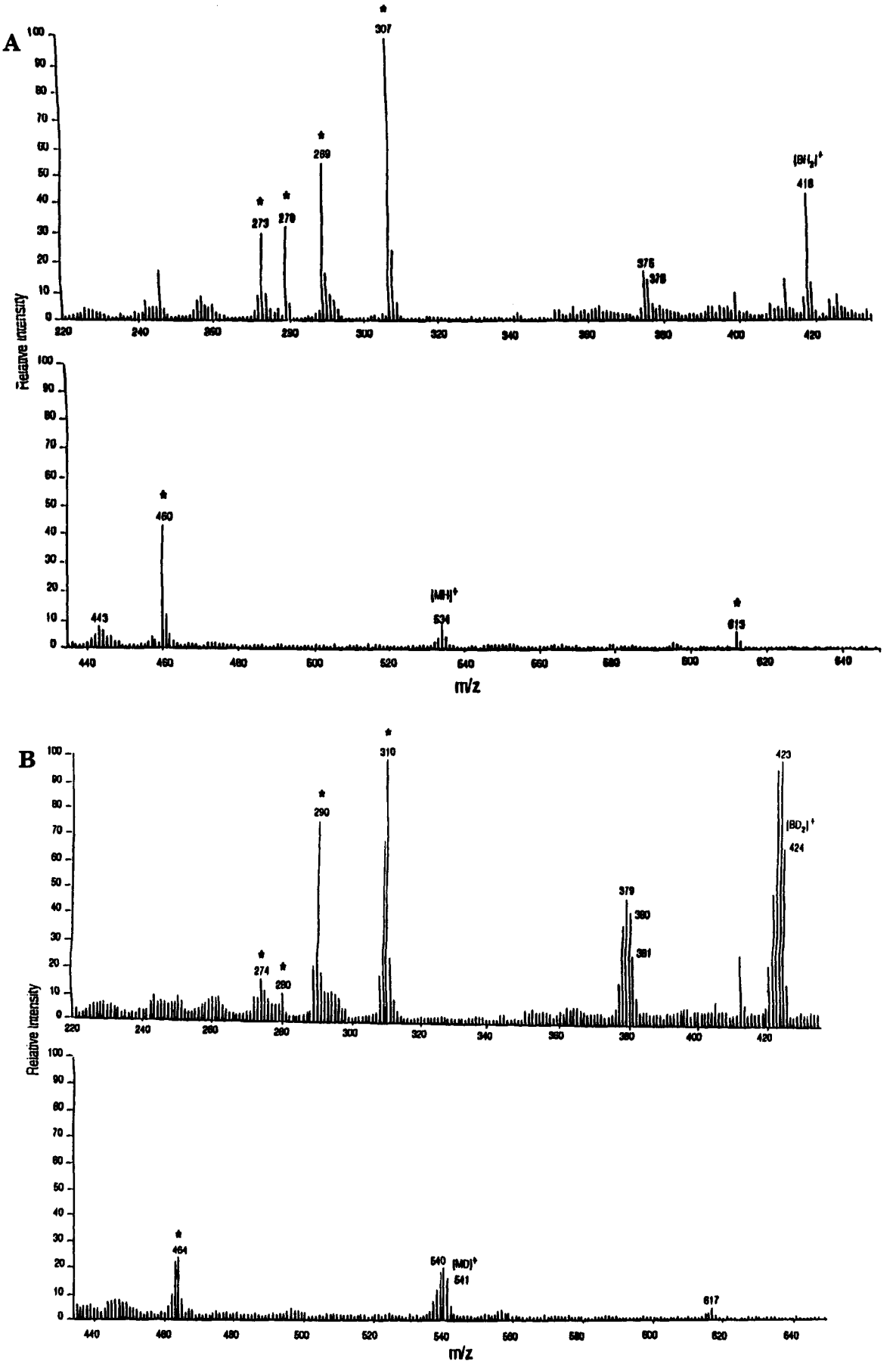

Figure 4. FAB mass spectra of synthetic *N*-(deoxyguanosin-8-yl)-N,N′-diacetylbenzidine (B) obtained from NBA as the matrix (panel A) and hydrogen–deuterium exchanged NBA as the matrix (panel B).

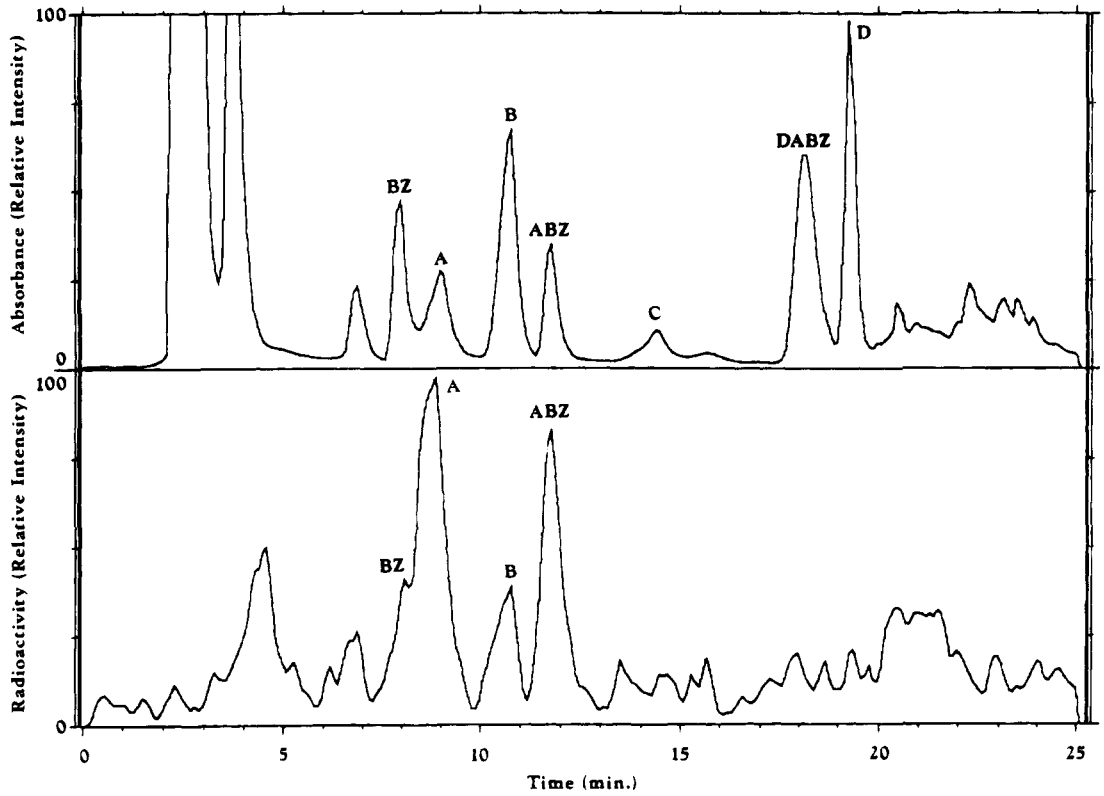

Figure 5. HPLC profile of nucleoside adducts from rat liver slices. Slices were incubated with 0.05 mM ^{3}H-benzidine for 4 h. The UV absorbance (280 nm) of cold carrier standards is illustrated on the top panel and the radioactivity of 1-butanol-extracted samples on the bottom. Benzidine, BZ; N-acetylbenzidine, ABZ; *N,N'*-diacetylbenzidine, DABZ; *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine, A; *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine, B; *N*-(deoxyguanosin-8-yl)benzidine, C; *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine, D.

observed (Figure 5, bottom panel). The major radioactive peak corresponded to the synthetic *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (**A**). Initial HPLC studies did not resolve these two supposed nucleoside adducts from peaks corresponding to benzidine and *N*-acetylbenzidine. However, under the HPLC conditions selected for this study, [^{3}H]benzidine and [^{3}H]-*N*-acetylbenzidine were separated from synthetic nucleoside adducts **A** and **B** and were observed in the 1-butanol extract (Figure 5, bottom panel). The [^{3}H]benzidine and [^{3}H]-*N*-acetylbenzidine observed in Figure 5, bottom panel, were probably noncovalently bound to DNA. To further verify the structure of the nucleoside adducts, 1-butanol-extracted nucleosides were treated with potassium carbonate. An extracted sample was spiked with cold **A** and **B**, and an equal amount of this sample was treated with either water or potassium carbonate. HPLC with UV analysis revealed that base treatment converted the synthetic standards *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (**A**) and *N*-(deoxyguanosine-8-yl)-*N,N'*-diacetylbenzidine (**B**) to *N*-(deoxyguanosin-8-yl)benzidine (**C**) and *N'*-(deoxyguanosine-8-yl)-*N*-acetylbenzidine (**D**), respectively. However, the radioactive peaks from the base-treated liver samples were not hydrolyzed to the corresponding peaks of these standards. The recovery of radioactivity in the control (water-treated) and base-treated samples was similar. Thus, the rat liver slices do not contain *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine (**A**) or *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**). Because ^{32}P-postlabeling is reported to be more sensitive than radiolabeled nucleoside analysis, this method was utilized to further analyze rat liver for benzidine adducts. Nucleotide adduct standards for ^{32}P-postlabeling analysis were prepared with 2'-deoxyguanosine 3'-monophosphate instead of 2'-deoxyguanosine (Figure 1).

^{32}P-Postlabeled N-acetylated benzidine nucleotide standards were separated by PEI–cellulose using multicomponent solvent system 1 illustrated in Figure 6. Using this solvent system, *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine does not migrate very far from the origin. However, the other two adduct standards are well separated by this system. The spot near the origin in the top right panel of Figure 6 is probably not *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine. This spot is inconsistently observed in solvent system 1. When this standard is chromatographed using solvent system 2, a spot corresponding to *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine is not observed. Therefore, this spot appears to be an impurity. Solvent system 1 was used to assess *N*-(3'-monophosphodeoxyguanosine-8-yl)-*N*-acetylbenzidine and *N*-(3'-monophosphodeoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine formation by rat liver slices. Neither adduct was detected. To assess the formation of *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine, solvent system 2 was utilized (Figure 7). With this solvent system, *N*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine and *N*-(3'-monophosphodeoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine standards migrated into the wick. At least one adduct was detected in rat liver which cochromatographed with the *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine standard (Figure 7). The rat liver adduct was nuclease P_1 sensitive (not shown). DNA adduct formation was evaluated during 0.014 and 0.05 mM [^{3}H]benzidine incubations in four separate experiments (Table 1). Variation in *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine

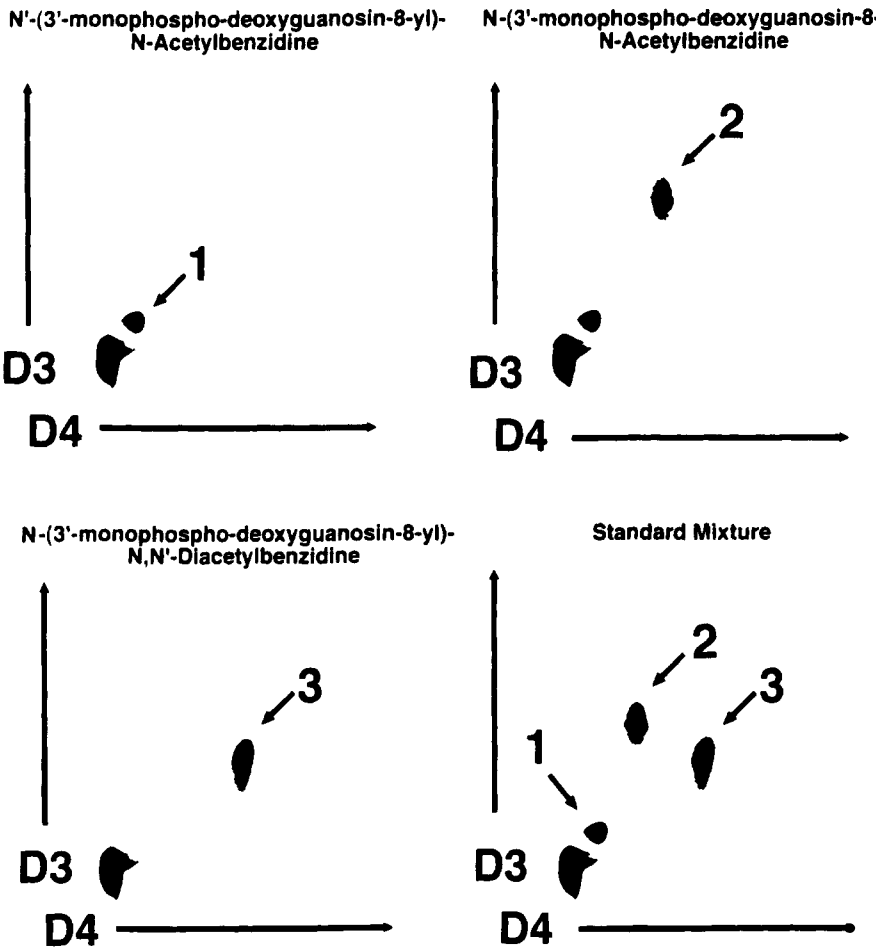

Figure 6. Separation of ^{32}P-postlabeled nucleotide adduct standards (see structures in Figure 1). Multicomponent solvent system 1 was used to separate adduct standards. Autoradiographs for representative PEI–cellulose sheets are depicted.

formation was observed. However, no other adduct was detected. After a 1-h incubation, the amounts of adduct detected at 0.014 and 0.050 mM [^{3}H]benzidine were 5 ± 0.6 and 66 ± 30 amol/μg of DNA, respectively. Similar results were also observed after 4 h. In general, the more benzidine added the more adduct detected. These results are consistent with rat being an acetylating species.

Dog liver samples were also analyzed by HPLC and ^{32}P-postlabeling. In Figure 8, the minor peak of material eluting at 10.7 min does not correspond to any of the synthetic benzidine nucleoside standards. Using ^{32}P-postlabeling, no adducts were detected using either solvent system (not shown). Thus, no adducts corresponding to the three acetylated benzidine adduct standards were detected in dog liver. This is consistent with dog being a nonacetylator with respect to benzidine.

Discussion

Results are consistent with the Fisher 344 rat being a rapid acetylator (*24*) and the dog a nonacetylator (*5*, *6*). In rat, benzidine was rapidly converted to *N*,*N*′-diacetylbenzidine with little *N*-acetylbenzidine detected. The only adduct detected in rat was *N*′-(3′-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine. This adduct can be formed from *N*′-hydroxy-*N*-acetylbenzidine by O-esterification or hydroxy abstraction (*8*, *9*). In addition, it can be formed from *N*′-hydroxy-*N*,*N*′-diacetylbenzidine by intramolecular *N*,*O*-acyl transfer. If *N*′-hydroxy-*N*,*N*′-diacetylbenzidine was, in part, responsible for producing the liver adduct, one would also expect O-esterification of this compound to yield *N*-(3′-monophosphodeoxyguanosin-8-yl)-*N*,*N*′-diacetylbenzidine. However, the latter was not detected. Possible reasons for this adduct not being detected include *N*′-hydroxy-*N*,*N*′-diacetylbenzidine may not be involved in adduct formation, O-esterification may not occur, or the adduct may be formed, but rapidly excised.

During synthesis of adduct standards, *N*-acetoxy-*N*,*N*′-diacetylbenzidine (**E**) was found to be relatively stable. After a 6-h incubation with 2′-deoxyguanosine in an aqueous solution at 37 °C, the ratio of *N*-acetoxy product to adduct was only 1:3. In an effort to increase adduct yields, the acetoxy was replaced with a 2,6-dichlorobenzoyloxy group. This modification has been shown to increase the yields of the C-8 adducts of *N*-acetyl-4-aminobiphenyl (*25*). However, the benzoyloxy analogue of *N*,*N*′-diacetylbenzidine was not reactive. The *N*-acetoxy analogue would be derived from O-esterification of *N*′-hydroxy-*N*,*N*′-diacetylbenzidine. Therefore, the O-esterification pathway could be active, but its product may not be sufficiently reactive to form adducts in vivo. On the other hand, *N*′-acetoxy-*N*-acetylbenzidine, the *O*-acetyltransferase product of *N*′-hydroxy-*N*-acetylbenzidine, may be quite reactive and responsible for the adduct observed in rat liver. It is also possible that *N*-acetoxy-*N*,*N*′-diacetylbenzidine (**E**) is more efficiently detoxified by conjugation, i.e., with glutathione.

N-Acetylation is thought to compete with N-oxidation. Because primary arylamines are easier to oxidize than secondary amines, acetylated products are less likely to

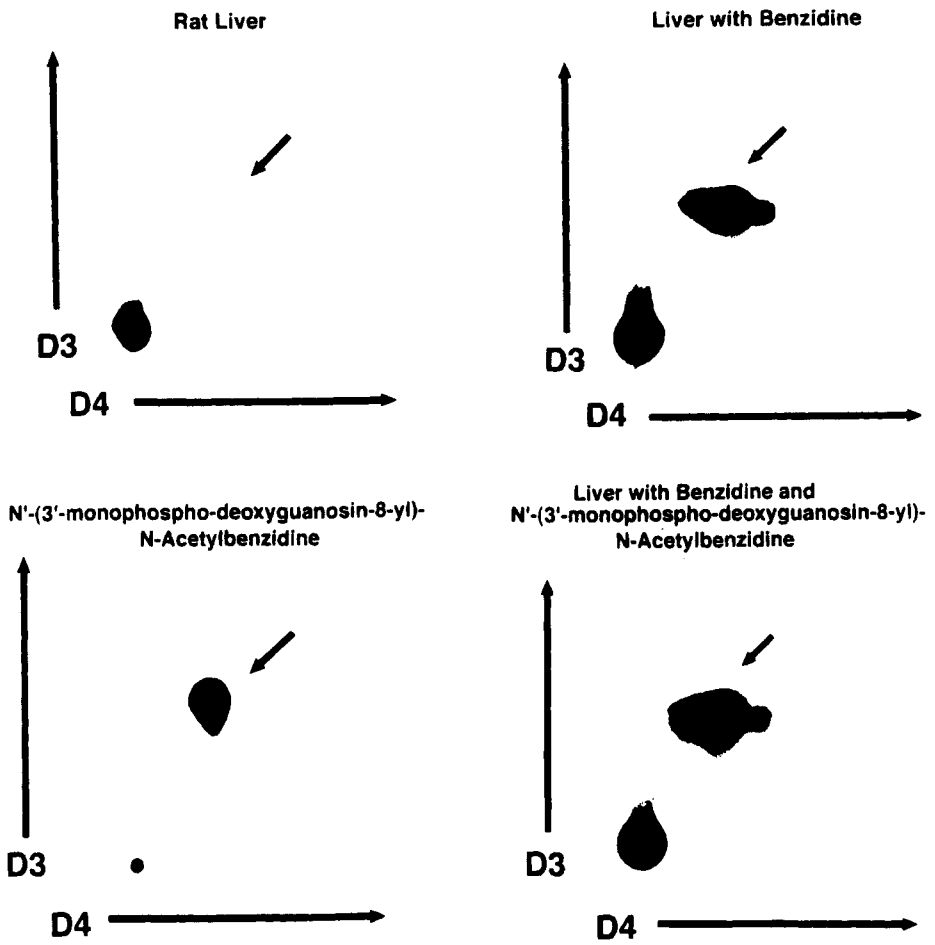

Figure 7. Identification of the benzidine nucleotide adduct in rat liver slices by ^{32}P-postlabeling. Slices were incubated with 0.05 mM benzidine for 1 h. Adducts were sensitive to nuclease P_1 treatment. Only 1-butanol-extracted samples are illustrated. These autoradiographs depict PEI–cellulose sheets developed with solvent system 2.

Table 1.
***N'*-(3'-Monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine Formation by Rat Liver Slices Incubated with Benzidine[a]**

expt	benzidine (mM)	adduct Content (amol/μg of DNA)
1	0.014	5
	0.050	35
2	0.014	4
	0.050	38
3	0.014	6
	0.050	36
4	0.050	157

[a] Rat liver slices (100 mg) were incubated with two different concentrations of [^{3}H]benzidine, in α-MEM (pH 7.4) for 1 h at 37 °C. DNA was prepared from the slices and ^{32}P-postlabeled. The DNA adduct content was calculated by determining the amount of radioactivity obtained in the TLC spot. *N'*-(3'-Monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine adduct content was based on recovery of radioactivity and the specific activity of ATP.

be oxidized than parent compound. Epidemiologic studies suggest that the slow acetylator phenotype in humans is associated with an increased incidence of bladder cancer (*6*, *26*). Recent molecular epidemiologic studies also support this conclusion (*27*). Hemoglobin adducts of 4-aminobiphenyl are higher in slow acetylators. Thus, N-acetylation is considered a detoxification step for aromatic amine-induced bladder cancer in humans.

In contrast to most aromatic amine bladder carcinogens, benzidine is a diamine, rather than a monoamine. Benzidine is not oxidized by mixed-function oxidases or flavin monoxygenases (*28*–*30*). In contrast, N-acetylated benzidine analogues undergo N-oxidation. N-Oxidation of *N*-acetylbenzidine is 48-fold faster in rat than that of *N,N'*-diacetylbenzidine (*31*). A recent study demonstrated greater than a 100-fold increase in the incidence of bladder cancer in Chinese workers with high exposure to benzidine (*32*). However, when members of the benzidine-exposed cohort were screened for their acetylator phenotype and genotype, no positive association was found between N-acetylation phenotype or genotype and bladder cancer risk in workers due to benzidine exposure (*33*). Therefore, the initial acetylation of benzidine to *N*-acetylbenzidine could be considered an activation rather than an inactivation step. Further acetylation of *N*-acetylbenzidine to *N,N'*-diacetylbenzidine may be an inactivation step.

The adducts and mechanism of metabolism of benzidine by dog liver remain largely undetermined. Metabolism does not appear to involve peroxidation. Peroxidation has been shown to yield *N*-(deoxyguanosin-8-yl)benzidine (**C**) as a major product (*12*). The latter was not detected in liver (Figure 8). The corresponding nucleotide adduct standard was not available for ^{32}P-postlabelling analysis. N-Acetylation does not appear to be a significant pathway in dog. *N*-Acetylbenzidine and *N,N'*-diacetylbenzidine were not detected in media, and acetylated adducts were not observed. Compared to dog and human, the rat liver exhibits a reduced ability to glucuronidate benzidine or *N*-acetylbenzidine (*18*, *34*). Glucuronidation results in hepatic detoxification and

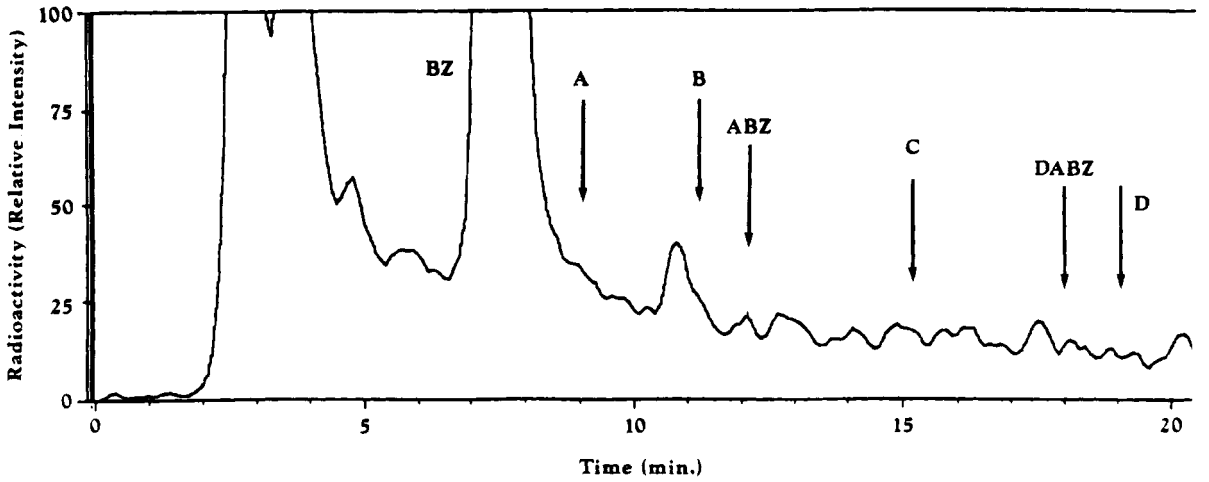

Figure 8. HPLC profile of nucleoside adducts from dog liver slices. Slices were incubated in a manner identical to that in Figure 4 with 0.05 mM ^{3}H-benzidine. The radioactivity of 1-butanol-extracted samples is illustrated. Arrows indicate elution time of synthetic standards. Benzidine, BZ; *N*-acetylbenzidine, ABZ; *N,N'*-diacetylbenzidine, DABZ; *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine, A; *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine, B; *N*-(deoxyguanosin-8-yl)benzidine, C; *N'*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine, D.

excretion into the bladder lumen. The *N*-glucuronides of benzidine and *N*-acetylbenzidine have $t_{1/2}$ values of 3–5 min at pH 5.3 and 37 °C. According to a new hypothesis, *N*-acetylbenzidine and benzidine are released from their glucuronides by acidic urine and further activated within the bladder to initiate carcinogenesis (*34*). The rats' reduced ability to form *N*-glucuronides and then excrete these arylamine carcinogens may contribute to their high incidence of benzidine-induced liver tumors and low incidence of bladder tumors (*1*, *2*). The dogs' ability to form benzidine *N*-glucuronide may contribute to their high incidence of bladder rather than liver cancer.

The ^{32}P-postlabeling analysis of nucleotides were more sensitive, and also more selective than nucleoside analysis of benzidine adducts. One problem with nucleoside analysis is the large amount of radioactivity incorporated into ^{3}H-benzidine metabolites relative to the very small amount of radioactivity incorporated into nucleoside adducts and recovered during HPLC analysis. Some of these metabolites coelute during HPLC with synthetic nucleoside standards. To overcome this problem, alkaline hydrolysis was used to convert the presumed tissue adducts to other known nucleoside adducts. This hydrolysis added specificity to the analysis and indicated that the radioactive peaks did not correspond to the standards. Unless HPLC separations are carefully monitored, *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**) can overlap with *N*-acetylbenzidine (Figure 5). Because the latter is a constant contaminant of 1-butanol extracts, this problem needs to be recognized or a false positive may be recorded for *N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**). It is not clear if this possible contaminant was considered in the study which observed rat liver slice [^{3}H]-*N*-(deoxyguanosin-8-yl)-*N,N'*-diacetylbenzidine (**B**) (*9*). Because the latter study purified DNA by hydroxyapatite chromatography, this may have reduced the amount of *N*-acetylbenzidine contamination.

Although other studies have assessed benzidine adducts using ^{32}P-postlabeling (*14*, *35*), the identity of the adduct was not unambiguously determined. That is, the standard used for analysis was prepared by reacting *N'*-hydroxy-*N*-acetylbenzidine with DNA. While this reaction would yield *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine, it is not known if the multiple TLC systems used adequately separated this adduct from two other potential N-acetylated adducts. For this reason, three N-acetylated benzidine C-8 deoxyguanosine nucleotide adducts were synthesized (Figure 1). Multiple TLC systems separated these standards, allowing unambiguous determination of *N'*-(3'-monophosphodeoxyguanosin-8-yl)-*N*-acetylbenzidine as the only rat liver adduct. Since similar TLC systems were used in other studies (*14*, *35*), it is likely that this is the same adduct they identified.

In conclusion, the results provide support for the dog being a nonacetylator with respect to benzidine. However, other aromatic amines, such as 2-aminofluorene, may be substrates for dog *N*-acetyltransferase (*10*). The availability of acetylated benzidine nucleotide standards and their separation by thin-layer chromatography using multicomponent solvent systems allowed unambiguous proof that *N'*-(3'-monophosphodeoxyguanosine-8-yl)-*N*-acetylbenzidine was the adduct present in rat liver slices. These acetylated nucleotide adduct standards will be useful in subsequent studies in animals and human. N-Acetylation of benzidine, but not of *N*-acetylbenzidine, may be an activation step for benzidine-induced carcinogenesis in rat.

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