Br. J. Pharmac. Chemother. (1966), **28**, 348–359.

THE BILIARY EXCRETION OF NEOSTIGMINE IN THE RAT

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(Received September 19, 1966)

The metabolism and excretion of (^{14}C)-neostigmine in urine has been studied recently in the rat. After parenteral administration, most of the dose is rapidly eliminated in the urine by renal tubular secretion, both as unchanged drug and as its metabolic products (Roberts, Thomas & Wilson, 1965a). Although the urinary metabolites have not been unequivocally identified, circumstantial evidence suggests that the principal metabolite is an unconjugated phenol, hydroxyphenyltrimethylammonium (Roberts, Thomas & Wilson, 1965b). Small amounts of another unidentified metabolite have also been isolated from urine after oral administration of (^{14}C)-neostigmine (Roberts, Thomas & Wilson, 1966).

In contrast, little is known of the extent or nature of the excretion of neostigmine in bile. Studies on other quaternary amines have shown that biliary excretion varies greatly from compound to compound. Some quaternary amines, for example benzomethamine and procainamide ethobromide, are excreted in high concentrations, and as much as 30% of the dose may be detected in bile (Levine & Clark, 1955 ; Schanker & Solomon, 1963). On the other hand, tetraethylammonium, hexamethonium, and decamethonium are only present in trace amounts in bile (Levine, 1960 ; Schanker, 1962 ; Lüthi & Waser, 1965). Although metabolites of quaternary compounds have not been identified in bile, both the unchanged compounds and their conjugates have been detected, and it has been suggested that they may form complexes *in vivo* with bile salts (Levine & Clark, 1955, 1957 ; Schanker & Solomon, 1963).

In the present paper, the excretion of neostigmine and its metabolites in bile has been studied in the rat. The initial experiments were concerned with the biliary excretion of radioactivity in normal rats after intravenous administration of (^{14}C)-neostigmine iodide. The effect of renal function on biliary excretion was also investigated. Subsequent experiments were concerned with the characterization by paper electrophoresis of the radioactivity excreted in bile. The bile/plasma concentration gradient after injection of (^{14}C)-neostigmine iodide was also studied.

METHODS

Experimental procedure

Ten male Wistar rats (200–400 g) were used in each type of experiment. Animals were anaesthetized with a 20% solution of urethane in distilled water (7.0 ml./kg I.P.). The trachea was intubated, and respiration was assisted when necessary. A polyethylene cannula was inserted in

the common bile duct through a mid-line abdominal incision. A similar cannula was inserted in the femoral or jugular vein. In some experiments, the renal vessels were ligated before the incision was closed.

Drugs

(^{14}C)-Neostigmine iodide (specific activity 15 μc/mg) was injected intravenously over a 5-min period. Doses ranging from 50 μg/kg to 200 μg/kg were contained in 0.3 ml. of 0.9% saline, and washed in with 0.1 ml. of saline.

Collection of bile and plasma

Bile was collected in graduated conical tubes (10 ml.). Collections were timed at hourly intervals for 6 hr after the injection of neostigmine, and the volumes were measured to the nearest 0.05 ml. Blood (approximately 2 ml.) was removed by cardiac puncture 30 min after the injection of neostigmine and added to dried heparin (0.1 mg) in a centrifuge tube. Plasma was removed after centrifugation and stored in the refrigerator.

Determination of radioactivity

Measurements of the radioactivity in bile and plasma were made in a series 3002 Packard Tri-Carb liquid scintillation spectrometer, using the scintillation fluid described by Kinard (1957). All results were corrected for quenching. After the radioactivity in the hourly collections of bile had been measured, the total bile secreted in the 6-hr period was pooled and extracted. In initial experiments, extracts of bile and plasma collected after low doses of (^{14}C)-neostigmine (50 or 100 μg/kg) resulted in unsatisfactory electrophoretograms. Subsequent experiments were therefore solely concerned with the extraction and electrophoresis of bile and plasma collected after doses of 200 μg/kg of (^{14}C)-neostigmine iodide.

Extraction of plasma and bile

Deproteinized extracts of plasma and bile were prepared by adding trichloracetic acid (20% w/v; 0.25 ml.) to plasma (1 ml.). After centrifugation, the supernatant fluid was washed twice with ether (4 ml.) and evaporated almost to dryness on a steam-bath.

Electrophoresis

Samples of deproteinized bile or plasma containing 20 c/s of radioactivity were applied as a narrow band, 5 mm long, in the centre of a strip of Whatman 3 MM paper (26 × 3 cm). The papers were moistened to within 1 cm of the origin by alternately immersing both ends in borate buffer, pH = 9.2 (Clark & Lubs, 1917), and blotting between sheets of filter paper. The labelled components of the specimens were resolved by electrophoresis in borate buffer for 4 hr (300 v, 1.1 mA/cm width). The electrophoretograms were then blotted, dried in a hot air oven, and cut transversely into serially numbered strips, 0.5 cm wide. Each paper strip was then transferred to a vial containing 2 ml. scintillation fluid and counted (Roberts *et al.*, 1965b).

Identification of neostigmine and hydroxyphenyltrimethylammonium conjugates

Bile (0.5 ml.) was added to β-glucuronidase (0.5 ml. Ketodase, William R. Warner; 5,000 u./ml.) or sulphatase (20 u. Sulfatase, Limpets Type III, Sigma Chemical Co.) and the pH of the solution was adjusted to 5.5 with sodium acetate buffer (0.2 M; pH = 5.0). Control specimens were prepared by adding equivalent volumes of acetate buffer to bile. After incubation for 24 hr at 37°, samples containing 20 c/s of radioactivity were resolved by electrophoresis.

Ethylene chloride extraction

(^{14}C)-Neostigmine iodide (1 μg) was added to 1% aqueous sodium glycocholate (2 ml.), 1% aqueous sodium taurocholate (2 ml.), or to normal rat bile (2 ml.). Control solutions were prepared by adding (^{14}C)-neostigmine iodide to distilled water. Ethylene chloride (1:2-dichlorethane; 10 ml.)

was added to 1 ml. of the above solutions or to bile collected after (^{14}C)-neostigmine injection. After shaking mechanically for 30 min, the specimens were centrifuged. The concentration of radioactivity in the aqueous and the organic phase was then determined.

RESULTS

Rate of biliary secretion

The rate of bile flow in control and experimental animals is shown in Table 1. In control animals the mean rate of bile secretion was initially 0.60 ml./hr. In subsequent collections, the volume was reduced, and after 6 hr the rate was 0.50 ml./hr. Similar results have been reported in rats anaesthetized with ethylurethane (Krayer, 1928).

During the second, third and fourth hours after injection of (^{14}C)-neostigmine iodide alone, biliary secretion was greater than in control animals, and the increase in flow was directly proportional to the dose of drug administered (Table 1 (i), (ii), (iii)). In contrast, during the other collection periods no similar relationship was observed. Differences in bile flow between control and neostigmine-treated animals were not statistically significant ($P>0.05$).

TABLE 1

THE RATE OF BILE FLOW IN CONTROL AND (^{14}C)-NEOSTIGMINE-TREATED RATS

The common bile duct was cannulated approximately 15 min before the intravenous injection of 0·3 ml. saline (control animals) or (^{14}C)-neostigmine iodide (experimental animals). Roman numerals in parentheses represent results from rats given (^{14}C)- neostigmine iodide: (i) 50 μg/kg, (ii) 100 μg/kg, (iii) 200 μg/kg, (iv) 50 μg/kg after ligation of the renal vessels. Bile was collected at hourly intervals after the injection of saline or neostigmine. Each result represents the mean and standard deviation of ten experiments.

Mean values were rounded off to the nearest 0·05 ml.

Time after injection (hr)	(Bile flow ml./hr)				
	Controls	(i)	(ii)	(iii)	(iv)
1	0·60±0·10	0·50±0·07	0·50±0·08	0·60±0·07	0·50±0·09
2	0·45±0·06	0·50±0·05	0·55±0·09	0·60±0·11	0·50±0·08
3	0·45±0·08	0·50±0·11	0·55±0·08	0·60±0·09	0·45±0·08
4	0·45±0·08	0·50±0·06	0·50±0·09	0·55±0·11	0·50±0·09
5	0·50±0·06	0·45±0·07	0·45±0·07	0·45±0·09	0·50±0·06
6	0·50±0·11	0·45±0·10	0·45±0·08	0·55±0·03	0·40±0·11

Ligation of the renal vessels before (^{14}C)-neostigmine iodide injection (Table 1 (iv)) caused little or no variation in bile flow, when compared with results in normal rats given an equivalent dose of the drug (Table 1 (i)).

Excretion of radioactivity in bile

Table 2 shows the excretion of radioactivity in bile after (^{14}C)-neostigmine iodide (50, 100 or 200 μg/kg, intravenously). Each result is expressed as a percentage of the dose administered, and represents the mean and standard deviation of 10 experiments. Only a small proportion of the administered radioactivity was recovered from the bile within 6 hr, suggesting that biliary excretion is of little quantitative importance in the elimination of neostigmine or its metabolic products. The percentage of the dose present in bile appears to be dependent on the amount of neostigmine given. Thus, after 50 μg/kg, only 1.45% of the administered radioactivity was recovered from the bile in 6 hr, but when the dose was increased to 200 μg/kg the proportion of radioactivity excreted was almost doubled, to 2.57%, within the same period. The differences between the mean

excretion of radioactivity after 50 μg/kg and 200 μg/kg (^{14}C)-neostigmine iodide were statistically significant (Table 2).

TABLE 2

THE EXCRETION OF RADIOACTIVITY IN BILE AFTER (^{14}C)-NEOSTIGMINE IODIDE

(^{14}C)-Neostigmine iodide (50, 100 or 200 μg/kg) was injected intravenously, and the total radioactivity excreted in bile was summated at hourly intervals and expressed as a percentage of the dose. Each figure represents the mean and standard deviation of 10 experiments. P is the probability that differences between the mean excretion after 50 μg/kg and 200 μg/kg are due to chance

Time after injection (hr)	Radioactivity in bile (% of dose) after (^{14}C)-neostigmine iodide			
	50 μg/kg	100 μg/kg	200 μg/kg	P
1	0·15±0·03	0·20±0·05	0·27±0·17	<0·05
2	0·35±0·06	0·55±0·14	0·59±0·34	<0·01
3	0·64±0·18	1·02±0·27	1·07±0·45	<0·02
4	0·94±0·28	1·40±0·33	1·60±0·52	<0·005
5	1·22±0·34	1·65±0·34	2·20±0·63	<0·001
6	1·45±0·39	1·86±0·39	2·57±0·61	<0·001

Ligation of the renal pedicles

Many quaternary amines are rapidly eliminated from the body by renal tubular secretion (Peters, 1960). In the rat, as much as 50% of a dose of parenteral neostigmine may be detected in the urine within 2 hr, either as the unchanged drug or as its metabolites (Roberts *et al.*, 1965b). Consequently, both the metabolism of the drug by the liver, and its biliary excretion, may be indirectly influenced by the absence of renal function.

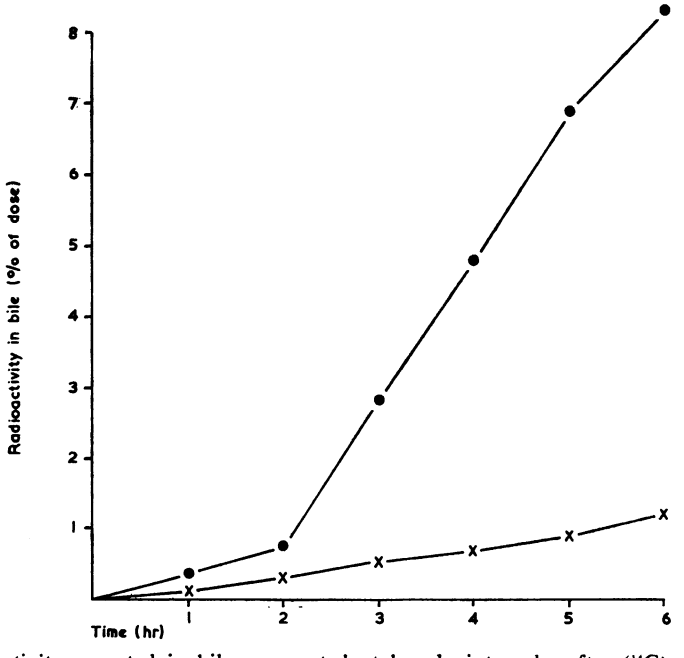

Fig. 1. Radioactivity excreted in bile, summated at hourly intervals, after (^{14}C)-neostigmine iodide (50 μg/kg, I.V.). Each point on the graph represents the mean of 10 experiments. (^{14}C)-neostigmine alone ×——×; (^{14}C)-neostigmine in rats with ligated renal vessels ●——●.

The results of experiments in which the renal pedicles were tied before the injection of (^{14}C)-neostigmine iodide (50 μg/kg, I.V.) are shown in Fig. 1. Compared with control animals, the proportion of the dose excreted in bile is greatly increased by ligation of the renal vessels, particularly during the later collection periods. For example, in the first 2 hr only 0.8% of the administered radioactivity was excreted in bile; however, during the remaining 4 hr a further 7% of the dose was recovered (Fig. 1). Analysis of the excretion of radioactivity in relation to time suggests that there are qualitative as well as quantitative differences between renal and hepatic excretion. Thus, although neostigmine and its principal metabolite are maximally excreted in the urine in the first 2 hr after parenteral administration of the drug (Roberts *et al.*, 1965b), ligation of the renal vessels does not markedly increase the biliary excretion of radioactivity during this period. Enhanced excretion of radioactivity in bile is most evident after 2 hr, when the maximal excretion in urine is normally declining. The delay suggests that metabolites and conjugates of neostigmine, rather than the drug itself, may be responsible for the increased excretion in bile after ligation of the renal vessels.

Electrophoresis of bile

Figure 2 illustrates the results of a typical experiment. When solutions of (^{14}C)-neostigmine and (^{14}C)-hydroxyphenyltrimethylammonium were added to normal rat bile *in vitro* and resolved, two distinct peaks of radioactivity were observed (Fig. 2a),

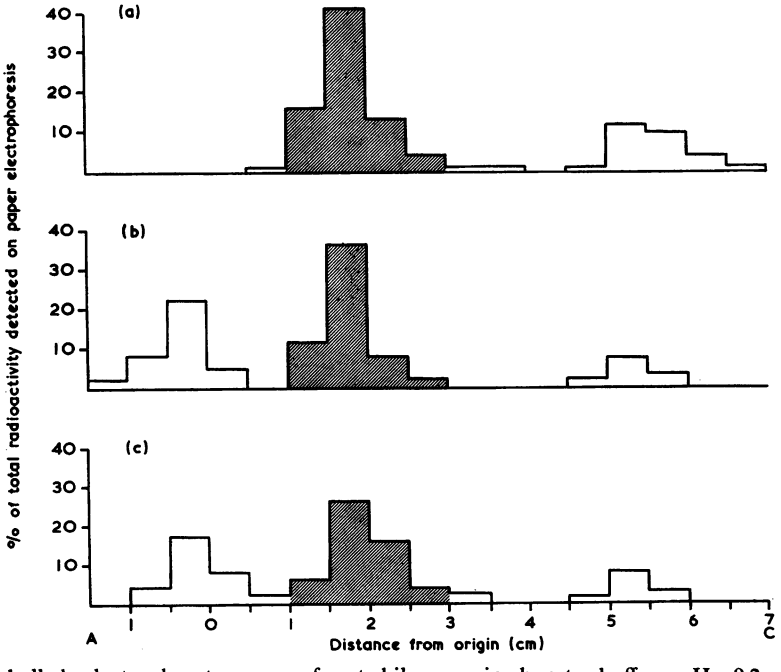

Fig. 2. Labelled electrophoretograms of rat bile run in borate buffer, pH = 9.2. A = anode, C = cathode. (a) Separation of (^{14}C)-hydroxyphenyltrimethylammonium (shaded area) from (^{14}C)-neostigmine (unshaded area) when added *in vitro* to normal rat bile. (b) Rat bile after (^{14}C)-neostigmine injection. (c) Rat bile incubated with β-glucuronidase after (^{14}C)-neostigmine injection.

Hydroxyphenyltrimethylammonium (shaded area) migrated towards the cathode less rapidly than neostigmine (unshaded area). The resolution of the two compounds from bile was less satisfactory than their electrophoretic separation from aqueous solutions (Roberts *et al.*, 1965b).

When rat bile collected after injection of (^{14}C)-neostigmine (200 μg/kg) was resolved, radioactivity was detected in three zones of the electrophoretogram (Fig. 2b). Significant quantities of radioactivity migrated slowly towards the anode, and were detected within 1 cm of the origin. Approximately half of the radioactivity moved 2–3 cm towards the cathode, in a similar position to concurrently run authentic standards of hydroxyphenyltrimethylammonium (shaded area). Small amounts of radioactivity were identified in a similar position to standards of neostigmine. Nevertheless, there was a marked variation from experiment to experiment in the amount of unchanged neostigmine present. Table 3 shows the percentage of neostigmine and hydroxyphenyltrimethylammonium in extracts of bile after injection of (^{14}C)-neostigmine, expressed as the mean and standard deviation of six experiments. In bile, $5.1 \pm 3.2\%$ of the radioactivity was due to free neostigmine. Hydroxyphenyltrimethylammonium accounted for $54.0 \pm 5.3\%$ of the radioactivity.

TABLE 3

THE IDENTIFICATION OF HYDROXYPHENYLTRIMETHYLAMMONIUM AND NEOSTIGMINE IN RAT BILE AND PLASMA

Blood was collected 30 min after injection of (^{14}C)-neostigmine iodide (200 μg/kg, I.V.). Plasma was removed after centrifugation and extracted. Bile was collected for 6 hr after injection of (^{14}C)-neostigmine and extracted. Hydroxyphenyltrimethylammonium and neostigmine were identified in extracts of plasma and bile by electrophoresis in borate buffer, pH=9·2. Values represent the mean and standard deviation of six experiments

	% radioactivity on electrophoretogram as	
	Hydroxyphenyltrimethylammonium	Neostigmine
Bile	54·0±5·3	5·1±3·2
Plasma	91·3±5·8	8·7±2·9

When specimens of rat bile collected after injection of (^{14}C)-neostigmine were incubated with β-glucuronidase before electrophoresis, no evidence of the presence of glucuronide conjugates of neostigmine or hydroxyphenyltrimethylammonium was obtained. The electrophoretogram (Fig. 2c) was similar to that observed when bile was not incubated (Fig. 2b), or when bile was incubated with acetate buffer alone. Similar results were obtained when bile was treated with sulphatase before electrophoresis.

Electrophoresis of plasma

The results of electrophoresis of extracts of plasma collected 30 min after injection of (^{14}C)-neostigmine are shown in Table 3. $91.3 \pm 5.8\%$ of the radioactivity had a similar electrophoretic mobility to concurrently run solutions of (^{14}C)-hydroxyphenylytrimethylammonium. Less than 10% of the radioactivity present ($8.7 \pm 2.9\%$) was identified as unchanged neostigmine. No radioactivity migrated towards the anode.

Ethylene chloride extraction

The effect of bile salts and bile on the extraction of radioactivity by ethylene chloride is shown in Table 4. When aqueous solutions of (^{14}C)-neostigmine were shaken with

ethylene chloride, approximately 10% of the radioactivity was removed. In the presence of bile salts or normal rat bile, the proportion of the drug extracted was increased three to five times; with sodium glycocholate, for example, almost half the total radioactivity was removed (Table 4). In several experiments, bile collected from rats after (^{14}C)-neostigmine injection was extracted with ethylene chloride. Only 8% of the total radioactivity present was removed. Ethylene chloride thus extracts a similar proportion of radioactivity from bile collected after (^{14}C)-neostigmine injection, and from aqueous solutions of (^{14}C)-neostigmine iodide (Table 4).

TABLE 4

THE EXTRACTION OF RADIOACTIVITY BY ETHYLENE CHLORIDE

All values are means of four experiments. Ethylene chloride (10 ml.) was shaken for 30 min with test solution or bile (1 ml.). Radioactivity in the aqueous and ethylene chloride layers was then determined

Solution extracted	% activity in aqueous phase	% activity in ethylene chloride phase
(^{14}C)-Neostigmine iodide in distilled water (0·5 μg/ml.)	89·7	10·3
(^{14}C)-Neostigmine iodide in normal rat bile (0·5 μg/ml.)	70·1	29·9
(^{14}C)-Neostigmine iodide in 1% aqueous sodium taurocholate (0·5 μg/ml.)	68·7	31·3
(^{14}C)-Neostigmine iodide in 1% aqueous sodium glycocholate (0·5 μg/ml.)	51·2	48·8
Bile collected after (^{14}C)-neostigmine iodide injection	91·5	8·5

TABLE 5

THE CONCENTRATION GRADIENT OF RADIOACTIVITY BETWEEN BILE AND PLASMA

Animals were given (^{14}C)-neostigmine iodide intravenously and blood was removed by cardiac puncture 30 min later. The concentration of radioactivity (counts/sec/ml.) in plasma was compared with that of bile in the first hour after neostigmine injection. Each figure represents the mean and standard deviation of 10 experiments. Roman numerals in parentheses correspond to results from rats given (^{14}C)-neostigmine iodide: (i) 50 μg/kg, (ii) 50 μg/kg after ligation of the renal pedicles, (iii) 100 μg/kg, (iv) 200 μg/kg. P is the probability that differences in the mean bile/plasma concentration ratio are due to chance

	Mean concentration counts/sec/ml.		Mean bile/plasma Concentration ratio	P
	Bile	Plasma		
(i)	18·0±5·6	4·7±1·7	3·9±0·8	
(ii)	39·1±10·8	9·2±3·1	4·1±1·2	>0·05
(iii)	60·5±21·6	12·4±4·7	5·0±1·2	>0·05
(iv)	90·4±65·3	17·3±11·4	5·5±2·0	>0·05

Bile/plasma concentration gradient

In these experiments, blood was obtained by cardiac puncture 30 min after the injection of (^{14}C)-neostigmine, and the concentration of radioactivity in plasma was estimated. The values obtained were compared with the concentration of radioactivity in bile during the first hourly collection. Table 5 shows the bile/plasma gradient of radioactivity, calculated for three different doses of neostigmine; data concerning the gradient after ligation of the renal pedicles was also included. Although the mean bile/plasma ratio increased from 3.9 to 5.5 as the dose of neostigmine was raised from 50 μg/kg to 200 μg/kg, the difference between the means was not significant. There was a consider-

able individual variation in the concentration gradients achieved, as indicated by the large standard deviations (Table 5). Ligation of the renal pedicles did not significantly influence the bile/plasma gradient of radioactivity after (^{14}C)-neostigmine iodide injection (50 μg/kg).

The bile/plasma gradient of radioactivity (Table 5) is not referable to either neostigmine or hydroxyphenyltrimethylammonium, since paper electrophoresis (Fig. 2; Table 3) demonstrated that the proportion of the two compounds in bile and plasma was different. The gradient for neostigmine and hydroxyphenyltrimethylammonium was therefore calculated from the concentration of radioactivity in bile and plasma and the results of paper electrophoresis after injection of (^{14}C)-neostigmine iodide (200 μg/kg). (This data involves the assumption that the relative proportions of neostigmine and hydroxyphenyltrimethylammonium excreted in bile are constant over the 6 hr collection period.)

TABLE 6

THE CONCENTRATION GRADIENT OF NEOSTIGMINE AND HYDROXYPHENYLTRIMETHYLAMMONIUM BETWEEN BILE AND PLASMA

Animals were given (^{14}C)-neostigmine iodide (200 μg/kg, I.V.), and blood was removed by cardiac puncture 30 min later. The concentration of neostigmine and hydroxyphenyltrimethylammonium was calculated from the total radioactivity (counts/sec/ml.) in bile and plasma and the results of subsequent paper electrophoresis. Each figure represents the mean and standard deviation of six to ten experiments

	Mean concentration counts/sec/ml.		Mean bile/plasma Concentration ratio
	Bile	Plasma	
Total radioactivity	90·4±65·3	17·3±11·4	5·5±2·0
Neostigmine	4·6±1·8	1·5±0·6	3·1±1·1
Hydroxyphenyltrimethylammonium	49·2±17·2	15·8±3·1	3·1±0·9

Table 6 shows the concentration of both compounds in bile and plasma, and the mean concentration ratio. The values shown (mean ± standard deviation) are based on the results of six experiments. The gradient for neostigmine (3.1 ± 1.1) was identical with that for hydroxyphenyltrimethylammonium (3.1 ± 0.9). However, in both cases the gradient was lower than the ratio of total radioactivity between plasma and bile, since a significant proportion of radioactivity in the latter was not identified as either neostigmine or hydroxyphenyltrimethylammonium.

DISCUSSION

The relative importance of renal and biliary secretion in the elimination of drugs varies greatly. Although active transport in biliary canaliculi is in general similar to that in the proximal renal tubule, there are marked differences in affinity for foreign organic compounds (Sperber, 1965). It has been suggested that these differences in substrate transport may be related to molecular size. Compounds of low molecular weight are mainly excreted in urine, while those of higher molecular weight are predominantly excreted in bile (Williams, Smith & Millburn, 1965). However, in the case of quaternary nitrogen compounds, there is considerable evidence that molecular structure as well as molecular size may influence the proportion of the drug excreted in bile. Thus quaternary amines with two polar groups at opposite ends of the molecule are excreted in bile in small amounts; in contrast, quaternary amines with one or more non-polar rings at one end of the molecule are excreted in bile in large amounts (Schanker, 1965).

These considerations suggest that only small amounts of neostigmine will be excreted in bile, since the drug has a relatively small molecule (mol. wt. = 223), with a polar amide group at the non-quaternary end of the molecule. Indirect evidence also supports the view that the biliary excretion of neostigmine is quantitatively insignificant. Roberts *et al.* (1965a) showed that after parenteral administration of (^{14}C)-neostigmine in the rat, radioactivity was rapidly concentrated in the liver, and slowly declined in the course of 24 hr. Nevertheless, only 6% of the administered radioactivity was recovered from the faeces during this period. Since there is no evidence that either neostigmine or its metabolites are significantly reabsorbed in the intestine, these results suggested that biliary excretion is of little importance in the elimination of the drug. The present results are consistent with this view. Even after large doses of (^{14}C)-neostigmine, less than 3% of the administered radioactivity was recovered from the bile within 6 hr. When these results are considered in conjunction with those of Roberts *et al.* (1965a), it is clear that little or none of the radioactivity excreted in the bile is reabsorbed from the gut, and that any entero-hepatic circulation of neostigmine must be extremely limited.

In the present experiments, the excretion of radioactivity in bile after (^{14}C)-neostigmine iodide injection was dependent on the dose of the drug administered. Thus, when the dose of neostigmine was raised from 50 μg/kg to 200 μg/kg, the percentage radioactivity excreted in bile was increased after 1 hr, and after 6 hr the proportion eliminated was almost doubled. Doses of 100 μg/kg had an intermediate effect. There are at least two possible explanations for the dose-dependence of biliary excretion. Firstly, large doses of neostigmine may saturate the renal tubular secretory capacity, so that increased amounts of the drug are metabolized by the liver and excreted in the bile; however, saturation must occur at extremely low plasma levels of neostigmine to account for the differences observed. Secondly, the binding capacity of plasma protein may be saturated by the low doses of neostigmine (Goldstein, Krayer, Root, Acheson & Doherty, 1949). Consequently, increasing the dose may enhance the rate of drug metabolism by raising the concentration of the diffusible fraction in plasma (Brodie, 1965).

Neostigmine, in common with many other quaternary amines is rapidly excreted by the renal tubules, and as much as 50% of the administered dose may be recovered from the urine within 2 hr (Peters, 1960; Roberts et al., 1965a). The present experiments have confirmed the pre-eminent importance of the kidney in the elimination of the drug, since ligation of the renal vessels before (^{14}C)-neostigmine injection markedly increased the percentage radioactivity excreted in bile. Differences in biliary excretion between rats with normal and impaired renal function were most evident in the second to sixth hours after (^{14}C)-neostigmine injection, suggesting that the increased biliary excretion of radioactivity in the latter was not due to the simple elimination of unchanged neostigmine in the bile as a consequence of its diversion to the liver, but to the delayed excretion of metabolites and conjugates of the drug. The results of paper electrophoresis confirm that only small amounts of radioactivity are excreted in bile as unchanged neostigmine. Approximately 5% of the total radioactivity in bile was present as the unaltered compound. Williams, Millburn & Smith (1965) have proposed that unless drugs are metabolized or conjugated by the liver they are not excreted in the bile, and the results of the present experiments are in accordance with this view.

More than half of the radioactivity present in bile was isolated as hydroxyphenyltrimethylammonium. Roberts *et al.* (1965b) have previously identified this compound as

the major breakdown product of neostigmine in urine. It is nevertheless surprising that no evidence of the presence of glucuronide or sulphate conjugates was obtained, since other phenolic compounds are frequently excreted in bile in this manner. However, the present evidence does not entirely exclude the existence of conjugates of hydroxyphenyltrimethylammonium, since both the conjugate and the parent phenol may have a similar electrophoretic mobility. Approximately 40% of radioactivity in bile remained at or near the origin. Its poor mobility suggests that it may represent a demethylation product of neostigmine. Birtley, Roberts, Thomas & Wilson (1966) presented evidence that the closely related compound pyridostigmine is partly demethylated in the rat, and it is possible that neostigmine is degraded in a comparable manner.

The results of electrophoresis of extracts of plasma show that in the rat, neostigmine is rapidly broken down to hydroxyphenyltrimethylammonium; within 30 min of intravenous injection, only 9% of radioactivity in plasma was present as unchanged neostigmine. Similarly, when human plasma is incubated with neostigmine *in vitro*, the drug is hydrolysed to the same phenolic metabolite (Nowell, Scott & Wilson, 1962). However, this reaction, catalysed by plasma cholinesterase, is extremely slow, and incubation for at least 8 hr is necessary before hydroxyphenyltrimethylammonium can be identified. It is therefore unlikely that the appearance of the metabolic products of neostigmine in plasma is due to the breakdown of the drug *in situ*. Probably the prompt appearance of the phenol in plasma after neostigmine injection is a reflection of the rapid metabolism by the liver (Roberts *et al.*, 1965b). Hydrolysis of neostigmine to hydroxyphenyltrimethylammonium in liver is almost complete within 10 min of parenteral administration, and is probably responsible for the rapid and evanescent action of the drug.

Several quaternary nitrogen compounds appear to combine *in vitro* with bile salts, forming complexes that are more readily extracted by organic solvents—for example, ethylene chloride, than aqueous solutions of the parent compounds (Levine & Clark, 1955, 1957; Schanker & Solomon, 1963). It has been suggested that the excretion of quaternary amines in bile might be dependent on the formation of similar compounds *in vivo*. Thus, bile salts, secreted by the biliary canaliculus, might function as a "carrier" by facilitating the transport of quaternary amines across the otherwise impermeable epithelium. However, the evidence that procainamide ethobromide is transported in this manner is unconvincing, although the drug is excreted in bile in extremely high concentrations (Schanker & Solomon, 1963). Furthermore, some compounds—for example, hexamethonium—are excreted in bile in low concentrations, although bile salts have no effect on their extraction by ethylene chloride (Levine & Clark, 1955). In the present experiments, the extraction of radioactivity by ethylene chloride *in vitro* was clearly influenced by the presence of bile salts. Solutions of sodium glycocholate and sodium taurocholate increased the extraction of (^{14}C)-neostigmine 3–5 times, and this effect was most marked with sodium glycocholate. However, when rat bile collected after (^{14}C)-neostigmine injection was treated in a similar manner, the radioactivity extracted from bile and aqueous solutions of (^{14}C)-neostigmine was similar. The results suggest that although neostigmine, like other quaternary amines, may form complexes with bile salts *in vitro*, these complexes are not an important factor in the excretion of radioactivity in bile after (^{14}C)-neostigmine injection.

Only low concentration gradients of radioactivity were observed between the bile and blood. The ratio ranged from 3.9 to 5.5, and was not significantly influenced by the dose of neostigmine administered or by ligation of the renal pedicles. However, this ratio is not directly referable to either neostigmine or hydroxyphenyltrimethylammonium, since electrophoresis showed that the proportion of radioactivity present as these compounds varied. When account was taken of this factor, the bile/blood concentration gradient of both compounds was 3.1. This gradient is considerably lower than that for most drugs excreted in bile by active mechanisms; in these cases, the bile/blood ratio usually ranges from 10 to 1000 (Brauer, 1959). Nevertheless, the low gradient does not exclude the possibility that both neostigmine and hydroxyphenyltrimethylammonium are actively transported from blood to bile against a low concentration gradient. Since the gradient in both cases is similar, if active transport occurs both compounds appear to have a similar affinity for the carrier.

SUMMARY

1. The biliary excretion of radioactivity after (^{14}C)-neostigmine iodide was studied in the rat. After 50 μg/kg, 1.45% of the radioactivity was detected in bile within 6 hr, but when the dose was increased to 200 μg/kg, the proportion excreted was 2.57%. Ligation of the renal pedicles greatly increased biliary excretion of radioactivity.

2. Electrophoretograms of bile after neostigmine injection showed that only trace amounts of free drug were present. More than half the radioactivity in bile was due to hydroxyphenyltrimethylammonium, while a further 40% was due to an unidentified metabolite. No evidence of conjugation was obtained. Similar studies on plasma showed that only 9% of radioactivity was present as free drug 30 min after injection.

3. The extraction of (^{14}C)-neostigmine by ethylene chloride was enhanced by bile salts. Similar amounts of radioactivity were extracted from bile collected after (^{14}C)-neostigmine, and from aqueous solutions of drug.

4. The bile/plasma gradient of radioactivity ranged from 3.9 to 5.5.

5. Differences in the biliary secretion rate of normal and neostigmine-treated animals were not significant.

I am most grateful to Professor A. Wilson for his advice and encouragement during this investigation. This work was in part supported by a grant from the Medical Research Council.

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