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# Primary and Secondary Metabolism of Pentamidine by Rats

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**The antiprotozoal drug pentamidine [1,5-bis(4′-amidinophenoxy)pentane] has been previously shown to be metabolized by rat liver microsomes, and five of the seven putative primary metabolites have been identified. With the synthesis and identification of 5-(4′-amidinophenoxy)pentanoic acid and 5-(4′-amidinophenoxy)-1-pentanol as the remaining two metabolites, the primary metabolism of pentamidine in rats appears fully characterized. Use of [$^{14}$C]pentamidine with rat liver microsomes confirms this conclusion, since no unidentified radioactive peaks were detected by high-performance liquid chromatography (HPLC). Isolated, perfused rat livers were used with [$^{14}$C]pentamidine to identify secondary metabolites. Only two novel radioactive peaks were detected by HPLC analysis of perfused liver samples. The treatment of liver samples with sulfatase or β-glucuronidase resulted in the reduction or elimination of these peaks and gave rise to peaks identified as *para*-hydroxybenzamidine and 5-(4′-amidinophenoxy)pentanoic acid. It was concluded from these results that only these two primary metabolites were conjugated with sulfate or glucuronic acid. After 4 h of incubation in the perfused liver system, approximately 15% of the recovered radiolabel was pentamidine. These results suggest that pentamidine metabolism can be rapid and extensive in rats.**

Pentamidine [1,5-bis(4′-amidinophenoxy)pentane] has been used for decades in the prophylaxis and treatment of African trypanosomiasis and treatment of antimony-resistant leishmaniasis and *Pneumocystis carinii* pneumonia (11, 20). The increased incidence of *P. carinii* pneumonia associated with the AIDS epidemic has brought about an increase in the clinical use of pentamidine in North America (15). Despite several early studies of the pharmacological properties of the drug (14, 16, 17, 22, 23), little was known about the compound. With the development of sensitive and accurate high-performance liquid chromatography (HPLC) assays (1, 9, 18), more detailed studies on the distribution and pharmacokinetics of pentamidine have been conducted (2, 7, 8). However, these studies have been performed with the misconception that pentamidine is metabolically inert, a conclusion primarily based on the work of Launoy et al. (16, 17).

We have previously demonstrated that pentamidine is readily converted to seven putative metabolites by rat liver microsomes (3, 4). Five of the seven primary metabolites have been identified as hydroxylated derivatives of the parent compound (see Fig. 1). The cytochrome P-450-dependent mixed-function oxidases have been identified as the enzyme system responsible for this activity (3). In this paper, we describe the characterization of the two remaining primary metabolites of pentamidine. In addition, isolated, perfused rat livers were used to determine the secondary metabolic pathways and to determine the extent of pentamidine metabolism in a model in which both primary and secondary metabolic systems were active.

## MATERIALS AND METHODS

**Compounds.** Pentamidine isethionate was obtained from Fujisawa USA, Inc. (Rosemont, Ill.), and [$^{14}$C]pentamidine (labelled in the amidino carbons; 32 mCi/mmol) was graciously provided by the National Institutes of Health (Bethesda, Md.). NADPH tetrasodium salt, 1-heptane sulfonic acid sodium salt, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, saccharic lactone, sulfatase, and β-glucuronidase were acquired from Sigma Chemical Co. (St. Louis, Mo.), and tetramethyl ammonium chloride and HPLC-grade acetonitrile were obtained from Fisher Scientific Co. (Fair Lawn, N.J.). All water was deionized and filtered by a water purification system (Darco, Durham, N.C.). The structures of all pentamidine metabolites are shown in Fig. 1.

**Synthesis of 5-(4′-amidinophenoxy)pentanoic acid.** A solution of 5-bromovaleric acid (Aldrich Chemical Co., Milwaukee, Wis.) (6.0 g, 0.03 mol) in absolute ethanol (150 ml) and a few drops of $H_2SO_4$ was heated under reflux for 24 h. The ethanol was removed under a vacuum to afford 6.7 g (97%) of ethyl-5-bromo-pentanoate as an oil. Sodium (0.63 g, 27.5 mmol) was dissolved in absolute ethanol (20 ml), and 4-cyanophenol (3.0 g, 25 mmol) was added. The solution was heated at reflux for 30 min before ethyl-5-bromo-pentanoate (6.67 g, 32 mmol) in absolute ethanol (10 ml) was added and the solution was heated under reflux for 60 h. The ethanol was removed under reduced pressure, and the resulting ethyl-5-(4′-cyanophenoxy)pentanoate was recrystallized from ethanol-$H_2O$ to afford 4.5 g (73%) of the desired product as a white solid.

A suspension of ethyl-5-(4′-cyanophenoxy)pentanoate (2.0 g, 8.1 mmol) in dry dioxane (100 ml) and dry methanol (2 ml) was chilled to 5°C, and the solution was saturated with HCl (gas) for 1.5 h, with the temperature maintained below 10°C. The flask was stoppered, and the reaction mixture was stirred at room temperature for 9 days. During this time, the solution was resaturated twice and additional methanol (4 ml) was added. After an infrared spectrum indicated that the nitrile had disappeared, the dioxane was removed under reduced pressure and the resulting white solid was suspended in anhydrous diethyl ether and collected by filtration. This imidate was immediately suspended in anhydrous meth-

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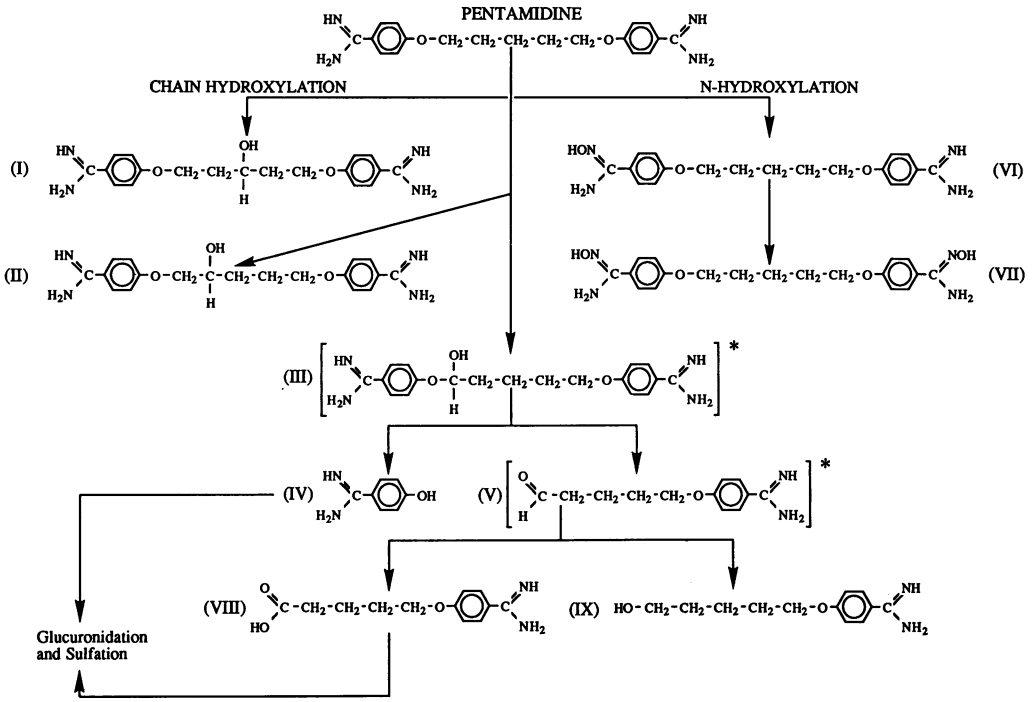


FIG. 1. Metabolism of pentamidine. Compounds: I, 1,5-bis(4'-amidinophenoxy)-3-pentanol; II, 1,5-di(4'-amidinophenoxy)-2-pentanol; III, 1,5-di(4'-amidinophenoxy)-1-pentanol; IV, *para*-hydroxybenzamidine; V, 5-(4'-amidinophenoxy)-1-pentanal; VI, 1-(4'-hydroxyamidinophenoxy)-5-(4'-amidinophenoxy)pentane; VII, 1,5-bis(4'-hydroxyamidinophenoxy)pentane; VIII, 5-(4'-amidinophenoxy)pentanoic acid; IX, 5-(4'-amidinophenoxy)-1-pentanol. Brackets and asterisks indicate that compounds III and V have not been isolated.

anol saturated with ammonia (75 ml), the flask was stoppered, and the solution was heated to an oil bath temperature of 80°C for 6 h. The heat was removed, and the solution was stirred at room temperature for 18 h. The solvent was removed under reduced pressure, and the resulting solid was dissolved in 2 N HCl (50 ml) and heated at reflux for 22 h. The solution was cooled to room temperature, and the solid was removed by filtration. The product remained predominantly in the aqueous solution, so the solvent was removed under reduced pressure to afford 482 mg of the desired product. The solid collected by filtration was heated with additional water and filtered while hot. Removal of the solvent under reduced pressure yielded 455 mg of additional product for a total of 937 mg (42%) of 5-(4'-amidinophenoxy)pentanoic acid. The melting point was 219 to 221°C. Nuclear magnetic resonance (90 MHz, dimethyl sulfoxide-$d_6$) 1.7 (m, 4H, internal $CH_2CH_2$); 2.25 (t, 2H, $CH_2CO$); 4.2 (t, 2H, $CH_2O$); 7.2 (d, 2H, Ar-H); 7.9 (d, 2H, Ar-H); 9.3 (d, 4H, Am-H). Elemental analysis was calculated for $C_{12}H_{16}N_2O_3 \cdot HCl$. Calculated: C, 52.84; H, 6.28; N, 10.27. Found: C, 52.75; H, 6.33; N, 10.17.

**Synthesis of 5-(4'-amidinophenoxy)-1-pentanol.** Sodium (0.43 g, 18.5 mmol) was dissolved in dry ethanol (15 ml), and 4-cyanophenol (Aldrich Chemical Co.) (2.0 g, 16.8 mmol) in ethanol (5 ml) was added. The solution was heated under reflux for 30 min. 5-Bromo-1-pentene (4.0 ml, 5.0 g, 33.6 mmol) was added, and the reflux was continued for 40 h. The solution was cooled on an ice bath, and the inorganic material was removed by filtration and washed with methylene chloride. The methylene chloride was extracted several times with 15% NaOH to remove residual starting phenol. The ethanol and methylene chloride were combined and dried with $MgSO_4$. The drying agent was removed by filtration, and the mother liquor was evaporated to dryness to afford an oil. The oil was chromatographed on silica gel (80 g) by elution with chloroform. The appropriate fractions were combined, and the solvent was removed under reduced pressure to yield 2.4 g (78%) of 5-(4'-cyanophenoxy)-1-pentene. A solution of 5-(4'-cyanophenoxy)-1-pentene (2.5 g, 13.4 mmol) in dry tetrahydrofuran (50 ml) was chilled to 0°C in dry glassware under $N_2$. $BH_3$ (1.0 M) in tetrahydrofuran (6.7 ml) was added dropwise via a syringe at a rate sufficient to maintain the low temperature. After the addition was complete, the solution was allowed to warm to room temperature and was stirred at room temperature for 3 h. Water (0.75 ml) was added slowly, the solution was chilled to 0°C, and 3.0 M NaOH (2.5 ml) was added all at once. The reaction mixture was warmed to room temperature, and 30% $H_2O_2$ was added at a rate that maintained the temperature of the reaction mixture below 40°C. After the addition was complete, the reaction mixture was warmed to 50°C for 1 h. The reaction mixture was cooled to room temperature, and the tetrahydrofuran was washed with $H_2O$ (25 ml). The $H_2O$ was extracted with diethyl ether (three 25-ml portions), and the organic fractions were combined and dried ($MgSO_4$). The inorganic material was removed by filtration, and the solvents were removed in vacuo to afford 3.1 g of impure material. The compound was purified by centrifugal chromatography by elution from silica gel plates with 4:1 ethyl acetate-hexanes. The appropriate fractions were combined, and the solvents were removed under reduced pressure to yield 2.35 g (85%) of 5-(4'-cyanophenoxy)-1-pentanol as a clear oil.

A solution of 5-(4'-cyanophenoxy)-1-pentanol (1.93 g, 9.4 mmol) in dry dioxane (100 ml) and methanol (2 ml) was cooled on an ice bath to a temperature of 10°C. The solution

was saturated with HCl (gas) for 1 h. The flask was stoppered, and the solution was stirred at room temperature for 20 h. The dioxane was removed in vacuo, and the resulting solid was stirred with diethyl ether and collected by filtration under $N_2$. The solid was dissolved in ethanol saturated with $NH_3$ (100 ml), the flask was stoppered, and the solution was heated to an oil bath temperature of 80°C for 6 h. The solution was stirred at room temperature for 14 h. The ethanol was removed under reduced pressure to afford 1.0 g (41%) of 5-(4'-amidinophenoxy)-1-pentanol. The melting point was 178 to 182°C. Nuclear magnetic resonance (300 MHz, dimethyl sulfoxide-$d_6$) 1.4 to 1.6 (m, 4H, HO-$CH_2\underline{CH_2}$, ArO-$\underline{CH_2}CH_2$); 1.75 (q, 2H, internal $CH_2$); 3.43 (t, $\overline{2H}$, $HOC\overline{H_2}$); 4.08 (t, 2H, $ArOCH_2$); 4.4 (br s, 1H, HO); 7.15 (d, 2H, Ar-H); 7.83 (d, 2H, $\overline{Ar\text{-}H}$); 8.98 (s, 2H, Am-H); 9.21 (s, 2H, Am-H). Elemental analysis was calculated for $C_{12}H_{18}N_2O_2 \cdot HCl$. Calculated: C, 55.70; H, 7.40; N, 10.83. Found: C, 55.71; H, 7.45; N, 10.74.

**Preparation of microsomes.** Male, barrier-raised Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottsdale, Pa.) were housed in a controlled environment with a 12-h light–12-h dark cycle and allowed free access to rat chow (Agway, Syracuse, N.Y.) and tap water. All food was removed 24 h before the animals were sacrificed by decapitation. The livers were excised immediately, rinsed with 50 mM phosphate ($KH_2PO_4$-$K_2HPO_4$) buffer (pH 7.4), and placed on ice. All subsequent steps were performed at 4°C. The livers were minced with scissors and homogenized with 2 volumes of 50 mM phosphate buffer (pH 7.4) with a Teflon pestle-glass tube homogenizer (A. H. Thomas Co., Philadelphia, Pa.). The homogenates were centrifuged at 9,000 × *g* for 20 min, and the supernatant was subsequently centrifuged at 100,000 × *g* for 60 min. The resulting microsomal pellet was resuspended in 50 mM phosphate buffer (pH 7.4).

**Incubation and assay system.** One milliliter of microsomal preparation was added to 3 ml of phosphate buffer (pH 7.4) and 1 ml of cofactor solution (2 mg of NADPH per ml, 1.3 mg of glucose-6-phosphate per ml, and 0.1 U of glucose-6-phosphate dehydrogenase per ml in phosphate buffer [pH 7.4]) and preincubated for 1 to 2 min at 37°C in a shaking water bath. One milliliter of pentamidine or [$^{14}$C]pentamidine (various concentrations) in phosphate buffer (pH 7.4) was added to start the incubation. The reactions were terminated by placement on ice.

Routine analysis of samples was performed as described by Berger et al. (3, 4). Briefly, a Hewlett-Packard 1090 or 1084B liquid chromatograph (Hewlett-Packard, Avondale, Pa.) was used with a Zorbax RX diisopropyl C-8 column (25 cm by 4.6 mm) (Mac-Mod Analytical, Chadds Ford, Pa.) kept at 40°C. Elution was performed with a gradient of 3.75 to 45% $CH_3CN$ (in 10 mM heptane sulfonate–10 mM tetramethylammonium chloride–4.2 mM $H_3PO_4$-$H_2O$) over 30 min. Detection was by UV spectrophotometry at 265 nm. This assay was sensitive to 1 ng of pentamidine per ml and was linear over the entire range of concentrations analyzed. The assay had an error of less than 5% on inter- and intraday analyses. Coelution of synthesized metabolites with suspected metabolite peaks from pentamidine incubations was detected by using the Zorbax RX column, the Supelco DB-C18 column (Supelco, Bellefonte, Pa.), the Keystone Scientific Hypersil2 phenyl column, and the Keystone Scientific Hypersil2 cyano column (Keystone Scientific, Bellefonte, Pa.) as described by Berger et al. (4).

Initially, samples were extracted over Prep-Sep C-18 solid phase extraction cartridges (Fisher Chemical Co., Fair Lawn, N.J.) prior to HPLC analysis (1). However, higher overall recovery was obtained through direct injection of microsomal incubations onto the HPLC. An in-line filter unit (Rheodyne 7335 inlet filter; Rheodyne, Cotati, Calif.) was used to protect the column from the increased particle load.

When microsomal samples were incubated with [$^{14}$C]pentamidine, 30-s fractions (0.75 ml) were collected from the HPLC after passing through the UV spectrophotometric detector. Aliquots of 200 μl from each fraction were then added to 3 ml of Cyto-Scint scintillation fluid (ICN Biomedicals, Costa Mesa, Calif.) and placed in a Beckman LS 7000 scintillation counter (Beckman, Norcross, Ga.) for measurement.

**Isolated, perfused rat liver incubations.** Male, viral-antigen-free, Sprague-Dawley rats (250 to 275 g) (Charles River, Kingston, N.Y.) were housed in well-ventilated cages and kept at 24 ± 2°C with 40 to 60% humidity while on a regular 12-h light-dark cycle for a minimum of 14 days before experiments. They were fed ad libitum on standard rat chow and tap water. At all times during this study, pain to animals was minimized through the use of anesthesia and analgesia.

Rats were anesthetized with ketamine (40 mg per kg of body weight) and xylazine (5 mg/kg) by intraperitoneal injection, and their livers were isolated according to a standard technique with modifications (19). The liver was placed on a glass platform inside a thermostatically controlled (37 ± 0.1°C) perfusion cabinet. Livers were perfused with Krebs bicarbonate buffer solution containing 20% fresh (1- to 5-day-old), washed sheep erythrocytes, 1% bovine serum albumin, and 0.1% glucose. This buffer was recirculated from a central reservoir by a roller pump (Minipuls-2; Gilson Int.) in a closed system, at a perfusion rate of 1.5 ml/g of liver weight per min. Prior to each experiment, all glassware and tubing were silanized with Aquasil (Pierce, Rockford, Ill.). Sodium taurocholate (30 μmol/h) was continuously infused (Syringe Infusion Pump no. 22; Harvard Apparatus) throughout the experiment to simulate enterohepatic bile acid cycling and to normalize the composition of bile (25). The perfusate was oxygenated with a mixture of humidified $O_2$-$CO_2$ (95/5) which was passed through an artificial lung composed of Silastic tubing (Dow Corning, Midland, Mich.). The perfusate pH was continuously monitored with a Radiometer ETS811,822 endpoint titration system (Radiometer, Copenhagen, Denmark), which maintained the pH at 7.4 ± 0.02 with sodium bicarbonate (2.5 M). Perfusate partial pressures of $O_2$ and $CO_2$ were measured with a Corning 168 blood gas analyzer at the beginning and end of each experiment. All experiments were performed after a 20-min stabilization period. The principal indices of liver viability were steady oxygen consumption (21), sustained bile production (6), stable potassium and alanine aminotransferase activity (12), stable pH, a portal pressure of less than 8 cm of $H_2O$, and normal visual appearance.

The metabolism and distribution of [$^{14}$C]pentamidine in the liver were studied for 4 h after a 5-mg/kg bolus injection ($n$ = 4). Labelled pentamidine (diluted with radioinert pentamidine to a specific activity of 27 μCi/mg) was added directly to the reservoir as a 5-mg/ml solution in saline. Samples (1 ml) were removed from the perfusate reservoir predose and at 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min postdose. The sample volume was replaced with an equal volume of fresh perfusate added directly to the reservoir. After centrifugation (1,100 × *g* for 3 min), the perfusate plasma was separated and stored at −70°C until it was assayed for pentamidine, its metabolites, and radioactivity. Bile was collected into preweighed vials at 30, 60, 90, 120, 150, 180, 210, and 240 min after the dose. The bile

volume was determined by weight (assuming a density of 1.0 g/ml) and stored at −70°C. The whole liver from each experiment was stored at −70°C until homogenization and fractionation.

Binding of the drug to the perfusion apparatus was evaluated after each experiment. The perfusate was pumped from the apparatus, and all glassware was washed with normal saline (twice), sodium hypochlorite solution, and methanol by forced syringe irrigation. Each washing solution was separately recirculated through the apparatus for 20 min. Samples of each type of tubing and of each of the washes were taken pre- and postperfusion for determination of radioactivity not removed by the washes.

Radioactivity was measured with a Packard 2500 liquid scintillation counter with correction for chemiluminescence and quench. Samples of whole perfusate, perfusate plasma, and washes (100 μl each) and tubing (3 cm) and bile (25 μl) were incubated with 600 μl of 30% $H_2O_2$ and 300 μl of 70% perchloric acid in a heated (60°C) water bath for 4 h. Scintillation fluid (10 ml) was added, and samples were cooled overnight and then assayed for radioactivity. Samples of whole liver homogenate and subsequent fractions were solubilized and decolorized as described above before being assayed for radioactivity.

**Analysis of radioactive rat liver samples.** Aliquots of samples from the isolated, perfused rat liver incubations were analyzed by HPLC with photodiode array UV and radiometric detection. The column (Zorbax RX), buffers, and run conditions were as described above for the microsomal HPLC method, with the column connected to a Waters 6000 solvent delivery system (Waters, Milford, Mass.) controlled by a Waters Expert Ease 860 operating system. A Waters temperature control system was used to keep the column at 40°C, and samples were injected by the Waters WISP 710 autoinjector. Effluent from the column passed through a Hewlett-Packard 1040 photodiode array detector controlled by the Hewlett-Packard Chemstation operating system. This detector scanned 180 to 400 nm every 640 ms. The effluent from the photodiode array detector then passed through a Ramona LS liquid cell radiochemical detector (Service Corporation, Fairfield, N.J.) with Hydrofluor (National Diagnostics, Manville, N.J.) liquid scintillation fluid.

**Identification of secondary metabolites.** The bile samples collected from the isolated, perfused rat livers were used to characterize conjugated metabolites. Aliquots of 200 μl from each of the bile samples were mixed with 400 μl of either 0.2 M acetate buffer (pH 5), 2,500-U/ml β-glucuronidase in 0.2 M acetate buffer (pH 5), or 660-U/ml sulfatase in 0.2 M acetate buffer (pH 5). Saccharic lactone (20 mM) was added to the stock sulfatase solution to inhibit any contaminating β-glucuronidase activity. The samples were then incubated at 37°C for 7 h in a shaking water bath. Portions of each sample were then analyzed by the radioactive HPLC method described above.

## RESULTS

**Identification of primary metabolites.** A chromatogram from the HPLC analysis of a 1-h incubation of pentamidine with rat liver microsomes is shown in Fig. 2. The peaks labelled a to g are the putative metabolites of pentamidine (peak p). Peaks a, d, e, f, and g have been previously identified as compounds IV (Fig. 1), I, II, VII, and VI, respectively (3, 4). Since metabolite IV likely resulted from hydroxylation of pentamidine at the carbon adjacent to the ether bond followed by cleavage of the unstable hemiacetal moiety, it was possible that the two unidentified metabolite peaks (b and c in Fig. 2) represented the remaining portion of the molecule. For this reason, attempts to synthesize compound V and its two potential conversion products, VIII and IX, were made.

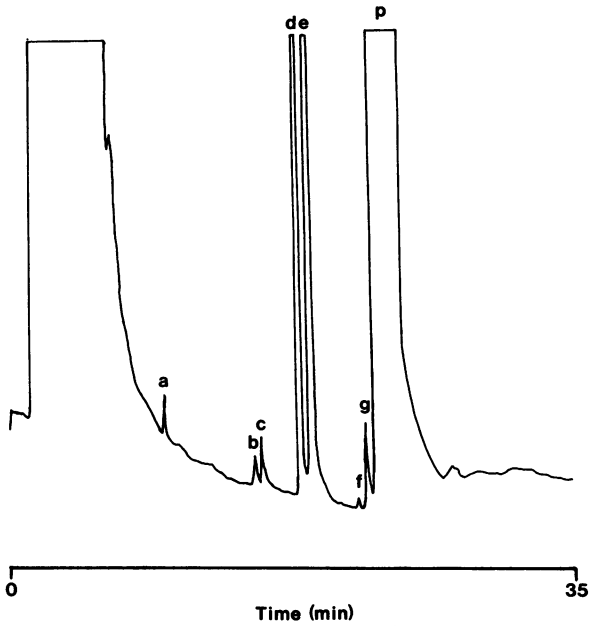


FIG. 2. Chromatogram of a microsomal pentamidine incubation. Pentamidine (0.17 mM) was incubated with rat liver microsomes for 1 h at 37°C. HPLC analysis was as described in Materials and Methods. Labelled peaks: a, *para*-hydroxybenzamidine (IV); b and c, unidentified metabolites; d, 1,5-bis(4′-amidinophenoxy)-3-pentanol (I); e, 1,5-di(4′-amidinophenoxy)-2-pentanol (II); f, 1,5-bis(4′-hydroxyamidinophenoxy)pentane (VII); g, 1-(4′-hydroxyamidinophenoxy)-5-(4′-amidinophenoxy)pentane (VI); p, pentamidine.

The last two compounds were synthesized and found to coelute with the two unidentified metabolite peaks as shown in Fig. 3. The coelution analyses were also performed on three other columns (octadecyl silane, phenyl silane, and cyano silane), and the two compounds were found to coelute in each case. These results strongly indicate that the remaining two primary metabolites of pentamidine are compounds VIII and IX.

**Mass balance of microsomal metabolism.** [$^{14}$C]pentamidine was used with rat liver microsomes to determine whether the seven metabolites identified to date accounted for the entirety of the primary metabolism of the drug. Preliminary experiments were performed to quantify the losses during the C-18 extraction process by monitoring the extraction of a [$^{14}$C]pentamidine incubation. Of the starting radioactivity, 69% was recovered in the eluate and 31% was recovered elsewhere (5% during loading, 1% in the $CH_3CN$ wash, 2% in the $H_2O$ wash, and 23% bound to the cartridge itself). Since a significant portion of the parent compound and its metabolites could be lost by extraction, samples were no longer subjected to C-18 extraction and were directly injected onto the HPLC. Table 1 shows the results of two different [$^{14}$C]pentamidine incubations with the same preparation of rat liver microsomes as the enzyme source. The samples were analyzed by HPLC followed by liquid scintillation of 30-s fractions collected from the column. While 2.5% of the total radioactivity was found as background (low-level radioactivity found in all the fractions), no addi-

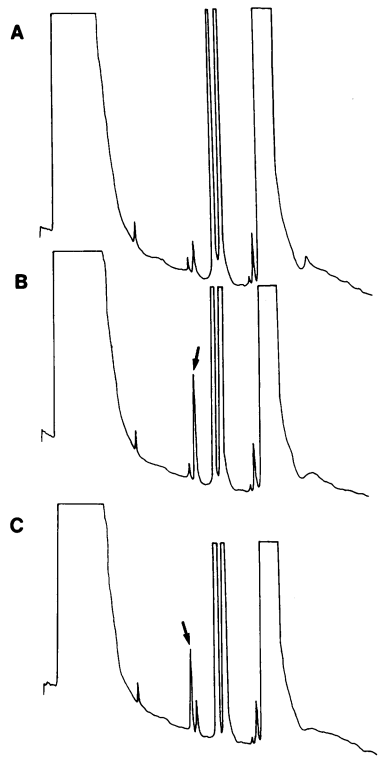


FIG. 3. Coelution of compounds VIII and IX with metabolite peaks. Pentamidine (0.17 mM) was incubated with rat liver microsomes for 1 h at 37°C and analyzed by HPLC as described in Materials and Methods. (A) Chromatogram of the pentamidine incubation; (B) chromatogram of a sample of the same incubation with synthesized compound IX added immediately before injection; (C) chromatogram of the same incubation with synthesized compound VIII added immediately before injection. Arrows indicate the peaks increased by adding standard compounds.

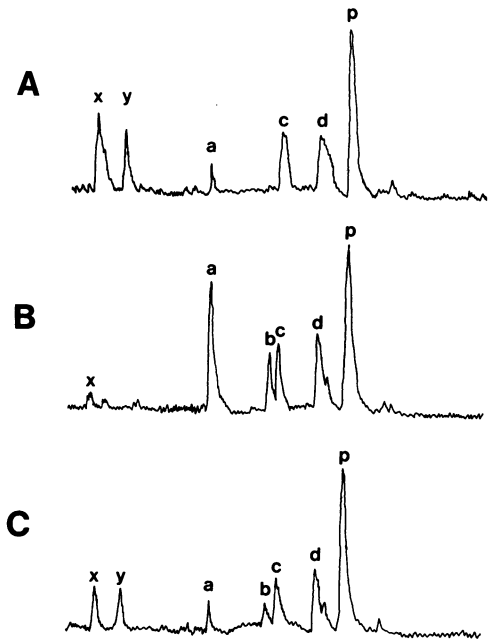


FIG. 4. Identification of secondary metabolites in a bile sample. [$^{14}$C]pentamidine (5 mg/kg) was recirculated in isolated, perfused rat livers, and 30-min bile samples were collected for 4 h. The bile from three livers was pooled to create composite samples, and aliquots from each time point were analyzed by HPLC-radiochemical detection as described in Materials and Methods. The chromatograms shown are from the 180- to 210-min composite bile sample. Samples of 200 μl of bile and 400 μl of either acetate buffer alone (control) (A), β-glucuronidase in acetate buffer (B), or sulfatase in acetate buffer (C) were incubated for 7 h at 37°C. Labelled peaks: x and y, secondary metabolites; a, compound IV; b, compound VIII; c, compound IX; d, compounds I and II; p, pentamidine compounds 6 and 7.

tional radioactive peaks beyond those associated with pentamidine and its known metabolites were discovered. Therefore, it is unlikely that there are any remaining unidentified primary metabolites of pentamidine.

**Secondary metabolism of pentamidine.** After determination of the primary metabolism of pentamidine, it was important to determine whether the primary metabolites were further metabolized. To accomplish this aim, [$^{14}$C]pentamidine was added to the perfusate of the isolated, perfused rat liver system. Under these conditions, for a period of 4 h, the drug was subject to active primary and secondary metabolic enzymes. By comparing chromatograms of bile or perfusate from the livers to chromatograms of microsomal incubations, it is possible to identify novel HPLC peaks that may be conjugated metabolites. Analysis of samples from perfused livers ($n$ = 8) showed two novel radioactive peaks in both bile and perfusate (Fig. 4A). Preincubation of the samples with sulfatase or β-glucuronidase decreased or eliminated these peaks (Fig. 4B and C). The conjugate peaks detected (peaks x and y in Fig. 4) appear to be mixtures of sulfate and glucuronide conjugates. As these conjugate peaks decreased, there was a corresponding increase in the peaks associated with metabolites IV and VIII. In all the samples analyzed (bile, perfusate, and liver fractions), only these two primary metabolites appeared to be conjugated.

Bile and perfusate samples were collected every 30 min for 4 h and were analyzed by HPLC after treatment with β-glucuronidase or sulfatase. As shown in Fig. 5, approximately 90% of the dosed pentamidine is rapidly cleared from the perfusate. A small portion of the radiolabel is detected in the bile samples over time, but it appears that the majority of the radioactivity remains in the liver. Figure 6 shows the relative excretion of conjugates and intact pentamidine in the

TABLE 1. Metabolism of pentamidine by rat liver microsomes

| Sample | Pentamidine concn (mM) | Incubation time (h) | dpm[a] | | % of recovered radiolabel identified as: | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Injected | Recovered | Pentamidine + VI + VII | I + II | VIII + IX | IV | Background radioactivity |
| 1 | 0.13 | 4 | $5.35 \times 10^7$ | $5.23 \times 10^7$ | 85.08 | 11.32 | 0.70 | 0.55 | 2.35 |
| 2 | 0.03 | 2 | $1.07 \times 10^7$ | $1.55 \times 10^7$ | 69.46 | 26.14 | 0.81 | 0.89 | 2.70 |

[a] dpm, disintegrations per minute.

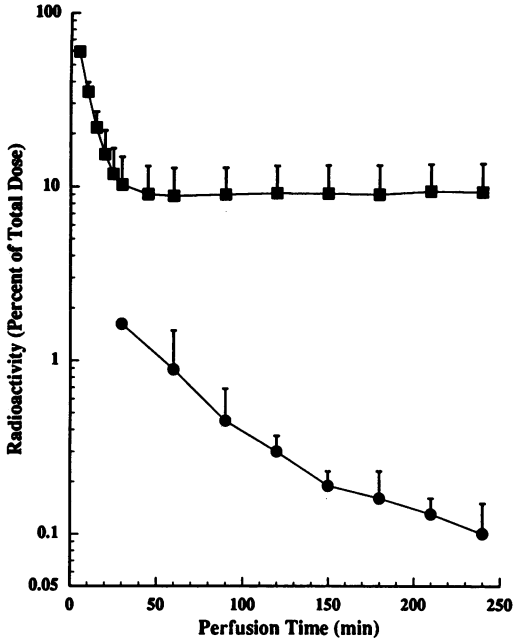


FIG. 5. Uptake of pentamidine by perfused livers. [$^{14}$C]pentamidine (5 mg/kg) was injected into isolated, perfused rat livers, and perfusate and bile samples were collected periodically. Both bile and perfusate samples were analyzed for radiolabel content by liquid scintillation as described in Materials and Methods. Squares, perfusate radioactivity; circles, bile radioactivity. Bars, standard deviations.

bile over time. Almost immediately, pentamidine is found in the bile, and its concentration drops over time as the concentration of conjugates excreted rises. The concentrations of primary metabolites in bile also rose over time (data not shown).

**Metabolism in isolated, perfused rat livers.** In addition to identifying potential secondary metabolites, use of the isolated, perfused rat liver allows a more complete model of metabolism than microsomes without the complications associated with whole-animal studies. All of the fractions from several perfused livers ($n$ = 4) were analyzed by HPLC to determine the overall metabolism of pentamidine. The percentages of the radiolabel recovered from the analyzed fractions were as follows: bile, 5.39 ± 0.45; perfusate, 11.17 ± 1.35; liver pellet (9,000 × $g$), 35.28 ± 8.78; liver supernatant (100,000 × $g$), 19.40 ± 1.29; and liver pellet (100,000 × $g$), 12.15 ± 2.70. (Values are averages ± standard deviations for four livers.) The overall recovery of the radiolabel was high (87.85% ± 10.06%), and most of the radioactivity was associated with liver samples, rather than with bile or perfusate. Sample loss due to binding to the perfusion apparatus was very low (approximately 3.5%).

Pentamidine is extensively metabolized in this model, with only 15% of the recovered radiolabel being the parent compound. In the recovered radiolabel, the percentages of various compounds found were as follows: pentamidine plus compound VI plus compound VII, 15.46 ± 3.04; compound I plus compound II, 61.75 ± 5.14; compound VII plus compound IX, 0.47 ± 0.16; and compound IV, 6.80 ± 3.50. (Values are averages ± standard deviations for four livers.) The majority of the radioactivity was recovered as metabolites I and II, and a significant proportion of the radiolabel (15.49% ± 1.69%) was detected as conjugated metabolites. Therefore, pentamidine can be rapidly metabolized by rat livers. No radioactive peaks which did not correspond to the known primary metabolites or to the conjugates of compounds IV and VIII were detected in these livers.

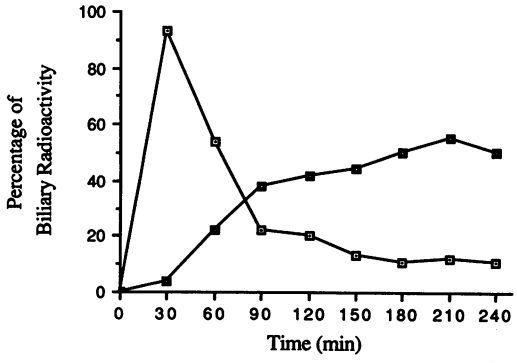


FIG. 6. Excretion of pentamidine and conjugated metabolites in rat bile. [$^{14}$C]pentamidine (5 mg/kg) was injected into isolated, perfused rat livers, and 30-min bile samples were collected. The individual bile samples were analyzed by HPLC-radiochemical detection as described in Materials and Methods. Lines show the relative percentage of biliary radioactivity present as pentamidine (open squares) or conjugated metabolites (closed squares).

## DISCUSSION

Pentamidine has been widely reported to be metabolically inert (5, 10, 11, 13, 20), partly on the basis of interpretations of research conducted by Launoy et al. (16, 17) and Waalkes et al. (22). However, in this study and in previous work (3, 4), we have demonstrated that pentamidine can be rapidly and extensively metabolized by the rat cytochrome P-450, glucuronyl transferases, and sulfotransferases. Figure 1 summarizes the metabolic pathway of pentamidine. In rats, the two major metabolites (quantitatively) are compounds I and II, which do not appear to be conjugated with glucuronic acid or sulfate. Studies conducted with [$^{14}$C]pentamidine in isolated, perfused livers and with rat liver microsomes seem to confirm that the scheme proposed in Fig. 1 represents the complete metabolism of pentamidine, since no radioactive peaks which do not correspond to a known metabolite were found by HPLC. It is possible, though unlikely, that other metabolites may coelute with the identified compounds. To date, we have not been able to synthesize compounds III and V.

Knowledge of the metabolic transformation of pentamidine leads to several questions, all of which are currently under investigation. Most important are the toxicity and antimicrobial activities of each of the metabolites, since pentamidine is utilized clinically and is quite toxic (15). More than 50% of all patients suffer from adverse effects, ranging from mild neutropenia to severe nephrotoxicity (24). We have previously demonstrated that N-hydroxylation of pentamidine leads to a drop in antimicrobial activity (3), and the remaining primary metabolites are currently being tested in a similar manner. Therefore, it will soon be determined whether pentamidine metabolism is an activating or a deactivating system with respect to toxicity and antimicrobial effectiveness.

Another important question relates to comparative metabolic studies. All work to date has been performed with rats, and there is no knowledge as to the potential pathways in

other species, particularly humans. We are presently intensively studying the metabolism of pentamidine in mice, rabbits, and humans. Finally, we reported (4) that the cytochrome(s) P-450 responsible for pentamidine metabolism in rats was not inducible by seven different compounds. We are presently involved in purifying and identifying the pentamidine hydroxylase from rat liver and determining whether the same isozyme is responsible for pentamidine hydroxylation in mouse and rabbit liver and lung tissue.

There is a great need for better compounds in the treatment of *P. carinii* pneumonia and African trypanosomiasis. Knowledge of pentamidine metabolism, its effect on toxicity and antimicrobial activity, and its enzymology will play a large role in the rational development of improved analogs for clinical use.

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