Differences between the Biliary Excretion of Tri[¹⁴C]methyl-(3-hydroxyphenyl)ammonium Iodide in Wistar and Gunn Rats

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1. The biliary excretion of [¹⁴C]trimophonium iodide [tri[¹⁴C]methyl(3-hydroxyphenyl)ammonium iodide] was studied in normal Wistar animals and in jaundiced homozygous Gunn rats. 2. In normal Wistar rats small amounts of radioactivity (approx. 3% of the dose in 4 h) were excreted in bile as two glucuronide conjugates, i.e. [¹⁴C]trimophonium glucuronide [tri[¹⁴C]methyl-(3-oxyphenyl)ammonium glucuronide] (85%) and 3-di[¹⁴C]methylaminophenyl glucuronide (10–15%). Only minor amounts of the unchanged drug were detected in bile. 3. In the homozygous jaundiced Gunn rat large amounts of radioactivity (26% of the dose in 4 h) were eliminated in bile as [¹⁴C]trimophonium glucuronide alone. The quantitative excretion of this metabolite in Gunn rat bile was about ten times that in normal animals. 4. It is proposed that the biochemical lesion in the homozygous Gunn rat may indirectly affect the biliary transport of exogenous glucuronides across the canalicular membrane.

Many phenolic compounds, e.g. morphine (Woods, 1954) and paracetamol (Brodie & Axelrod, 1948), are eliminated as glucuronide conjugates. Under these conditions only minor amounts of the unchanged phenol are excreted in bile or urine. It is generally recognized that conjugation with glucuronic acid increases the acidity and water-solubility of these compounds, and that the reaction is catalysed by the enzyme UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) in the endoplasmic reticulum of the mammalian liver.

It is uncertain whether these considerations also apply to the metabolism and conjugation of phenolic quaternary amines. Indeed, it is commonly assumed that quaternary compounds are not metabolized by the liver, since breakdown products have not been generally detected. The lipoprotein nature of endoplasmic membranes may restrict the penetration and limit the metabolism of most polar compounds (Gaudette & Brodie, 1959; MacMahon & Easton, 1961; Mazel & Henderson, 1965). In addition, glucuronide conjugation cannot significantly enhance the water-solubility of quaternary amines, so that synthetic reactions serve no useful purpose.

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In the present experiments the metabolism and biliary excretion of the phenolic quaternary amine trimophonium iodide [trimethyl-(3-hydroxyphenyl)ammonium iodide] were compared in Gunn's strain of jaundiced rats and normal Wistar animals. Homozygous Gunn rats are jaundiced throughout life, since the genetic lesion in UDP-glucuronyltransferase prevents the formation of bilirubin glucuronide, and the conjugation of many phenolic compounds is also depressed to a variable extent (Schmid, Axelrod, Hammaker & Rosenthal, 1957; Schmid, Axelrod, Hammaker & Swarm, 1958).

EXPERIMENTAL

Experimental procedure. Fed normal Wistar rats (250–300 g) or homozygous jaundiced Gunn rats (240–550 g) of either sex were anaesthetized with urethane (14%, w/v, in deionized water; 10.0 ml/kg body wt., injected intraperitoneally). The trachea was intubated and respiration was assisted when necessary. The abdomen was opened by a mid-line incision, and a polyethylene cannula (0.4 mm internal diam., 0.8 mm external diam.) was inserted in the common bile duct approx. 1 cm above its junction with the duodenum. A similar cannula was placed in a femoral vein.

[Me-¹⁴C]Trimophonium iodide (The Radiochemical Centre, Amersham, Bucks., U.K.), specific radioactivity 10.2 μ Ci/ μ mol, was dissolved in 0.9% NaCl. The dose administered to each rat (2.0 μ mol/kg body wt. in approx. 0.3 ml of 0.9% NaCl) was standardized by weight and

usually injected into a femoral vein over a 1 min period. In some experiments sulphobromophthalein sodium ($1.0 \mu\text{mol/kg}$; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and [^{14}C]trimophonium iodide ($2.0 \mu\text{mol/kg}$) were directly injected into the portal vein. Both drugs were injected into the same animal; injections were separated by an interval of 1–2 h.

Collection of bile. Samples of bile were usually collected at 1 h intervals after the intravenous injection of [^{14}C]trimophonium iodide. Volumes were calculated by dividing the weight of each sample by a previously determined value for the specific gravity of bile (1.011).

Measurement of radioactivity in bile. Radioactivity in bile was assayed by liquid-scintillation spectrometry. Samples of bile ($50\text{--}100 \mu\text{l}$) were directly added to scintillation fluid (10.0 ml) in low-potassium glass vials. The composition of the scintillation fluid (per l) was as follows: naphthalene (80 g), 2,5-diphenyloxazole (5 g), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (110 mg), ethanol (230 ml), dioxan (385 ml) and toluene (385 ml). The radioactivity of the samples was usually counted at an efficiency of 66–74% in a Packard Tri-Carb model 3002 liquid-scintillation spectrometer. Counting efficiency was determined by the addition of an internal standard of $n\text{-}[^{14}\text{C}]\text{hexadecane}$ (The Radiochemical Centre).

Separation of metabolites. Samples of bile containing approx. 10^4 d.p.m. of radioactivity were applied as a narrow band to one end of a strip of Whatman no. 1 paper ($9 \text{ cm} \times 50 \text{ cm}$). Labelled components in bile were resolved by descending chromatography in butan-1-ol-water-ethanol-acetic acid (32:12:8:1, by vol.) for 18 h at room temperature. After resolution, radiochromatograms were usually dried and cut transversely into serially numbered strips approx. 1.0 cm wide; each strip was then transferred to a vial containing scintillation fluid and its radioactivity counted as described above. Radioactive zones on paper chromatograms were sometimes identified by means of a Tracerlab 4π scanner. In some experiments, samples of bile were collected and directly chromatographed at 1 min intervals after the injection of [^{14}C]trimophonium iodide into the portal vein. The presence of sulphobromophthalein was detected at similar time-intervals by allowing bile to collect on a pellet of NaOH.

In other experiments [^{14}C]trimophonium iodide ($100 \mu\text{l}$, $2.0 \mu\text{mol/ml}$) was added to sodium glucuronide ($100 \mu\text{l}$, $2.0 \mu\text{mol/ml}$) or UDP-glucuronic acid ($100 \mu\text{l}$, $2.0 \mu\text{mol/ml}$), and the volume of the mixture was adjusted to 1.0 ml by the addition of 0.2 M -sodium acetate buffer, pH 3.6–5.6. Studies were carried out at three different pH values (pH 3.6, 4.6 and 5.6). After incubation for 2 h at 38°C the labelled components were separated by paper chromatography as described above. Radioactive zones were identified by scanning the paper chromatograms with a Tracerlab 4π scanner.

RESULTS

Excretion of radioactivity in bile. The excretion of radioactivity in the bile of normal Wistar and homozygous Gunn rats after intravenous injection of [^{14}C]trimophonium iodide is shown in Fig. 1.

There were marked differences in the biliary excretion of radioactivity between normal Wistar

Fig. 1. Excretion of radioactivity in bile after intravenous injection of [^{14}C]trimophonium iodide ($2.0 \mu\text{mol/kg}$ body wt.). Results were summated at 1 h intervals. Each point and vertical line represents the mean and standard error of ten experiments. ●, Normal Wistar rats; ○, homozygous Gunn rats.

animals and homozygous Gunn rats. In normal rats biliary excretion was of little or no importance in the elimination of [^{14}C]trimophonium iodide and its metabolites. Only small amounts of radioactivity were detected in bile; roughly 3% of the dose was recovered in 4 h. Thus the biliary excretion of [^{14}C]trimophonium iodide is quantitatively similar to that of the parent compound neostigmine (Calvey, 1966). In contrast, jaundiced homozygous Gunn rats eliminated large amounts of radioactivity in bile after intravenous injection of [^{14}C]trimophonium iodide. In the first hour $9.0 \pm 1.4\%$ (mean $\pm$ s.e.m.) of the dose was present in bile; more than one-quarter of the drug ($26.4 \pm 3.0\%$) was excreted by the end of the experiment (Fig. 1). The proportion of the drug eliminated in Gunn rat bile was eight to ten times that in normal animals.

Separation of metabolites. When [^{14}C]trimophonium iodide was administered to normal rats and samples of bile were collected and resolved, radioactivity was detected in four zones of the chromatogram (M1, M2, M3 and M4; Table 1). The radioactive areas corresponded to R_F values of 0.07–0.13 (M1), 0.38–0.46 (M2), 0.65–0.72 (M3) and 0.81–0.88 (M4). Identification of the chemical nature of these metabolites has been described by Somani, Wright & Calvey (1970). Prior incubation of bile with β -glucuronidase [controlled by the addition of the inhibitor glucaro-(1 $\rightarrow$ 4)-lactone] established that compounds M1 and M2 were the

Table 1. *Metabolites of [¹⁴C]trimophonium in Wistar and Gunn rat bile*

The labelled metabolites M1 (trimophonium glucuronide), M2 (3-dimethylaminophenyl glucuronide), M3 (trimophonium) and M4 (3-dimethylaminophenol) were separated by chromatography as described in the text. The values, expressed as the means $\pm$ s.d., correspond to the proportion of the total radioactivity (corrected for background) detected at each R_F value.

		Distribution of radioactivity (%)			
R_F value	...	M1 0.07–0.13	M2 0.38–0.46	M3 0.65–0.72	M4 0.81–0.88
(a) Normal Wistar rat bile					
Hour 1		84 $\pm$ 6	10 $\pm$ 4	6 $\pm$ 3	Trace
Hour 2		84 $\pm$ 5	15 $\pm$ 4	1 $\pm$ 0	Trace
Hour 3		86 $\pm$ 3	14 $\pm$ 3	Trace	Trace
Hour 4		86 $\pm$ 3	14 $\pm$ 3	Trace	Trace
(b) Homozygous jaundiced Gunn rat bile					
Hour 1		98 $\pm$ 2	1 $\pm$ 1	1 $\pm$ 0	Trace
Hour 2		99 $\pm$ 1	1 $\pm$ 0	Trace	Trace
Hour 3		99 $\pm$ 1	1 $\pm$ 0	Trace	Trace
Hour 4		99 $\pm$ 1	1 $\pm$ 0	Trace	Trace

glucuronides of compounds M3 and M4 respectively; in addition, concurrent chromatography of bile with authentic standards of [¹⁴C]trimophonium iodide and 3-dimethylaminophenol in five different solvent systems suggested that compounds M3 and M4 represented the unchanged drug and its *N*-demethylated metabolite (Somani *et al.* 1970). In other studies it has been shown that the u.v.-absorption spectrum of compound M3 is identical with that of authentic trimophonium iodide (Somani, 1969). Table 1 shows the excretion of trimophonium and its metabolites in normal rat bile during the 4h experiment. Most of the drug eliminated in bile (i.e. 84–86%) was present as trimophonium glucuronide [trimethyl-(3-oxyphenyl)ammonium glucuronide or *O*-(3-trimethylamino)phenyl glucuronide]; a further 10–15% was excreted as 3-dimethylaminophenyl glucuronide. Excretion of the unchanged drug in bile rapidly declined during the first hour of these experiments.

Different results were obtained in the homozygous Gunn rat (Table 1). After intravenous injection of [¹⁴C]trimophonium iodide 98–99% of the radioactivity in bile was eliminated as trimophonium glucuronide (M1). 3-Dimethylaminophenyl glucuronide (M2) only accounted for 1% of the total biliary excretion. Only trace amounts of other metabolites were present in bile.

Appearance of metabolites in bile. In some experiments sulphobromophthalein sodium or [¹⁴C]trimophonium iodide was directly injected into the portal vein of normal Wistar rats, and samples of bile were collected and resolved at 1min intervals. Unchanged [¹⁴C]trimophonium was detected in bile within 5min; sulphobromophthalein and [¹⁴C]-

trimophonium glucuronide appeared 2–3min later. 3-Dimethylaminophenyl glucuronide was not detected in bile until 18min after the intraportal injection of [¹⁴C]trimophonium iodide.

Studies in vitro. When [¹⁴C]trimophonium iodide was incubated *in vitro* with sodium glucuronide or UDP-glucuronic acid no evidence of non-enzymic formation of [¹⁴C]trimophonium glucuronide was obtained. Only unchanged [¹⁴C]trimophonium was identified on the radiochromatograms.

DISCUSSION

In the present experiments there were marked differences between the excretion of [¹⁴C]trimophonium and its metabolites in Wistar and Gunn rat bile. In normal Wistar animals small amounts of the radioactive drug were eliminated as [¹⁴C]-trimophonium glucuronide and 3-di[¹⁴C]methylaminophenyl glucuronide. In contrast, homozygous Gunn rats excreted a much greater proportion of the dose as [¹⁴C]trimophonium glucuronide alone; only trace amounts of other metabolites were detected in bile.

Some of these differences (e.g. the absence of 3-di[¹⁴C]methylaminophenyl glucuronide from Gunn rat bile) can be readily explained by the genetic defect in the jaundiced animals. Many drugs and foreign compounds (e.g. pethidine, ephedrine, imipramine, mepacrine and diphenhydramine) are *N*-demethylated by microsomal enzymes (La Du, Gaudette, Trousof & Brodie, 1955; Gaudette & Brodie, 1959; Clouet, 1964; Clark, 1967), and [¹⁴C]trimophonium may be *N*-demethylated by a similar mechanism. The present results suggest

that conjugation of the demethylated metabolite may be dependent on a microsomal glucuronyltransferase, since the proportion of biliary radioactivity excreted as 3-di ^{14}C methylaminophenyl glucuronide was much greater in normal animals (10–15%) than in the homozygous Gunn rat (1%). In these experiments 3-di ^{14}C methylaminophenyl glucuronide was not detected in bile until 18 min after the intraportal injection of ^{14}C trimophonium iodide. The slow appearance of this metabolite in bile is consistent with demethylation and conjugation by the endoplasmic reticulum.

Different considerations apply to the conjugation of unchanged ^{14}C trimophonium. In the homozygous Gunn rat, as well as in normal Wistar animals, most of the radioactivity identified in bile was present as ^{14}C trimophonium glucuronide. It is possible that the conjugation of ^{14}C trimophonium to form a 3-*O*-phenyl glucuronide is dependent on a microsomal glucuronyltransferase present in both strains of animals. Thus there is considerable evidence that several glucuronyltransferases are present in the endoplasmic reticulum (Dutton, 1966), and that homozygous Gunn rats are deficient in only some of these enzymes (Gartner & Arias, 1969). Alternatively, the conjugation of ^{14}C trimophonium may not be dependent on the endoplasmic reticulum. It is unlikely, for instance, that any quaternary amine can readily penetrate the phospholipid barrier imposed by endoplasmic membranes, which limits the metabolism of most polar compounds (Gaudette & Brodie, 1959). In addition, the rapid elimination of ^{14}C trimophonium glucuronide in bile is inconsistent with the conjugation of an ionic compound by the endoplasmic reticulum. In the present studies non-enzymic conjugation of ^{14}C trimophonium was not demonstrated, although some *N*-glucuronides may be synthesized in this manner (Bridges & Williams, 1962). It is therefore tentatively suggested that the conjugation of ^{14}C trimophonium is dependent on an enzyme system outside the endoplasmic reticulum.

These experiments suggest that the uptake, metabolism and biliary excretion of trimophonium in normal animals occurs in the following manner. After intravenous administration the labelled drug is readily transferred from the sinusoids to the hepatic parenchyma, since liver cells (unlike cells in many other tissues) are exceptionally porous (Schanker, 1962) and may not present a barrier to the inward diffusion of quaternary amines. In the liver cell approx. 15% of trimophonium slowly penetrates the phospholipid membrane of the endoplasmic reticulum, and is subsequently demethylated and conjugated to 3-dimethylaminophenyl glucuronide; the remaining 85% is rapidly metabolized to trimophonium glucuronide outside the reticulum by

an alternative mechanism. (The proposed partition of trimophonium may be comparable with the slow, restricted and unpredictable penetration of most cellular membranes by quaternary amines.) In the Gunn rat the partition of trimophonium between the reticular (15%) and the extrareticular system (85%) may be generally similar, but in the absence of UDP-glucuronyltransferase the demethylated metabolite is not conjugated. Excretion of the tertiary amine 3-dimethylaminophenol is limited by the unfavourable pH gradient between the liver cell (pH 7.6) and bile (pH 7.6).

In the present experiments there were marked differences between the proportion of radioactivity excreted in normal Wistar animals and in homozygous Gunn rats. It is suggested that the diminished excretion in normal animals is dependent on competition between the glucuronide conjugates and bilirubin glucuronide for receptor sites on the canalicular membrane. Presumably bilirubin glucuronide has a greater affinity for the transport system than the exogenous glucuronides, so only small amounts of drug metabolites enter bile; the rest leaves the liver cell via the sinusoids and is subsequently excreted in the urine. In the homozygous Gunn rat the canalicular transport of glucuronides is apparently unaffected by the genetic defect in conjugation, and the maximum excretion rate of preformed bilirubin glucuronide is similar to that of normal animals (Schmid *et al.* 1958; Arias, Johnson & Wolfson, 1961). In jaundiced animals receptor sites on the cellular aspect of the canalicular membrane (identical with those concerned with the active transport of bilirubin glucuronide in normal animals) are presumably unoccupied by substrate molecules; in these conditions glucuronides (e.g. ^{14}C trimophonium glucuronide) formed by Gunn rats may be excreted in bile in high concentrations, since these conjugates do not compete with bilirubin glucuronide for transport from the liver cell to bile. Recent studies in the homozygous Gunn rat suggest that the biliary excretion of ^{14}C trimophonium glucuronide is decreased by 80–90% after intravenous injection of bilirubin glucuronide; a similar depression was not observed in normal animals (T. N. Calvey & D. Back, unpublished work). Although some quaternary amines are actively transported from blood to bile by a specific carrier (Schanker & Solomon, 1963), there is no evidence that ^{14}C trimophonium is excreted in this manner. In both strains of animals only small amounts of unchanged trimophonium were excreted in bile. Excretion of the unchanged drug was greatest in the first hour, and rapidly declined as the compound was cleared from the plasma; thus its elimination may well reflect direct diffusion from plasma to bile in the peribiliary vascular plexus (i.e. downstream from the biliary canaliculus).

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