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METABOLISM AND BILIARY EXCRETION OF PHENANTHRIDINIUM SALTS—I

NATURE OF THE BILIARY METABOLITES*

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Abstract—The nature of the biliary metabolites of three phenanthridinium salts—3,8-diamino-6-*p*-aminophenyl-5-methylphenanthridinium chloride (150C47), 3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide (ethidium), and 2-amino-6-*p*-carbethoxyaminophenyl-5-methylphenanthridinium sulfate (carbidium)—has been investigated in rats with ligated renal pedicles. Up to 50 per cent of a small intravenous dose of each of these compounds may be excreted in the bile of rats with ligated renal pedicles in 1 hr depending on the dose and individual variation. At the dose selected, about 15 per cent of the dose was excreted in the first hour after injection. Sixty-five per cent of a 15 mg/kg dose of 150C47 was recovered in the bile in 11–21 hr; 85 per cent of this total was excreted within 5 hr. Of the material recovered, approximately 15 per cent was unchanged 150C47. The major metabolite present in bile was a monoacetyl amino conjugate and this accounted for about 50 per cent of the material recovered. Two other metabolites were also shown to be present and these are also believed to be acetyl conjugates. One of these is probably a diacetylated compound and accounted for about 25 per cent of the material recovered. The other accounted for the remaining 10 per cent. During the first hour after injection, the material was almost entirely in the form of the major metabolite plus the unchanged compound. After injection of the major monoacetyl metabolite, only the injected monoacetyl compound and the diacetylated one could be detected in bile, and the rate of excretion exceeded the rate of excretion of all forms of 150C47 in control rats given the same dose of 150C47.

Of a 15 mg/kg dose of ethidium, 50–55 per cent was recovered in the bile in 16–18 hr. Unchanged ethidium accounted for 20–25 per cent of the material present. Two metabolites were found and these are both believed to be monoacetyl amino conjugates. They accounted for 65–70 per cent and 10 per cent of the material recovered; 85–90 per cent of this total was excreted within 5 hr. Carbidium is excreted in bile as the unchanged compound and its acetyl conjugate. Only one acetyl conjugate is possible for this compound and it was the only metabolite found in bile. Of a dose of 15 mg/kg, 60–65 per cent was recovered in 10–16 hr. About 20 per cent of the material recovered was unchanged carbidium; the remaining 80 per cent was the acetyl derivative. During the first hour after injection, 55 per cent of the material excreted was unchanged carbidium.

THE PRESENT work was prompted by two separate lines of interest. First, it has been reported that, in addition to the generally accepted phenomenon of active secretion of organic anions into bile, there are also organic cations which may be actively secreted by an independent mechanism.[1,2] The biliary excretion of few organic cations has been studied in detail and, since a number of phenanthridinium compounds are known to be

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rapidly excreted in bile,[3,4] it was felt that they might represent a class of organic cations with numerous available analogs which would be useful in the investigation of organic cation transport into bile.

Second, phenanthridinium compounds, originally of interest as trypanocidal agents,[5] are now being investigated from a number of different viewpoints. Ethidium (3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide), which has been marketed for some time as a drug to treat trypanosomal infections in African cattle, is probably the most studied of these compounds. To cite a few examples of studies involving ethidium: it has been shown to intercalate between the bases of DNA and to cause uncoiling and reverse coiling of circular DNA;[6,7] it causes a specific loss of kinetoplastic DNA in trypanosomes;[8] it specifically inhibits exogenous but not *de novo* synthesized purines from being incorporated into nucleic acids;[9,10] it alters purine metabolism in Ehrlich ascites tumor cells[11] and prolongs the survival time of mice implanted with these cells;[12] it causes a specific inhibition of mitochondrial as opposed to nuclear DNA synthesis;[13] it inhibits cell-free aminoacylation;[14] it transforms yeast cells into respiratory-deficient mutants;[15] and it is used as a probe to investigate red cell membranes.[16] Several phenanthridinium compounds are also known to possess antibacterial activity.[17,18]

Earlier investigations of phenanthridinium compounds indicated that they were not biotransformed.[4,19,20] These results suggested that the above effects may be attributed to the unchanged phenanthridinium compounds. The present work reports the isolation of metabolically altered ethidium, 150C47 (3,8-diamino-6-*p*-aminophenyl-5-methylphenanthridinium chloride), and carbidium (2-amino-6-*p*-carbethoxyamino-5-methylphenanthridinium sulfate) from rat bile.

MATERIALS AND METHODS

Animal preparation. Sprague–Dawley rats, 200–300 g, were obtained from Blue Spruce Farms, Altamont, N.Y. Animals were anesthetized with pentobarbital and their tracheas cannulated. The renal pedicles were ligated and the bile ducts cannulated with Clay–Adams PE-10 polyethylene tubing through an abdominal incision. Rectal temperature was maintained at 36–38° with a heat lamp. Drugs were administered via a cannula in the left external jugular vein and washed in with isotonic sodium chloride solution. Carbidium sulfate and ethidium bromide were administered in doses of 15 mg/kg. 150C47 was administered in the same dose but was sometimes followed by an infusion of 500 or 750 μg/hr.

Chemical determination. Ethidium, carbidium, and 150C47 were determined by the method of Bratton and Marshall.[21] Bile samples were either diazotized directly after dilution with water or were first precipitated in 5% trichloroacetic acid and then made 1 N in hydrochloric acid and hydrolyzed at 80–90° for 1 hr before analysis. Column eluates were diazotized directly or after appropriate dilution with water or the buffer employed. If it was desired to hydrolyze the eluate fractions, 0·5 ml of 12 N HCl was added to 5 ml of the fraction and hydrolysis and analysis were then carried out as above. Appropriate standards in the fluid being analyzed were run with each determination. When whole bile was analyzed for 150C47 or ethidium without hydrolysis, a problem was presented by the presence of a diazotizable metabolite whose azo derivative had a slightly different absorption maximum from that of the parent compound. Since acid hydrolysis was found to convert this metabolite back into the

original compound with little or no change in the total measured material, it was concluded that the extinction coefficients of the azo derivatives of the different forms must be close to identical. This was confirmed for 150C47 for which the metabolite was identified and synthesized. Total diazotizable material, in terms of microgram equivalents of the parent compound, was therefore measured by reading the absorption midway between the maxima of the two forms, where the per cent maximum absorption of each form is identical, and comparing with standards of the parent compound at the same wavelength. This procedure was necessary only for unhydrolyzed samples of 150C47 or ethidium, which contained appreciable quantities of two diazotizable forms. The maximal absorptions of the azo derivative of 150C47 and its acetyl derivative are 530 and 570 mμ respectively. The respective maxima for ethidium and its metabolite are 565 and 550 mμ. The small amounts of other metabolites known to be present do not interfere appreciably in the experiments reported here.

Synthesis of acetyl derivatives. The amino groups of 150C47 were acetylated by one of the following methods.

(1) Approximately 70 mg (0·2 m-mole) 150C47 was dissolved in 4 ml water and warmed to 40°. Pyridine (0·04 ml) was added, followed by 25 μl acetic anhydride (0·265 m-mole), and the solution was allowed to stand in a beaker of warm (40°) water.

(2) In order to completely acetylate the compound, a 100-fold molar excess of acetic anhydride was used. Substituting approximately 35 mg 150C47 and 1 ml acetic anhydride into the above procedure yielded a product which is thought to be totally acetylated, plus a small amount of material which was not totally acetylated (see Fig. 1, F and G). The small amount of material which was not totally acetylated could be eliminated by adding 4 ml of a 1:1 mixture of benzene–nitrobenzene to the solution before the addition of acetic anhydride and shaking the mixture for the first 30 min of the reaction. This apparently slows the conversion of the anhydride to acetic acid by the water present and allows more complete acetylation to take place. The product was then extracted into 30 ml of the ammonium formate buffer described below.

The reaction products were separated on 1·5 × 30 cm columns of Sephadex G-15 eluting with ammonium formate buffer, after suitable dilution in the buffer so that about 2 mg equivalents 150C47 of the mixture could be added in 1 ml. When it was desired to prepare larger amounts, 2·5 × 45 cm columns were used.

Carbidium and ethidium were acetylated by essentially the same procedure described above. Molar ratios of carbidium:acetic anhydride used were 1:4, 1:16 and 1:800. Molar ratios of ethidium:acetic anhydride were 1:10 and 1:800. Pyridine (1%) was used as a catalyst in all cases.

The acetyl derivative of carbidium was separated on Silica gel thin-layer plates as described below. The acetylated derivatives of ethidium were separated on 1·5 × 30 cm columns of Sephadex G-25 eluting with 0·056 M phosphate buffer.

Thin-layer chromatography. Thin-layer chromatography was carried out on plastic-backed Silica gel plates (Eastman Chromatogram Sheet 6061; Distillation Products Industries, Rochester, N.Y.) using butanol–acetic acid–water (100:30:80) as solvent and on glass-backed cellulose plates (Analtech Inc., Wilmington, Del.; 250 μ precoated avicel uniplate) using acetone–water–concentrated hydrochloric acid (100:20:1) as solvent. The R_f values in the cellulose system were heavily dependent on whether non-fluorescent or fluorescent-backed plates were used. The 150C47 and its diazotizable

metabolites were more strongly bound to the nonfluorescent plates and the non-diazotizable material separated nicely on these plates.

Paper electrophoresis. Paper electrophoresis was carried out in a Beckman model R series D electrophoretic cell on No. 320046 Beckman paper using 0·075 ionic strength barbiturate buffer (pH 8·6) at 200 V for 20 hr.

Sephadex chromatography. Sephadex chromatography was carried out on 1·5 × 30 cm columns using either Sephadex G-15 and eluting with 0·08 M ammonium formate buffer (pH 3·4) or using Sephadex G-25 and eluting with 0·056 M phosphate buffer (pH 7·0). Fractions (10·8 ml) were collected with the aid of a dropcounter and automatic fraction collector. The ultraviolet absorption of the effluent at 280 mμ was monitored continuously using an optical flow cell. A larger column (2·5 × 45 cm) was used for the preparation of acetyl 150C47 on the G-15 system.

Infrared spectroscopy. Samples of bile peaks I and II, standard 150C47 with and without added bile, and monoacetyl 150C47 were all obtained in ammonium formate eluate fractions from Sephadex G-15. The volatile buffer was removed by lyophilizing and 2 ml of 15% KBr was added to an aqueous solution of 250–1000 μg of the residue, expressed as microgram equivalents of 150C47. These solutions were lyophilized and 300-mg KBr pellets, 0·5 in. diameter, were prepared under vacuum at 6000 lb pressure in a hydraulic press. Control pellets from lyophilized buffer as well as from aqueous standards not carried through the Sephadex procedure were also prepared. Spectra were obtained on a Perkin–Elmer model 21 dual-beam recording spectrophotometer versus a KBr blank. The instrument was calibrated with a polystyrene standard and found to be in good agreement with the calibration peaks.

Gas chromatography. Acetic acid was determined by gas chromatography using a 4 mm × 180 cm column of 10% Carbowax on Chromosorb W (60/80 mesh; Applied Science Laboratories, Inc., State College, Pa.) The analysis was carried out isothermally at 70° using a hydrogen flame ionization detector. The inlet chamber and detector were maintained at 100°. Helium was used as the carrier gas at a flow rate of 55 ml/min. It was found to be necessary to flush the column with acetic acid before use to eliminate variable adsorption of the sample to the column. Acetic acid standards were prepared in 1 N sulfuric acid, with and without a 10-fold excess of 150C47. The presence of 150C47 had no effect on the results. Ten-μl samples containing 0·9–3·5 × 10^{-8} moles of acetic acid or the material to be analyzed were added to the column.

Samples of bile peak I and II material, synthetic acetyl 150C47, and standard 150C47, all prepared from Sephadex G-15 eluate fractions as described above, were hydrolyzed in 0·2 ml of 1 N sulfuric acid for 2 hr at 80° in sealed tubes and assayed for acetic acid. The tubes were then resealed and the process was repeated the following day to be certain hydrolysis was complete. The diazotizable material present both before and after hydrolysis was measured as described above, and the content of each tube was expressed in terms of equivalent moles of 150C47.

RESULTS

150C47. Paper electrophoresis of bile collected for 2 half-hr periods following 150C47 administration revealed the presence of yellow-orange material moving slower in the negative direction than a red-orange spot, which apparently consisted of unchanged 150C47. This yellow-orange spot yielded a product which was much more blue after treatment with the Bratton and Marshall reagents than the reddish purple

of the parent compound. 150C47 added to control bile behaved identically to standard aqueous solutions of 150C47 and also to the red-orange material found in rat bile.

Cellulose thin-layer chromatography gave tailing and poor resolution of the yellow-orange material, but revealed the presence of a fluorescent metabolite which was bright green after spraying with 1 N HCl. The green fluorescent metabolite was not affected by spraying with the Bratton and Marshall reagents.

It was found to be possible to resolve the compounds in bile into four fractions using Sephadex column chromatography. Both Sephadex G-25 using 0·056 M phosphate buffer, pH 7·0, as eluent and Sephadex G-15 using 0·08 M ammonium formate, pH 3·4, as eluent were found to be useful systems. The result of a typical separation using the G-15 ammonium formate system is shown in Fig. 1. A similar picture is obtained using the G-25–phosphate system, except that the peaks shown in Fig. 1 are all obtained in a total elution volume of 250 ml rather than 600 ml. The latter system is thus faster, but the former has the advantage that the buffer is volatile and may be removed from the eluted material. The two major forms are designated peaks I and II in Fig. 1. Peak II, with the larger elution volume, appeared identical with unchanged

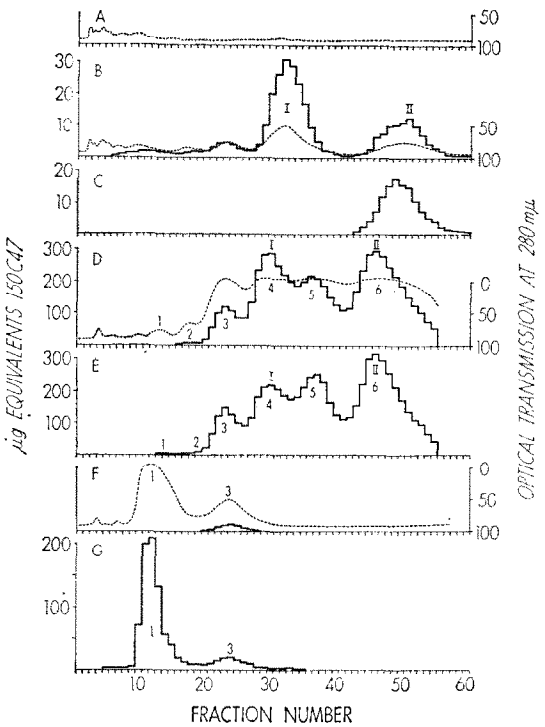

FIG. 1. (a–c). Sephadex chromatography of bile after 150C47 administration; 1·5 × 30 cm column of G-15 eluted with 0·08 M ammonium formate. Diazotizable material, ————; optical transmission, – – – – –. (a) Bile, 0·5 ml; (b) 0·5 ml bile from a 290 g female rat given 15 mg/kg of 150C47 followed by a 500 μg/hr infusion for 2·5 hr; (c) standard 150C47 or standard 150C47 plus 0·5 ml bile. d–g. Separation of acetyl 150C47 reaction mixtures on Sephadex G-15 eluted with 0·08 M ammonium formate. (d and e) Molar ratio of acetic anhydride: 150C47, 1:1; (f and g) molar ratio of acetic anhydride: 150C47, 100:1; (d and f) direct Bratton and Marshall analysis of buffer fractions; (e and g) Bratton and Marshall analysis after acid hydrolysis of buffer fractions (0·5 ml of 12 N HCl added to 5 ml of fraction and heated 1 hr at 85°).

150C47, as evidenced by its elution volume, color and fluorescent appearance, chromatography of the eluate fractions, and the color of its azo derivative and diazotized intermediate, as well as other evidence to follow. In addition to these two major peaks, there was a small peak of diazotizable material preceding peak I. The green, nondiazotizable metabolite cannot be identified as a unique band after doses of 15 mg/kg, and was thought to elute in the same region as the fast-moving bile constituents (Fig. 1). If the dose of 150C47 is increased to 50 mg/kg, a small peak which can be diazotized only after hydrolysis in 1 N HCl could be distinguished in this region, and this probably corresponds to the green, nondiazotizable material isolated by cellulose thin-layer chromatography after the lower dose of 150C47. About 90–100 per cent of the diazotizable material present in bile was recovered with the G-25–phosphate system and about 80 per cent with the G-15–ammonium formate system. Recoveries of standards added to the columns were comparable.

Since 150C47 has three aromatic amino groups, it was thought possible that peak I might consist of an amino conjugate of 150C47 which was coupling with one of the other amino groups during the assay procedure. This would explain the shift in the absorption maximum of the azo derivative and would also explain the observation that the spectrum of the diazotized material from bile becomes identical to authentic 150C47 if the bile is subjected to acidic hydrolysis before diazotization is carried out. To test this possibility, the peak I material from bile was eluted from a Sephadex G-15 column with ammonium formate buffer. The volatile buffer was removed by lyophilizing, and the material was hydrolyzed for 1 hr at 90° in 1 N hydrochloric acid. The solution was then neutralized with 1 N NaOH and rechromatographed with an internal 150C47 standard on Sephadex G-15. As expected, the material behaved identically with the 150C47 standard after acidic hydrolysis.

It was felt that infrared spectroscopy would be the simplest method of gaining further information on the nature of the material in peaks I and II. Peaks I and II were prepared in ammonium formate buffer from Sephadex G-15, lyophilized, and 300-mg KBr pellets prepared for spectroscopy as described in Methods. The spectra of these fractions were compared to the spectra of similar eluate fractions of aqueous 150C47 and 150C47 plus rat bile, as well as standard 150C47 not subjected to the Sephadex procedure. These spectra are shown in Fig. 2.

It was found that control pellets prepared from buffer residues had some absorption peaks and that the intensity varied from pellet to pellet. This accounts for the variable nature of the peaks in the 1050–1100 cm^{-1} region and at 800 cm^{-1}. The broadening of the 3280 cm^{-1} peak of 150C47 in buffer eluates is explained by the presence of a buffer absorption at 3400 cm^{-1}. There is also a buffer peak at 1600 cm^{-1}, but it is relatively weak and is overridden by the strong absorption of 150C47 and its derivative in this region.

The spectra obtained from 150C47 alone, 150C47 after Sephadex chromatography and 150C47 added to bile and then chromatographed were the same. This indicates that little, if any, material from bile is associated with 150C47 in these eluate fractions, and confirmed that no significant amounts of impurities are present in the stock material which are removed by the chromatographic procedure. Further, the spectrum of the peak II material from rat bile was identical with the spectrum of authentic 150C47, indicating that this peak contains only unaltered 150C47. The spectrum of the peak I material was similar to that of 150C47, except for a new sharp band at 1680 cm^{-1}

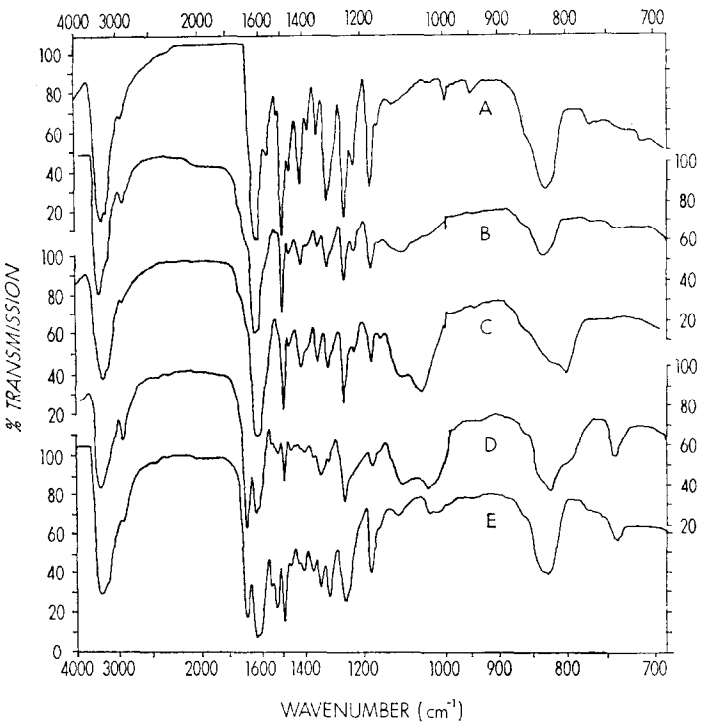

FIG. 2. Infra-red spectroscopy of biliary forms of 150C47. (a) Standard 150C47, 1·1 mg; (b) standard 150C47 plus rat bile carried through Sephadex separation procedure, 330 μg (spectrum identical if bile omitted); (c) peak II material from rat bile, 400 μg; (d) peak I material from rat bile, 250 μg; (e) synthetic monoacetyl 150C47, 800 μg.

and another band at 740 cm^{-1}. The 1680 cm^{-1} band was highly suggestive of a doubly bonded oxygen and suggested that the peak I material contained a metabolite consisting of an acid coupled to one or more of the amino groups of 150C47.

Since acetic acid seemed the most likely acid to be coupled with 150C47, acetylated derivatives of 150C47 were prepared as described in Methods. Since there are three amino groups on 150C47, each of which may form an acetyl conjugate, there are seven possible acetyl derivatives which might be obtained. Under the conditions described, five of the seven acetylation products could be obtained and separated on Sephadex G-15. Three of these corresponded in elution volume, color, and fluorescence to the three metabolites found in rat bile. Figure 1, d–g, illustrates the separation of the products from the reaction mixture.

When equimolar amounts of acetic anhydride and 150C47 were reacted, a large yield of a product which behaved identically on Sephadex to the peak I material from rat bile was obtained. This product also went through the same color changes during diazotization and exhibited the same absorption maximum of its azo derivative as the peak I material from rat bile. When a 100-fold excess of acetic anhydride was used to completely acetylate the 150C47, a derivative was obtained which eluted much more quickly than peak I. It is possible that this product could correspond to the small amount of nondiazotizable material found in rat bile, but this is not necessarily the

case. The small peak 3 in Fig. 1, f and g, was not obtained when the alternative method using the organic solvents was used. Thus, it is believed that peak I in Fig. 1, f and g, is the completely acetylated form of 150C47 and that only minor amounts, if any, of this material appear in rat bile under the conditions of our experiments.

The synthetic acetyl derivative corresponding to peak I from bile was purified on Sephadex G-15 in two batches. The first was prepared for infrared spectroscopy using the same method by which previous samples were prepared for spectroscopy. This particular sample contained an appreciable amount of the faster moving diazotizable material (about 20 per cent). The infrared spectrum of this material is shown in Fig. 2 (e). The presence of 1680 cm^{-1} and 740 cm^{-1} absorption bands confirms that the peak I material from rat bile is an acetyl conjugate of 150C47.

The other, larger batch was also prepared on Sephadex G-15 and this preparation contained only peak I material. The buffer fractions were lyophilized, redissolved in water, and lyophilized several times to remove all traces of the buffer. No ammonium ion could be detected in the residue by the method of Conway,[22] which would have detected 2 per cent ammonium formate contamination. After acid hydrolysis in 1 N hydrochloric acid, this acetyl derivative was reconverted to 150C47 and co-chromatographed with standard 150C47.

To determine the number of acetyl groups per mole of 150C47 and further confirm the presence of acetyl groups, the acetic acid released from the above acetyl derivative and similarly prepared peak I material from rat bile during acid hydrolysis was

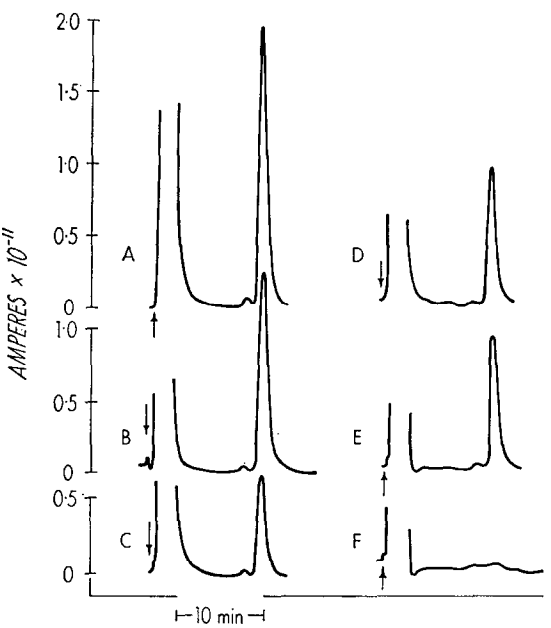

FIG. 3. Gas chromatographic identification and analysis of acetic acid released by acid hydrolysis of bile peak I and synthetic acetyl 150C47. Arrows indicate addition of sample to column. (a) Acetic acid, $3{\cdot}5 \times 10^{-8}$ moles; (b) $2{\cdot}5 \times 10^{-8}$ moles synthetic acetyl 150C47 (150C47 equivalents); (c) $1{\cdot}4 \times 10^{-8}$ moles acetic acid; (d) $2{\cdot}0 \times 10^{-8}$ moles peak I material (150C47 equivalents); (e) $1{\cdot}8 \times 10^{-8}$ moles acetic acid; (f) blank or $0{\cdot}65 \times 10^{-8}$ moles peak II material (150C47 equivalents) or $3{\cdot}5 \times 10^{-8}$ moles standard 150C47 prepared from Sephadex procedure.

TABLE 1. RECOVERY OF METABOLITES OF 150C47, CARBIDIUM AND ETHIDIUM IN BILE

Compound administered	Material recovered	Per cent of administered dose recovered		
		Rat 1	Rat 3	Rat 2
		(21 hr)*	(16 hr)	
150C47	Directly diazotizable†	62	55	
	Unchanged	10	9	
	Pre-peak I	19	14	
	Peak I (monoacetyl)	33	32	
	Diazotizable only after acid hydrolysis	6	7	
	Total (all forms)	68	62	
		(16·5 hr)	(9 hr)	(10 hr)
Carbidium	Unchanged	12	15	17
	Acetylated	48	46	50
	Total	60	61	67
		(18 hr)	(18 hr)	(16·5 hr)
Ethidium	Unchanged	14	10	
	Major metabolite	33	38	
	Minor metabolite	4	6	
	Total	51	54	51

* Total time of bile collection.

† In a total of five rats given 150C47, the directly diazotizable material recovered during periods ranging from 11 to 21 hr after administration was 64 ± 6·5 (S.D.) per cent of the administered dose.

measured by gas chromatography. The detector was relatively insensitive to formic acid, so there was no possibility of the small amount of formic acid which might remain from the buffer interfering with the analysis. Figure 3 confirms that acetic acid is released from the biliary peak I material. Quantitatively it was found that 1 mole of acetic acid was released from the hydrolysis of 1 mole equivalent of either the peak I material from bile or of the synthetic acetyl 150C47. Thus, peak I material from rat bile must be a monoacetyl amino conjugate of 150C47.*

The recovery of the different forms of 150C47 in bile after the injection of 15 mg/kg is summarized in Table 1. About 75–80 per cent of the material recovered is excreted within 4 hr.

To determine what compounds appear in bile after intravenous injection of the major monoacetyl metabolite, one female rat was given a dose of 5·2 mg/kg (150C47 equivalents) and bile was collected for one-half hr. A higher dose was not used because of the limited amount of material available. The rate of excretion during this time was about twice the excretion of all forms of 150C47 in control rats given the same dose of 150C47. Chromatography of this bile on Sephadex G-25 revealed that most of the material was present in the form of the injected monoacetyl compound. A small

* A small amount of acetylated 150C47 corresponding to the minor metabolite preceding the peak I metabolite (Fig. 1) was also prepared and the acetyl groups were measured by the method described above. Only a small amount of material was obtained and it is estimated that this analysis was subject to about 15 per cent erorr, but a ratio of 1·7 moles of acetic acid per mole equivalent 150C47 was obtained. This peak therefore contains either a diacetyl derivative of 150C47 or possibly a mixture of a mono- and a diacetyl derivative. Larger amounts could be prepared and a more accurate analysis obtained, but this has not been done.

amount was converted to the minor metabolite preceding peak I, which is thought to be deacetylated. No free 150C47 was present, so deacetylation, if it occurs at all, cannot be very rapid.

Carbidium. Silica gel thin-layer chromatography using butanol–glacial acetic acid–water (100:30:80) revealed the presence in bile of a blue fluorescent metabolite of carbidium which was not altered by spraying with the Bratton and Marshall reagents, in addition to a spot which appeared to be identical with carbidium on the basis of R_f, fluorescent appearance, and Bratton and Marshall color. The R_f values were 0·70 for carbidium and 0·65 for the metabolite.

This metabolite of carbidium could also be separated on Sephadex G-25 by eluting with 0·056 M phosphate buffer. The metabolite had an elution volume of 100 ml and could not be diazotized until it was hydrolyzed in acid as described in Methods. Standard carbidium had an elution volume of 150 ml.

Only unchanged carbidium and the single metabolite described above were observed in the bile of rats injected with carbidium. This would be expected if acetylation of the single free amino group were the only pathway of conversion in the animal.

To demonstrate that this metabolite was indeed the expected acetyl conjugate, carbidium was acetylated by reacting with various molar ratios of acetic anhydride as described in Methods. The acetylated product obtained behaved identically to the metabolite found in rat bile in the Silica gel chromatographic system.

This amino conjugate of carbidium could be hydrolyzed by heating to 80–90° for 1 hr in 1 N HCl. The difference in diazotizable material before and after acid hydrolysis therefore represents the amount of amino conjugate present in a sample of bile containing both carbidium and its acetyl conjugate. This procedure revealed that about 45 per cent of the carbidium present in bile collected for 1 hr after intravenous administration of 15 mg/kg was conjugated.

The recovery of carbidium and its metabolite in bile collected for 10 to 16·5 hr after the administration of 15 mg/kg is summarized in Table 1. About 75 per cent of the material recovered was excreted during the first 4 hr after injection of carbidium.

Ethidium. Sephadex G-25 chromatography of bile from rats given 15 mg/kg of ethidium using pH 7·0 phosphate buffer as eluent gave results analogous to those obtained with 150C47 (see Fig. 4). One-hundred per cent of the diazotizable material in bile was recovered from the column. The diazotizable groups in bile were found in two major peaks and one minor peak with colors and elution characteristics similar to those of the 150C47 peaks. The increase in diazotizable groups after acid hydrolysis is, like 150C47, very small. When part of the bile from the experiment shown in Fig. 4, a, was hydrolyzed in 1 N HCl for 2 hr at approximately 90° and subjected to chromatography on Sephadex G-25, the fraction I material was absent and material had appeared in fraction II (Fig. 4, c), indicating that fraction I was probably an acid-labile amino conjugate of ethidium.

Acetylated derivatives of ethidium were prepared as described in Methods and separated from the reaction mixture on Sephadex G-25. The acetylated product corresponding to peak 3 in Fig. 4, d and e, appears to be identical to the major metabolite found in rat bile. Since this metabolite can be diazotized and coupled without acid hydrolysis, it must be a monoacetyl conjugate. The diacetyl derivative was also prepared (Fig. 4, f) and is believed to correspond to the small peak I in Fig. 4, d. This compound, if present in rat bile after doses of 15 mg/kg, must be a very minor con-

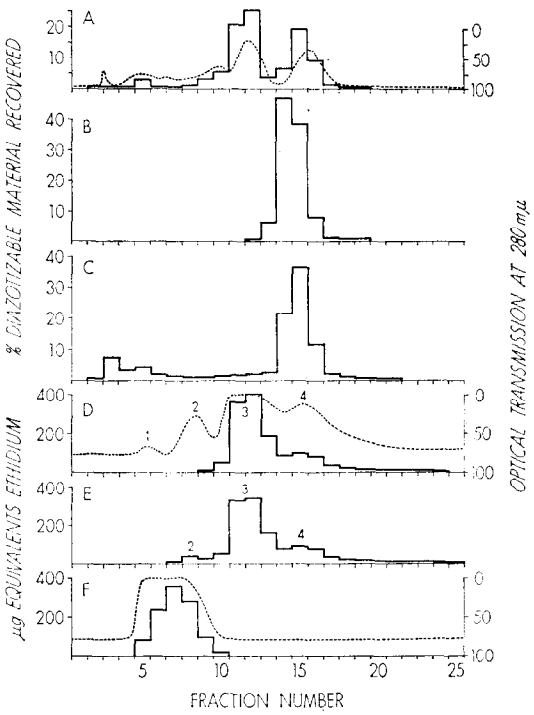

FIG. 4. (a–c). Sephadex chromatography of bile after ethidium administration; 1·5 × 30 cm column of G-25 eluted with 0·056 M phosphate buffer. Diazotizable material, ————; optical transmission, – – – – –. (a) Bile, 0·3 ml, from a 240 g female rat given 15 mg/kg of ethidium, 1·5 hr collection; (b) standard ethidium; (c) same bile as in (a) and (b) above, after hydrolysis for 2 hr at 90° in 1 N HCl. (d–). Separation of acetylated ethidium from reaction mixtures on Sephadex G-25 eluted with 0·056 M phosphate buffer. (d and e) Molar ratio of acetic anhydride: ethidium, 10:1; (f) molar ratio of acetic anhydride:ethidium, 800:1; (d) direct Bratton and Marshall analysis of buffer fractions; (e and f) Bratton and Marshall analysis after acid hydrolysis of buffer fractions. (No diazotizable material is present in the 800:1 reaction mixture before acid hydrolysis.)

stituent. The other metabolite of ethidium appears to correspond to peak 2 in Fig. 4, d and e, and must also be a monoacetyl conjugate, since there are only three possible acetylated derivatives. Peak 4 in Fig. 4, d and e, is unchanged ethidium. Thus, it appears that the observed metabolites of ethidium in bile are all acetyl amino conjugates.

The recovery of ethidium and its metabolites in bile collected for 16·5–18 hr after injection of 15 mg/kg of ethidium is summarized in Table 1. Of the material recovered, 85–90 per cent was excreted during the first 5 hr after injection of the ethidium.

DISCUSSION

Previous investigations have failed to reveal any evidence that phenanthridinium salts are biotransformed by animals. The only evidence of alteration *in vivo* is the cleavage of the acid-labile triazeno group of isometamidium after intragastric administration.[20]

Ethidium has previously been investigated in the mouse by Kandaswamy and

Henderson,[19] who claimed that all of the radioactive ethidium administered intraperitoneally could be accounted for as unaltered ethidium. Urine was subjected to paper chromatography, but only 51 per cent of the injected dose was recovered in urine. Although two ion-exchange chromatography systems were employed in an attempt to separate metabolites from liver extracts, both proved unsatisfactory. In one system, no radioactivity could be eluted from the column, even with 6 N HCl, while all the material passed through in the water wash in the other system. Although standard ethidium behaved identically to the liver extracts in these systems, such data should not be used to infer that metabolites are not present, as they would almost certainly fail to show up in these systems. It is possible that the 49 per cent not recovered in urine in their experiments consists largely of metabolized ethidium. In our experiments, urinary excretion was eliminated by ligating the renal pedicles.

The distribution and excretion of carbidium in rats and rabbits have been studied by Goodwin *et al.*[3] They determined the carbidium in terms of diazotizable amino groups, making no effort to determine if metabolites were present. They found that none of this compound was recovered in the urine of rats and about 10 per cent was recovered in bile in 75 min, using their assay procedure, which involved heating in 0·2 N HCl at 80° for 5 min. This may have hydrolyzed some of the amino conjugate. We recovered over 20 per cent of the injected dose in our preparation in 1 hr as free plus conjugated material when the same dose was given (7·5 mg/kg). They also reported that they sometimes observed a faint blue-green fluorescence in the urine of rabbits given very high doses of carbidium. This fluorescence was probably due to the metabolite we have described.

In the distribution and excretion studies of prothidium by Taylor,[4] attempts were made to locate metabolic products of the compound in various tissues and fluids, but the practice of homogenizing in 2 N sulfuric acid and of using the ultraviolet absorption in 1 N sulfuric acid as a criterion that the material in extracts was identical to prothidium would undoubtedly have hydrolyzed any amino conjugates of the type we found for 150C47, carbidium and ethidium. Thus, the possibility that there are acetyl or other acid-labile amino conjugates of this compound cannot be excluded.

On the basis of the results of the above authors, it is generally assumed that the effects of ethidium and other phenanthridinium compounds are due to the unaltered compound. The results of the present work have shown that ethidium, 150C47 and carbidium are all metabolized appreciably, apparently chiefly to acetylated conjugates. This suggests that the effect of the acetylated forms of these compounds must also be considered. The acetylated derivatives prepared by Walls[23] generally had less trypanocidal activity than the compounds from which they were derived. Work in this laboratory, however, has revealed that, although acetylation of the amino groups of ethidium and 150C47 generally decreases their inhibition of macromolecular synthesis in *Escherichia coli*, L5178Y mouse lymphoma cells and mitochondria, the monoacetylated forms of ethidium and 150C47 corresponding to the metabolites formed by the rat may in some cases be even more toxic than the parent compounds.*

The mechanism of acetylation of these compounds is not known. Weber and Cohen[24] have described an *N*-acetyltransferase which catalyzes the conjugation of isonicotinic acid hydrazide and several sulfonamides with acetyl coenzyme A. This enzyme is located in the particle-free supernatant fraction of liver. It has been sug-

* H. B. Bosmann and J. T. MacGregor, submitted for publication.

gested that phenanthridinium compounds are not metabolically transformed because their low lipid solubility excludes them from the microsomal drug-metabolizing enzymes.[19] The fact that the only metabolites found for these compounds seem to be acetyl conjugates and that there are acetylating enzymes which are apparently located in the cytoplasm of the cells would tend to support this idea.

These and other workers have also found other enzymes which utilize acetyl coenzyme A as a substrate for acetylation. Aas and Bremer[25] have demonstrated that acetyl coenzyme A synthetase activity is found both in mitochondria and in the particle-free supernatant fraction of rat liver. Guly *et al.*[26] have shown that acetyl phosphate can be synthesized in rat liver and utilized for the enzymatic acetylation of *p*-aminobenzoic acid. Acetyl coenzyme A is formed as an intermediate in this reaction. Whether these systems are involved in the acetylation of phenanthridinium compounds is unknown.

Although all of the material appearing in bile after the administration of the phenanthridinium compounds investigated is not unchanged, the excretion of both the free and the acetylated forms via bile is quite rapid and apparently against a large blood/bile concentration gradient (unpublished work). A comparison of the mechanism of excretion of phenanthridinium compounds with the active transport mechanism proposed by Schanker[1,27] for procaineamide ethobromide (a quaternary ammonium compound which is also rapidly excreted in bile, primarily in the form of amino conjugates) and other quaternary ammonium compounds including carbidium and prothidium will soon be reported.*

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