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METABOLISM AND BILIARY EXCRETION OF PHENANTHRIDINIUM SALTS—II

BILIARY EXCRETION*

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Abstract—The biliary excretion of phenanthridinium salts has been investigated in rats with ligated renal pedicles. The biliary excretion of the four phenanthridinium compounds investigated took place against large bile–blood concentration gradients and became constant with increasing dose. 150C47 (3,8-diamino-6-*p*-aminophenyl-5-methylphenanthridinium chloride) was investigated in detail to determine if it is excreted into bile by the specific metabolically dependent transport system that has been proposed for a number of other quaternary ammonium compounds, including certain phenanthridinium compounds. The excretion of total diazotizable material in bile reached a maximum at a dose of about 20 mg/kg and then remained constant as the dose was increased further. This maximum was not a result of either decreased bile flow or a limited ability of the liver to accumulate 150C47. The excretion of free 150C47 increased with dose over the entire dose range investigated (0–50 mg/kg), whereas its monoacetyl metabolite reached a maximum rate of excretion at 15–20 mg/kg and then decreased as the dose was increased further. The excretion of both forms was directly proportional to dehydrocholate-induced bile flow, but independent of the decrease in flow caused by the intravenous administration of hypertonic sucrose. The possible role of bile salts in the excretion of phenanthridinium compounds is discussed, and data *in vitro* on the binding of 150C47 to plasma proteins and liver homogenates and its complexing with bile are presented in connection with this point. The behavior of this compound is compared with that of procaineamide ethobromide, a quaternary compound for which a specific metabolically dependent transport system has been proposed.

ALTHOUGH it is well known that a number of organic anions are actively secreted into bile, the first evidence that the liver might possess a secretory mechanism for organic cations was the discovery by Schanker[1] in 1962 that in the rat, procaineamide ethobromide (PAEB) was transferred from blood to bile against very high concentration gradients. Further evidence led to the belief that PAEB and certain other quaternary ammonium compounds were secreted into bile by a specific and metabolically dependent transport system.[2,3] Similar evidence has now been accumulated that some highly ionized tertiary amines may also be secreted into bile by the same mechanism.[4,5] Much

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of the evidence supporting the specialized excretory mechanism described above has been reviewed by Schanker.[6,7]

In contrast to the highly specialized transport mechanism proposed by Schanker, it had previously been thought that most quaternary amines were excreted in bile chiefly as a result of their ability to form soluble complexes with the negatively charged bile salts. It is commonly known that quaternary amines form complexes with bile salts,[8] and Levine and Clark[9–11] have shown a strong correlation between the ability of various quaternary amines to form complexes with bile salts and the rate of excretion of the amines in bile.

A number of phenanthridinium compounds are known to be rapidly excreted in bile,[12–14] and Schanker[6,7] has suggested that they may be excreted by the same active mechanism responsible for the excretion of PAEB. The present work is an investigation of the biliary excretion of phenanthridinium compounds and a comparison of their excretion properties with those of PAEB. Four phenanthridinium compounds, 150C47 (3,8-diamino-6-*p*-aminophenyl-5-methylphenanthridinium chloride), ethidium (3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide), carbidium (2-amino-6-*p*-carbethoxyaminophenyl-5-methylphenanthridinium sulfate), and prothidium [3-amino-8-(2-amino-6-methylpyrimidin-4-ylamino)-6-*p*-aminophenyl-5,1′-dimethyl-phenanthridinium bromide] were considered. 150C47 was chosen for more detailed studies.

Ethidium, 150C47, and carbidium have previously been shown to be excreted in rat bile both unchanged and as amino conjugates.[13] 150C47 is present chiefly as a monoacetyl amino conjugate and free 150C47, plus small amounts of another metabolite, which is probably a diacetylated derivative. The major and minor metabolites are referred to as the peak I and pre-peak I metabolites, respectively, because of their elution characteristics from Sephadex columns.[13] The evidence indicates that the metabolites of ethidium and carbidium in bile also result from acetylation of the amino groups in these compounds.

METHODS

Animal preparation. Rats with ligated renal pedicles and cannulated bile ducts were prepared as described previously.[13]

Chemical determinations. 150C47, ethidium, carbidium and their metabolites in bile were determined as previously described.[13] Prothidium, PAEB and the totally acetylated derivatives of 150C47, ethidium and carbidium were determined by the same method.

The method of Goodwin *et al.*[12] was used for the determinations of the above compounds in liver. In the determination of the free and conjugated forms of PAEB and carbidium, livers were homogenized to 10 or 20 times their weight in distilled water and deproteinized with $CdSO_4$ as described by Smith.[15] Five ml of the homogenate was added to 9 ml of the $CdSO_4$ reagent plus 3 ml of water with constant agitation. Three ml of 1 N NaOH was added and, after thorough mixing, the flask was heated in a water bath at 80° for 3 min to help flocculate the protein. The filtrate from the above mixture was then analyzed by the procedure given above.

The recovery of the monoacetyl metabolite of 150C47 from liver was determined at concentrations of 150 μg/g of liver and 500 μg/g of liver and compared to the

recovery of 150C47 at the same concentrations. The recoveries found for 150C47 and its metabolite were 103 and 106 per cent (average of two determinations), respectively, at the lower concentration and 104 and 96·5 per cent, respectively, at the higher concentration. It was concluded that the recovery of the metabolite was similar to that of 150C47 and standard curves, prepared by adding known amounts of 150C47 to liver homogenates, were used to determine both forms in liver.

Totally acetylated 150C47, carbidium and ethidium were determined after the addition of an equal volume of 2 N HCl to the supernatant from the above method and hydrolysis at 85° for 1·5 hr. The recovery of totally acetylated 150C47 was found to be about the same as the recovery of 150C47 itself.

Appropriately diluted plasma samples were precipitated with an equal volume of 10% trichloroacetic acid and centrifuged. The supernatant was then analyzed by the method of Bratton and Marshall[16] both directly and after the addition of an equal volume of 2 N HCl followed by hydrolysis at 80° for 1·5 hr.

150C47 in red blood cells was determined by diluting 1 ml of cells (hematocrit approximately 78 per cent) to 5 ml with distilled water and analyzing this solution by the method described above for liver. The concentration obtained was corrected for the amount of 150C47 in plasma, assuming 25 per cent of the volume to be extracellular, to obtain the amount associated with the red blood cells.

Standard curves were prepared in each case by adding known amounts of the compound measured to the homogenate or fluid being analyzed, with the exception noted above. In the case of PAEB, the conjugated material has not been identified and is expressed as the increase in diazotizable material after acid hydrolysis.

All metabolites and acetylated derivatives are expressed as equivalents of the parent compound, unless otherwise noted.

Distribution and biliary excretion of 150C47 *and PAEB.* Groups of rats were administered a slow intravenous dose of 150C47 or PAEB, and bile was then collected for two 30-min periods. After the last bile collection, the blood and liver were removed for analysis. For each dose given another group was similarly treated, but the blood and liver were removed after dosing. The concentration of 150C47 or PAEB was determined in each of the bile, liver and blood samples, as described above.

Separation of 150C47 *and its metabolites from liver.* The concentration of 150C47, its major monoacetyl metabolite and the pre-peak I metabolite in liver was determined in female rats 30 min after the administration of doses of 5, 20 and 50 mg/kg of 150C47.

The liver was removed and immediately homogenized to 10 times its weight with ice cold distilled water. Twenty ml of this homogenate was then added to 20 ml of 0·022 M phosphate buffer (pH 7·0), followed by 40 ml of 10% TCA. After centrifugation, the supernatant was decanted and immediately neutralized with 3 M NaOH. After removal of an aliquot for the determination of diazotizable material, the solution was frozen and lyophilized. The residue was redissolved in about 3 ml of distilled water and applied to a 2·5 × 35 cm column of Sephadex G-25 for separation of the diazotizable material. Recoveries of the monoacetyl metabolite and 150C47 were determined at concentrations of 150 and 500 μg/g of liver. The recoveries of 150C47 and its metabolite were 101 and 106 per cent (average of two determinations), respectively, at the lower concentration and 104 and 92 per cent, respectively, at the higher concentration. The recovery of the pre-peak I metabolite (one determination)

at the higher concentration was 91 per cent. All three compounds were determined as micrograms per milliliter of 150C47 after the TCA supernatant was made 1 N in HCl and hydrolyzed at 85°.

Chromatography of the prepared metabolites on Sephadex G-25 revealed that the monoacetyl metabolite contained a small amount (less than 5 per cent) of pre-peak I metabolite. The pre-peak I metabolite eluted in a single peak. Sephadex chromatography of these metabolites recovered from liver by the above procedure showed that the metabolites were not altered by the procedure.

Effect of choleresis and anticholeresis on 150C47 *excretion.* The effects of sodium dehydrocholate-induced choleresis and hypertonic sucrose anticholeresis on 150C47 excretion were determined in 190–220 g female rats. The animals were divided into three groups, referred to as A, B and C. All groups received a priming injection of 25 mg/kg of 150C47 followed by an infusion of 700 μg/hr of 150C47. The infusion rate was chosen to produce a constant excretion of 150C47 during the experiment. After a 15-min equilibration period, two 20-min bile samples were collected. Group A (five rats) then received 50 mg/kg of sodium dehydrocholate, followed by an infusion of 25 mg/hr of sodium dehydrocholate and 700 μg/hr of 150C47. After 15 min, three 20-min bile samples were collected. Group B (six rats) received an injection of saline followed by resumption of the 700 μg/hr of 150C47 infusion. After 15 min, three 20-min bile samples were collected. Group C received 1·5 ml of 2 M sucrose, followed by an infusion of 700 μg/hr of 150C47. An equilibration sample of bile was then collected for 30–35 min (until at least four dead space volumes were excreted, i.e. approximately 0·2 ml)[17], and then two 20-min bile samples were collected. Bile flow was measured over intervals of 5–10 min by weighing the collection tubes to be certain no washout artifacts were obtained from changes in bile flow. Previous experiments had been carried out which showed that the above manipulations would double bile flow in Group A and decrease it by 50 per cent in Group C.

To determine if the excretion of both the unchanged 150C47 and its monoacetyl metabolite was similarly affected by the dehydrocholate infusion, a slightly different protocol was employed. The control rats were given a priming dose of 15 mg/kg of 150C47 followed immediately by an infusion of 700 μg/hr of 150C47. After 15 min, bile was collected for 1 hr and subjected to chromatography on Sephadex G-25, as previously described. The experimental rats received 50 mg/kg of sodium dehydrocholate immediately after the priming dose of 150C47 and an infusion of 25 mg/hr of sodium dehydrocholate and 700 μg/hr of 150C47 was then begun. After 15 min, bile was collected for 1 hr and subjected to chromatography on Sephadex G-25.

Dialysis. The distributions of 150C47 between buffer and bile, buffer and liver, and bile and liver were investigated using 0·25-in. dialyzer tubing of 48 Å average pore size (4465–A2, A. H. Thomas Co., Philadelphia, Pa.). The tubing was boiled in distilled water for 1–2 hr and then soaked for several hours in distilled water before use. A U-shaped loop of tubing extending to the bottom of a small vial was secured with a rubber stopper. The sac always contained 1 ml of either buffer or bile, while the outer solution consisted of 1 ml of bile or buffer or 2 ml of 20 per cent liver homogenate in buffer. The stopper could be removed and the inner and outer solutions sampled at any desired time. Fifty μl of buffer or bile and 100 μl of liver homogenate were removed for analysis at each sampling time. The buffer employed was 5·6 mM phosphate in 145 mM NaCl. All solutions were adjusted to pH 7·8.

Binding to plasma proteins was investigated using Fisher 0·75-in. cellophane dialyzer tubing (8-667 Fisher Scientific Co., Pittsburgh, Pa.). The tubing was prepared as described above. Eight ml of rat serum was dialyzed against 10 ml of 154 mM sodium chloride containing the desired concentration of 150C47. The 150C47 in the outer solution was measured until equilibrium was reached and then the 150C47 present both inside and outside was determined. In this experiment, the dialyzer tubing was secured with a double knot at both ends and shaken inside a stoppered tube. The sac was cut open after equilibrium was attained for analysis of the inside solution. The pH values of the inner and outer solutions were then determined. The protein concentration in the rat serum used was determined by the method of Lowry *et al.*[18]

Preparation of acetylated compounds. The totally acetylated derivatives of 150C47, ethidium and carbidium were prepared as previously described[13] using approximately a 700:1 molar ratio of acetic anhydride–phenanthridinium compound in each case. After completion of the reaction, the volatile material remaining was removed by lyophilizing, leaving only the acetylated compound.

The peak I and pre-peak I metabolites were prepared as previously described,[13] using 150 mg of 150C47, 5 ml of water, 50 μl of pyridine and 75 μl of acetic anhydride. This ratio of reactants was found to give an increased yield of pre-peak I metabolite. The acetylated compounds were separated from the reaction mixture on Sephadex G-15, as previously described.

RESULTS

Excretion as a function of dose in single animals. The excretion of the four phenanthridinium compounds considered, determined by giving increasing doses every 30 min, is shown in Fig. 1 as a function of cumulative dose. Experiments in which single doses were given and the excretion then followed as a function of time showed that the excretion never increased after the 30-min period following the injection. The free and conjugated forms of carbidium are reported separately. The excretion of the other phenanthridinium compounds is reported as total diazotizable material appearing in bile. Similar data for PAEB are shown in Fig. 2. The excretion of each phenanthridinium compound reached a maximum with increasing dose. The maximal rate

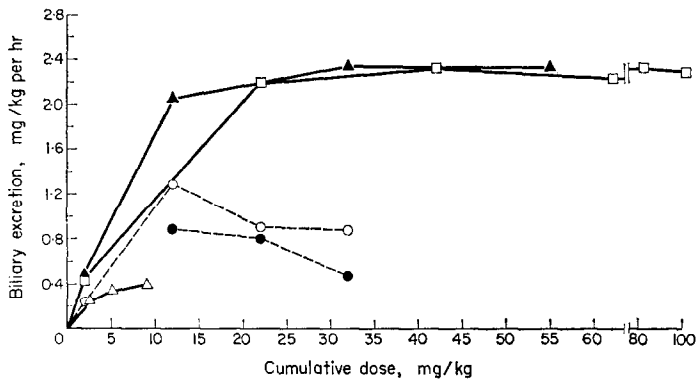

FIG. 1. Excretion of phenanthridinium compounds as a function of cumulative dose. All compounds are expressed as equivalents of the parent compound. Ethidium: ▲——▲; 150C47: □——□; carbidium, free: ○- - -○, conjugated: ●- - -●; prothidium △——△. Squares denote males; all other symbols denote females. Each curve represents a single animal.

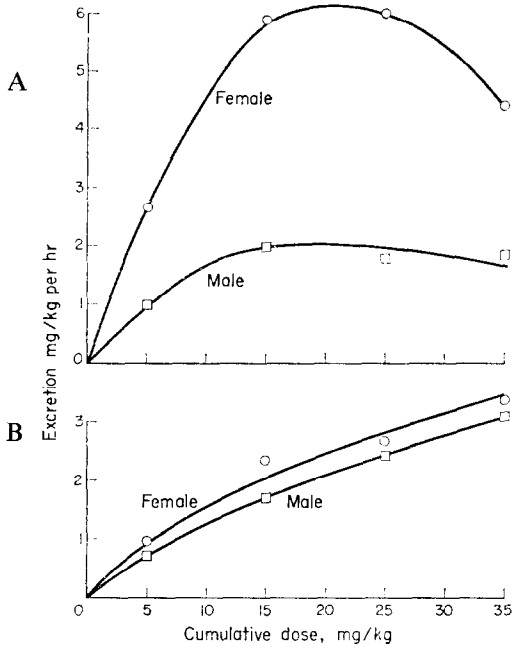

FIG. 2. Excretion of PAEB as a function of cumulative dose. (A) Unconjugated PAEB; and (B) conjugated PAEB (as equivalents PAEB). Each curve represents a single animal.

of excretion was in each case less than the maximal rates of PAEB excretion. (On a molar basis 150C47, ethidium and carbidium were excreted at roughly one-half the rate of excretion of PAEB in the male rat—Fig. 2.) Although 10 per cent or less of the administered dose is excreted in bile during the experiment, the plasma concentration at the end of the experiment was in every case less than 10 μg/ml, indicating that most of the material disappeared from the plasma during the experiment. Although Schanker and Solomon[2] have previously reported the excretion of both forms of PAEB to be constant above a dose of 5 mg/kg, the excretion of conjugated PAEB continued to increase in both rats over the entire dose range (0–35 mg/kg). The rates of excretion of PAEB reported here are also considerably higher than those reported by Schanker.

150C47 was chosen for further studies on the biliary excretion of phenanthridinium compounds. Although it is known that at least three metabolites of 150C47 are excreted in bile, only free 150C47 and its monoacetylated derivative constitute an appreciable percentage of the material appearing in bile during the period considered in this paper.[13] Evidence to be presented indicates that only these two forms are present in liver in appreciable concentrations during the period investigated. To determine the characteristics of the biliary excretion of 150C47 it is therefore necessary to consider only these two forms.

Distribution and excretion of 150C47. The distribution and biliary excretion of 150C47 as a function of dose is shown in Fig. 3. It should be noted that there are no significant changes in bile flow at any of these doses, indicating that the leveling off of 150C47 excretion is not due to a nonspecific inhibition of bile output. Figure 3C

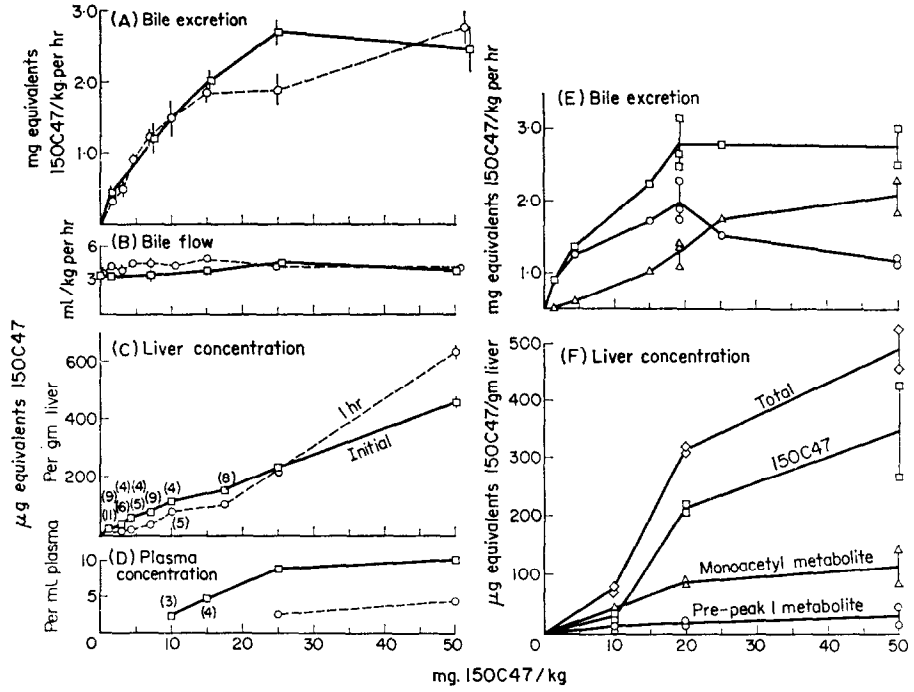

FIG. 3. Biliary excretion and distribution of 150C47 as a function of dose. (A) Excretion of total diazotizable material. Females: □——□; males: ○- - -○. One-hr average excretion ± S. E. M. Five rats per group unless otherwise noted. (B) Bile flow, same groups as in A, above. Values presented as mean ± S. E. M. (C) Concentration of diazotizable material in liver. After administration of 150C47: □——□; after 1-hr bile collection: ○- - -○. Pooled data from males and females, 10 rats per group unless otherwise noted. Values presented as mean ± S. E. M. (D) Concentration of diazotizable material in plasma. Same groups as in C, above. (E) Excretion of unchanged 150C47 and its monoacetyl metabolite. Data from Sephadex chromatography of bile from nine female rats. Bile was collected from 1 hr following 150C47 administration. Total diazotizable material: □——□; unchanged 150C47: △——△; monoacetyl metabolite: ○——○. (F) Liver concentration of 150C47 and its metabolites. Data from Sephadex chromatography of liver supernatants from six female rats. Livers were removed 30 min after 150C47 administration.

shows that the total diazotizable material in liver increases proportionally to the dose. There were no significant differences ($P > 0{\cdot}1$ by Student's t-test) between the liver concentrations of males and females either before or after bile collection, except at the 15 mg/kg dose where the females had a slightly higher concentration ($P < 0{\cdot}05$) both before and after bile collection. Liver concentrations are reported as grouped data. Note that at 25 mg/kg the rate of uptake by the liver exactly balances the rate of removal over the 1-hr time period. Since the liver concentration is proportional to the dose, it appears that the maximum biliary excretion observed is not determined by a limited ability of the liver to take up the compound. The plasma concentrations at the beginning and end of each collection period are shown in Fig. 3D. Because of the longer time of injection necessary to avoid respiratory failure at 50 mg/kg, the initial plasma concentration at this dose is not comparable to the other initial plasma levels reported, although it is connected with a solid line. It is perhaps worthy of note that an intravenous LD_{50} of 9·4 mg/kg has been reported for 150C47 in mice.[19] The

rats used in these experiments survived doses of 50 mg/kg or more with no apparent adverse effect provided the injection was carried out very slowly. This difference probably reflects the rapid removal of 150C47 from plasma. The plasma concentrations observed for males and females were not significantly different by Student's *t*-test ($P > 0{\cdot}1$) and are reported as grouped data. The plasma levels were always extremely low, even shortly after completion of the injection.

The 150C47 associated with the red cells was determined for two male rats and one female rat after a dose of 50 mg/kg both before and after bile collection. Immediately after administration of the dose, the diazotizable material associated with the red blood cells was about one-half the concentration in plasma. After the bile collection, the red cells from the males contained roughly twice the plasma concentration, and the female had the same concentration in both plasma and red cells. The optical densities obtained from the small amounts of diazotizable material in the red blood cells were only about twice the blank value and so are not known precisely, but it is apparent that no large amounts of diazotizable material are bound in or on the red cells.

To determine the relative amounts of each form of 150C47 in bile as a function of dose, the metabolites present in bile collected from female rats for 1 hr following the administraton of different doses of 150C47 were separated by Sephadex chromatography as previously described.[13] The excretion of free 150C47, its major monoacetyl metabolite, and total diazotizable material is given in Fig. 3E as a function of dose. The excretion of the pre-peak I metabolite is not shown, but was usually less than 10 per cent of the total material recovered. It can be seen that at doses below 5 mg/kg almost all the diazotizable material in bile was in the form of the monoacetyl metabolite. At 20–25 mg/kg approximately equal amounts of free 150C47 and its metabolite were excreted in the first hour, while at 50 mg/kg the ratio of free-metabolite was about 2:1. The excretion of the metabolite reached a maximum rate and then decreased, while the excretion of free 150C47 continued to increase as the dose was increased. This suggested that the large amount of free 150C47 present after large doses of 150C47 (see Fig. 3F) might be depressing the excretion of the monoacetylated form.

The concentrations of each form present in liver 30 min after administration of 5, 20 and 50 mg/kg of 150C47 are shown in Fig. 3F. It is not known why the concentration of diazotizable material after 20 mg/kg was so much higher than the results shown in Fig. 3C. The values obtained at 5 and 50 mg/kg are comparable to those presented in Fig. 3C. The data indicate that the concentration of free 150C47 increases with increasing dose, as expected. The increase in the major acetylated derivative with increasing dose was much less. Further, the low biliary excretion of the pre-peak I metabolite is due to the fact that little is formed in liver and not due to a low transfer rate from liver to bile.

Distribution and excretion of PAEB. Experiments with PAEB showed a distribution and biliary excretion similar to 150C47 as a function of dose. Figures 4A and 4B show the PAEB excretion as a function of dose for male rats. It can be seen that the excretion of PAEB was not a linear function of dose, but neither was a constant excretion found above 5 mg/kg, as reported by Schanker and Solomon.[2] The concentration of total PAEB in liver was found to be proportional to dose, as is shown in Fig. 4D. The plasma concentration as a function of dose is shown in Fig. 4E. Very

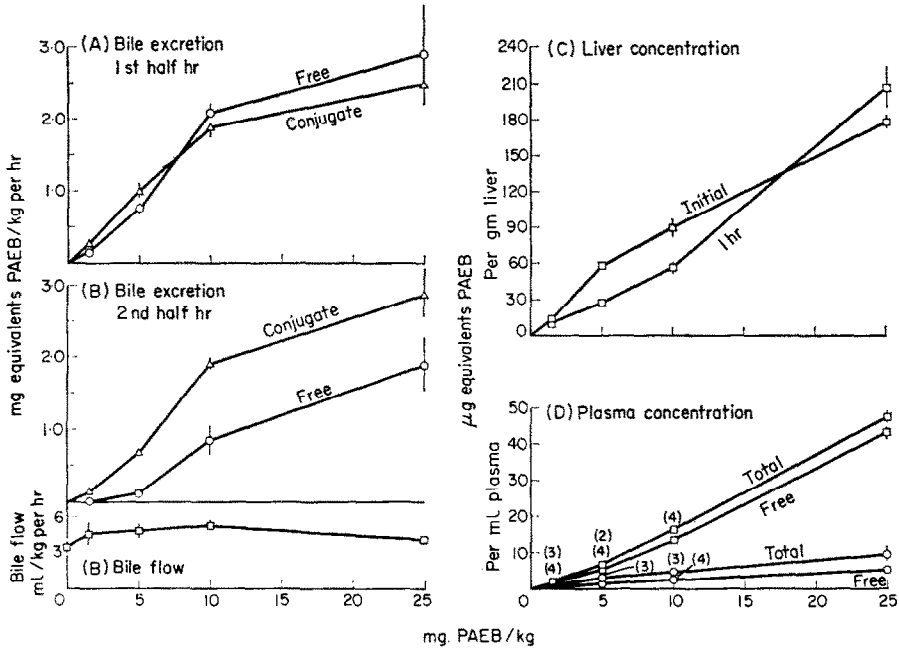

FIG. 4. Biliary excretion and distribution of PAEB as a function of dose. (A) Excretion during first half hour after administration. Unconjugated PAEB: ○——○; conjugated PAEB; △——△. (B) Excretion during second half hour after administration. Same groups as in A, above. (C) Bile flow, same groups as in A, above. (D) Concentration of diazotizable material in liver. (E) Concentration of diazotizable material in plasma. After administration: □——□; after 1-hr bile collection: ○——○. Values presented as means ± S. E. M. Data from male rats. $N = 5$ in all cases unless otherwise stated.

little conjugated PAEB was ever found in the plasma. Considerable concentrations of free PAEB were found in plasma after its injection, but the concentration fell rapidly during the first hour after injection. The excretion was also lower during the second collection period, but the decrease seems to be more closely correlated with the liver concentration than with the plasma concentration.

Of the two animals examined in the preliminary studies on PAEB excretion, the female seemed to excrete much more free PAEB than the male. To see if females actually excreted more free PAEB, a group of females was given 25 mg/kg, following the same protocol used for the males given this dose. The results of this experiment are given in Table 1. It can be seen that the excretion values compare very well with those found in the rats given cumulative doses of PAEB. The females did excrete more free PAEB. The reason for this was not a slower conjugation, since the females had as much or more conjugate both in blood and liver. As was found in the earlier experiment, the rate of excretion of conjugated PAEB was the same in the males and females.

Effect of choleresis and anticholeresis on 150C47 *excretion.* To try to elucidate the mechanism by which 150C47 and its metabolites gain entrance into the bile, the effects of choleresis and anticholeresis on the biliary excretion of 150C47 were investigated. As described in Methods, the bile flow was manipulated by the administration of sodium dehydrocholate or hypertonic sucrose, while 150C47 was infused at a constant rate. Table 2A compares the average excretion of total diazotizable material

TABLE 1. EXCRETION AND DISTRIBUTION OF PAEB IN MALE AND FEMALE RATS AFTER A DOSE OF 25 mg/kg*

		Excretion (mg/kg/hr)				Concentration (μg/ml)			
		First half hour		Second half hour		First half hour		Second half hour	
Sex	No.	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
Male	5	2·9 ± 0·67	2·5 ± 0·3	1·9 ± 0·36	2·9 ± 0·34	686 ± 125	606 ± 43	479 ± 76	706 ± 46
Female	5	4·5 ± 0·70	2·8 ± 0·33	3·6 ± 0·49	3·0 ± 0·33	1423 ± 156	901 ± 58	959 ± 75	807 ± 29
P		< 0·1	N.S.	< 0·025	N.S.	< 0·005	< 0·005	< 0·005	< 0·1
		Liver concentration (μg/g)				Plasma concentration (μg/ml)			
		After PAEB injection		After bile collection		After PAEB injection		After bile collection	
Sex	No.	Free	Conjugated	Free	Conjugated	Free	Conjugated	Free	Conjugated
Male	5	89 ± 5·0	86 ± 4·4	39 ± 5·4	155 ± 13	43 ± 1·7	4·5 ± 0·2	5·1 ± 1·1	4·4 ± 1·3
Female	5	81 ± 3·9	130 ± 10	33 ± 2·3	166 ± 10	51 ± 3·0	8·4 ± 1·1	6·8 ± 1·3	4·8 ± 1·3
P		N.S.	< 0·01	N.S.	N.S.	< 0·05	< 0·025	N.S.	N.S.

* Values are means ± S.E.M.; P values calculated by Student's *t*-test; N.S. = not significant.

during the two control periods with the average excretion during the experimental periods. The equilibration sample is not included for the sucrose-treated rats, but this would not have altered the results. The liver concentrations ± S. E. M. of diazotizable material at the end of the experiment were, 268 ± 34·4, 375 ± 18·5 and 414 ± 19·8 μg/g liver from groups A, B and C respectively.

TABLE 2. EFFECT OF CHOLERESIS AND ANTICHOLERESIS ON 150C47 EXCRETION

A. *Effect on excretion of total diazotizable material**

Group	No.	Bile flow (% control)	Biliary 150C47 excretion (% control)
A (dehydrocholate)	5	234 ± 9·3	216 ± 13·4
B (control)	6	93 ± 8·1	94 ± 8·3
C (sucrose)	6	48 ± 2·9	96 ± 8·3

B. *Effect on excretion of* 150C47 *and its monoacetyl metabolite*

Treatment	Excretion of diazotizable material (mg equiv. 150C47/kg/hr)	Ratio metabolite/150C47
Control	2·66	2·1
	1·99	2·3
Dehydrocholate	4·60	2·4
	3·61	1·7

* Values are means ± S.E.M.; 150C47 excretion measured as total diazotizable material.

The bile from two control and two sodium dehydrocholate-infused rats was subjected to column chromatography on Sephadex G–25 to determine if the excretion of unchanged 150C47 was affected differently than the excretion of its major metabolite. Table 2B shows that the increase in excretion of diazotizable material was due to a parallel increase in the excretion of both forms. A similar experiment using a different priming dose and collecting bile for 30 min gave analogous results.

It is evident from the above results that the biliary excretion of both forms of 150C47 is directly proportional to the bile salt-induced bile flow.

Binding of 150C47 *to liver, plasma and bile.* Since it appeared that the excretion of 150C47 might be dependent on bile salt excretion, it seemed of interest to determine if 150C47 could form complexes with bile. Furthermore, it was felt that knowledge of the extent of binding of 150C47 to liver homogenates and to plasma proteins might help clarify if the compound could be efficiently removed from plasma on the basis of its binding to liver and subsequently removed into bile by complexing with bile salts or some other complexing agent present in bile.

If 150C47 is dialyzed against bile as described in Methods, it is possible to demonstrate that the 150C47 accumulates on the bile side of the sac. The distribution of

TABLE 3. DISTRIBUTION OF 150C47 BETWEEN BILE AND BUFFER DURING DIALYSIS

Vial No.	Initial contents outside sac	Initial contents inside sac	Ratio of 150C47 outside/150C47 inside							
			1 hr	2 hr	6 hr	12 hr	18 hr	24 hr	28 hr	36 hr
1	Buffer + 100 μg/ml 150C47	Buffer + 100 μg/ml 150C47		1·0	1·0		1·0			
2	Buffer	Buffer + 200 μg/ml 150C47			1·0		1·1			
3	Bile + 100 μg/ml 150C47	Buffer + 100 μg/ml 150C47		3·4	2·6		2·3		2·0	
4	Bile + 100 μg/ml 150C47	Buffer + 100 μg/ml 150C47		3·6	2·9		2·4		1·9	
5	Bile	Buffer + 200 μg/ml 150C47	1·0			1·9		2·0		1·8
6	Bile	Buffer + 200 μg/ml 150C47	0·87			2·1		2·0		1·9

150C47 between bile and buffer is shown in Table 3. It can be seen that the bile–buffer ratio decreases with time, indicating that the bile constituent to which 150C47 binds moves through the sac, but much more slowly than 150C47 itself. It is clear that 150C47 is complexed by some bile constituent, and this constituent is most likely the bile salts.

The ability of bile to complex 150C47 initially present in a 20 per cent liver homogenate was determined by comparing the movement of 150C47 from a homogenate into bile to the movement from homogenate into buffer. Figure 5 shows that bile is more effective than buffer at removing 150C47 from the liver homogenate. The homogenate concentrations at 12 and 24 hr averaged 945 μg/ml for the dialysis against buffer and 930 μg/ml for the dialysis against bile, and did not change significantly between 12 and 24 hr. Thus, 95 per cent of the 150C47 in the liver homogenate was bound, and only 5 per cent was freely diffusable into the buffer.

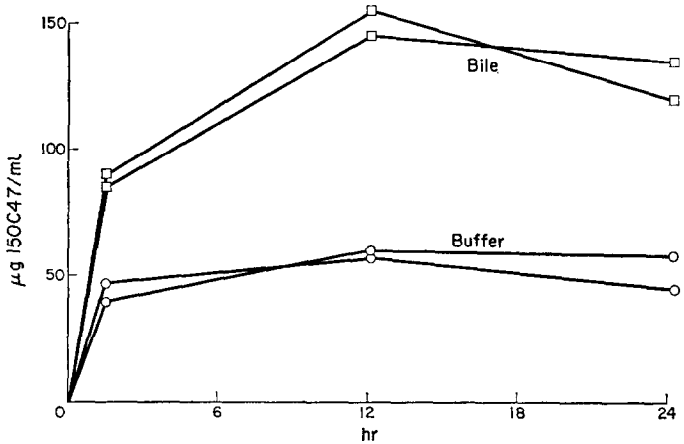

FIG. 5. Dialysis of 150C47 from 20 per cent liver homogenate into buffer or bile. See text for details.

The results of dialyzing various concentrations of 150C47 against rat serum are shown in Table 4. The ratio of bound–free 150C47 initially decreased as the amount bound increased, as is predicted by the law of mass action.[20] As the amount bound increased further, however, the ratio of bound–free increased as the amount bound increased. This increase in the ratio of bound–free with increasing bound 150C47 does not follow from the law of mass action, and suggests that 150C47 produced an alteration in the molecule to which it was bound, exposing binding sites not previously accessible to the compound. This change occurred at an amount bound between 1·8 and 4·9 μg of 150C47 per mg of protein. A 1 : 1 mole ratio of 150C47–albumin is equal to 5·1 μg of 150C47 per mg of albumin.

Excretion of acetylated 150C47, *ethidium and carbidium.* To determine if the totally acetylated derivatives of 150C47, ethidium, and carbidium are excreted in bile after intravenous administration, the totally acetylated derivatives of the compounds were prepared as described in Methods and administered to female rats.

After administration of 6 mg/kg of acetylated ethidium, the bile flow decreased rapidly and a precipitate was visible in the bile. The concentration of the compound measured in bile after acid hydrolysis was 645 μg/ml during the first 30 min after

TABLE 4. BINDING OF 150C47 TO SERUM PROTEINS

Free (μg/ml)	Bound (μg/ml)	Bound/free	Bound (%)	pH	Protein concn. (mg/ml)
7·9	33·3	4·2	81	8·0	83 ± 8
11·2	35·8	3·2	76	7·8	92 ± 4
33·4	98	2·9	75	7·9	92 ± 4
80	165	2·1	67	7·8	92 ± 4
159	416	2·6	73	7·6	92 ± 4
203	662	3·3	77	7·6	92 ± 4
76	824	10·8	92	10·8	83 ± 8
96	118	1·2	55	7·7	46
300	460	1·5	61	7·7	46

administration and 241 μg/ml during the following 60 min. The average excretion over the 90-min collection period was 0·2 mg/kg/hr, and 119 μg/g was found in the liver at the end of this time period. The rat given ethidium in Fig. 1 had 86 μg/g in its liver at the end of the experiment. Another rat given 3 mg/kg had a bile concentration of 603 μg/ml and an excretion of 0·54 mg/kg/hr during the first 30 min after injection, but the administration of an additional 6 mg/kg caused bile flow to cease entirely. The liver concentration in this rat was 190 μg/g 2 hr after administration of the initial dose. The bile flow had not resumed by this time. Mixing bile with an equal volume of a 1000 μg/ml solution of acetylated ethidium yielded a precipitate which looked like that obtained in bile. Chromatography of the bile collected on Sephadex G-25 indicated that the compound had not been deacetylated in liver, as none of the other metabolites were detected in bile.

After injection of 3 mg/kg of acetylated carbidium, a concentration of 695 μg/ml and an excretion of 1·2 mg/kg/hr were found during the 30 min following the injection. The dose was then repeated and bile was again collected for 30 min. A concentration of 670 μg/ml and an excretion of 1·2 mg/kg/hr were found during this time period. It thus appears that the excretion of the acetylated form of carbidium reaches a maximum with increasing dose. The maximum rate of excretion of acetylated carbidium found after the administration of carbidium itself was similar to that observed in this experiment (see Fig. 1). The rat given carbidium in Fig. 1 and 57 μg/g of free and 151 μg/g of conjugated carbidium in its liver at the end of the experiment. After collecting bile for a total of 1 hr after the second dose of the compound, the liver was analyzed and found to contain 35 μg/g of liver. Chromatography of the bile obtained during this experiment on Sephadex G-25 did not reveal any unconjugated carbidium, so the compound is apparently not deacetylated by the liver.

One rat given a dose of 5·6 mg/kg of totally acetylated 150C47 excreted 0·62 mg/kg/hr during the first 30 min after injection. As was the case with ethidium, a precipitate was visible in bile and the bile flow decreased after administering the compound. The concentration found during this time period was 545 μg/ml. Bile was collected for an additional hour and the liver then removed for analysis. The liver contained 92 μg/g at this time. Another rat given 3 mg/kg excreted 0·86 mg/kg/hr during the 30-min period following administration. During the 30 min following injection of an additional 6 mg/kg, the excretion was 1·05 mg/kg/hr. The concentrations

in bile during these two periods were 523 and 692 μg/ml respectively. Bile was collected for an additional hour and the liver then analyzed. The concentration in liver was found to be 132 μg/g. The plasma contained approximately 2 μg/ml. No free 150C47 or other deacetylated products were found when the pooled bile from these two rats was subjected to chromatography on Sephadex G-25.

A dose of 5·2 mg/kg of the major monoacetyl metabolite was administered to one female rat and the biliary excretion found to be 2·13 mg/kg/hr during the 30 min following the injection. This is a higher excretion than the excretion of total diazotizable material usually found during the same period following administration of the same dose of 150C47. The liver was found to contain 81 μg/g at this time. Chromatography on Sephadex G-25 of both bile and the material in the trichloracetic acid supernatant from liver indicated that no deacetylated 150C47 was present. Some of the pre-peak I metabolite which is believed to be diacetylated was, however, present in bile. The amount of this metabolite present in liver was too small to detect by the method used.

DISCUSSION

Although the available data do not provide conclusive evidence of the type of transport mechanism involved in the transfer of phenanthridinium compounds from blood into bile, some insight into the mechanisms which may be operating is provided.

The excretion of all the compounds investigated took place against large bile–blood concentration gradients and became constant with increasing dose. This behavior is clearly not consistent with simple diffusion or filtration.

The presence of free amino groups apparently plays no role in determining their excretion, since the concentrations of the totally acetylated compounds in bile were similar to those found for the unconjugated ones.

The limited ability of the liver to accumulate 150C47 is clearly not the reason that its excretion becomes constant at doses greater than 20 mg/kg, since the concentration found in liver after 150C47 administration was roughly proportional to dose over the range of doses investigated. Further, it is interesting that, although the excretion of total material is constant above 20 mg/kg, the excretion of the monoacetyl metabolite reaches a maximum at about 20 mg/kg and then decreases as the dose is increased further. This decrease in the excretion of the monoacetyl derivative at higher doses is not due to the fact that there is less present in the liver, since there is at least as much present after 50 mg/kg as after 20 mg/kg. This behavior would be consistent with a competition of 150C47 and its monoacetyl metabolite for binding to a fixed quantity of "complexing" agent in bile or to some "carrier" involved in their excretion. A likely candidate for such an agent would be the negatively charged bile salts, which are themselves secreted into bile and with which quaternary amines are known to form complexes.[8] Also consistent with this idea is the fact that the excretion of both forms of 150C47 is directly proportional to the sodium dehydrocholate-induced bile flow. It is unlikely that a reduced backflux of material due to the increased flow is the explanation for this phenomenon for two reasons. First, such large, positively charged ions would probably not be very permeable to the biliary tree, and one would have to postulate a very large backflux to explain the present data. (If it is assumed that doubling the flow halves the backflux, at least 67 per cent of the material entering the biliary tree would have to be reabsorbed.) Second, the excretion of 150C47 is unaffected

by altering the bile flow with hypertonic sucrose, a procedure which reduces the flow of fluid and electrolytes while having little effect on bile salt or bilirubin excretion.[21]

It was hoped that the dialysis experiments might clarify if 150C47 could be efficiently removed from plasma by binding and then subsequently be removed into bile by complexing with bile salts or some other complexing agent present in bile. Although direct determinations of binding to the various components of bile have not been carried out, it is clear that some components of bile are able to bind 150C47. Although it is possible to demonstrate binding, quantitative evaluation of these experiments is difficult because the agent to which 150C47 binds moves through the dialysis sac itself, although more slowly than 150C47. When a 20 per cent liver homogenate containing 150C47 was dialyzed against bile, the maximum concentration observed in bile was only about one-third the maximum concentration of unchanged 150C47 found in bile *in vivo*, even though the concentration in the liver homogenate was much higher than the liver concentration of 150C47 *in vivo* and even though a 20 per cent homogenate was used. It must be borne in mind, however, that this maximum concentration is not reached until 12 hr of dialysis and by this time the composition of bile has surely been altered. It was qualitatively noted that considerable surface active material had passed through the dialysis sac by this time, and it is likely that other small molecular weight constituents which might produce a marked effect on the physical properties of bile had also been lost. The above mechanism cannot be entirely eliminated at this time even though the bile–liver concentration ratios observed in the dialysis experiments were not large enough to explain the accumulation of 150C47 in bile *in vivo*.

The possibility that a coupling of the phenanthridinium transport at the primary site of bile salt transport is involved, rather than a simple diffusion of the substance into bile followed by complexing with the bile salts, should also be considered.

Whether the micelles in bile play a role in the complexing of phenanthridinium compounds is not known, but Gitler *et al.*[22] have shown that micelle formation of sodium lauryl sulfate coincided with an enhancement of the fluorescence of ethidium in solution, and this suggests that the micelles are capable of forming complexes with ethidium.

Although the distribution of 150C47 between liver and plasma was not actually measured, the maximum possible passive accumulation of 150C47 can be estimated from the data available. At a concentration of approximately 10 μg/ml of free 150C47 in plasma, about 77 per cent of the total 150C47, or 33 μg/ml, would be bound to plasma protein. If 150C47 distributes in equilibrium with the 43 mV membrane potential of the rat liver cell,[23] about 50 μg/ml of free 150C47 will be found inside the cell. At this concentration of free 150C47 about 95 per cent of the total 150C47, or 950 μg/ml, was found to be bound to a 20 per cent liver homogenate. If the binding is extrapolated to a 100 per cent homogenate by assuming the equation

$$[FB] = \frac{[FB][B_t]}{K + [F]}$$

[where F = free 150C47, B = unbound agent to which 150C47 binds, B_t = total (bound plus free) agent to which 150C47 binds, and K = the dissociation constant of FB], derived from the simple mass law interaction $F + B \leftrightarrows FB$, to be valid, it can be

seen that 4750 μg/ml of 150C47 would be bound to a 100 per cent homogenate. The liver–plasma concentration ratio predicted at this plasma level would therefore be (4800 μg/ml)/43 μg/ml), or about 110. This concentration ratio is sufficient to account for the liver–plasma ratios of free 150C47 observed *in vivo*. It is, therefore, not necessary at this time to invoke any mechanism other than the operation of these passive factors plus metabolic conversion to explain the observed accumulation of 150C47 and its metabolites in liver.

The behavior of 150C47 may be compared with that of PAEB, a quaternary ammonium compound which has been reported to be actively secreted into bile. As was found with 150C47, the nonlinearity of the dose excretion curve cannot be attributed to a limited ability of the liver to accumulate PAEB, since the amount found in liver was roughly proportional to dose over the dose range investigated. Solomon and Schanker[3] indicate that PAEB is accumulated by liver slices *in vitro* by a saturable process closely related to the biliary secretion *in vivo*. It should be noted that only a 2-fold accumulation was observed in the slices after 2–4 hr of incubation. Since the present results demonstrate that *in vivo* 80 per cent of the PAEB in liver was conjugated 1 hr after the administration of 25 mg/kg of PAEB, the possibility that the accumulation observed by these workers *in vitro* was due entirely to metabolic conversion of PAEB in the slices and not to the proposed transport process must be considered. In any case, the accumulation of PAEB by the liver does not appear to be the rate-limiting step in the transfer from blood to bile *in vivo*.

Although PAEB has been shown to be only slightly bound by liver homogenates,[3] the dose–liver concentration curves for PAEB are quantitatively similar to those for 150C47. The present work shows that 50–60 per cent of the PAEB in liver is already conjugated immediately after the administration of 25 mg/kg. The conjugated PAEB in liver after lower doses of PAEB was not measured, but it is likely that an even greater fraction might be conjugated at lower doses. The plasma concentrations of PAEB were considerably higher than the plasma concentrations of 150C47 corresponding to the same dose. PAEB, in contrast to 150C47, is probably only slightly bound to plasma proteins.[7]

It appears that passive distribution of PAEB by binding and equilibration with the membrane potential, combined with the observed rate of metabolism, might account for the liver–plasma distribution ratios observed immediately after the administration of 25 mg/kg of PAEB. Although the concentration of free PAEB has fallen considerably in both liver and plasma by the end of the 1-hr bile collection period, the plasma level has fallen to a relatively lower level, so that at this time the above factors can probably no longer account for the observed liver–plasma distribution ratio.

Whether similar mechanisms are responsible for the transfer of PAEB and 150C47 from liver into bile is difficult to evaluate. The excretion of PAEB is independent of sodium dehydrocholate-induced bile flow,[2] and this may indicate that the compounds are not excreted in the same manner. It would be of considerable interest to determine the effect of altering the excretion of the normally occurring bile salts on the excretion of both PAEB and 150C47.

Although the inhibition of PAEB excretion by other compounds has been taken as evidence that they are excreted by the same mechanism,[1-7] it is felt that the information gained from such an experiment could be misleading in the present case. If the 150C47

excretion depends on binding to bile salts or other complexing agents in bile, then even if PAEB enters the bile by a separate mechanism it could still influence the excretion of 150C47, since PAEB is known to complex with constituents in bile.[2] In this case interference of one compound with the transport of another one with similar binding properties would not be evidence that the primary mechanism by which they enter the bile is the same. In like manner, efficient transport of PAEB may depend on its concentration in bile being maintained at a low level by complexing with components in bile. Even though increasing the quantity of this material might not alter the rate of PAEB excretion, this could simply mean that an excess of the necessary complexing agent is already present, and decreasing the amount of this agent by competitive binding might cause this factor to become rate-limiting and decrease the rate of PAEB excretion. An effect of either compound on the excretion of the other might, therefore, result even though the two compounds gained entrance into the bile by entirely different mechanisms. Extreme caution should be employed in the interpretation of competition experiments in such complex systems until sufficient knowledge of the processes operating is available to permit a realistic evaluation of the results.

Of the quaternary ammonium compounds known to be excreted in bile,[6] few have been extensively studied. As more information on the manner in which these compounds accumulate in liver and bile becomes available, it may be possible to define the basic mechanisms underlying their biliary excretion more clearly.

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