Species Variations in the Threshold Molecular-Weight Factor for the Biliary Excretion of Organic Anions

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1. The excretion in the bile and urine after intravenous injection of 16 organic anions having molecular weights between 355 and 752 was studied in female rats, guinea pigs and rabbits. 2. These compounds were mostly excreted unchanged, except for three of them, which were metabolized to a slight extent (<7% of dose). 3. The rat excreted all the compounds extensively (22-90% of dose) in the bile. 4. In guinea pigs four of the compounds with mol.wt. 355-403 were excreted in the bile to the extent of 7-16% of the dose, four with mol.wt. 407-465 to the extent of 25-44% and eight compounds with mol.wt. 479-752 to the extent of 44-100%. 5. In rabbits four compounds with mol.wt. 355-465 were excreted in the bile to the extent of 1-8% of the dose, two compounds with mol.wt. 479 and 495 to the extent of 24 and 22%, and six compounds with mol.wt. 505-752 to the extent of 31-94%. 6. These results, together with those of other investigations from this laboratory, are discussed and the conclusion is reached that there is a threshold molecular weight for appreciable biliary excretion (i.e. more than 10% of dose) of anions, which varies with species: about 325 ± 50 for the rat, 400 ± 50 for the guinea pig and 475 ± 50 for the rabbit. 7. Anions with molecular weights greater than about 500 are extensively excreted in the bile of all three species. 8. That proportion of the dose of these compounds which is not excreted in the bile is excreted in the urine, and in the three species, bile and urine are complementary excretory pathways, urinary excretion being greatest for the compounds of lowest molecular weight and tending to decrease with increasing molecular weight. 9. Some implications of this interspecies variation in the molecular-weight requirement for extensive biliary excretion are discussed.

The extent to which an organic compound is excreted in the bile may be influenced by a number of physicochemical factors, which include molecular weight, polarity, chemical structure and lipid solubility (Millburn *et al.*, 1967*a,b*; Hirom *et al.*, 1972). Further, with some compounds the extent varies widely with species and this may result in a marked interspecies variation in the overall pattern of excretion of the compounds. Those compounds and their metabolites that have a relatively low molecular weight (below about 300) are excreted mainly in the urine rather than in the bile, irrespective of species, but with organic anions of higher molecular weight (300-500) extensive elimination in the bile occurs in the rat and dog but not in the guinea pig, rabbit and rhesus monkey (Abou-El-Makarem *et al.*, 1967*b*). It has been proposed that for appreciable biliary excretion (i.e. >5-10% of dose) to occur in the rat an organic anion requires a minimum molecular weight of at least 325 ± 50 (Millburn *et al.*, 1967*a*). The findings of Abou-El-Makarem *et al.* (1967*b*) suggest that the minimum or threshold molecular weight required for appreciable biliary excretion may vary with species and may be higher, for example, in the guinea pig and

rabbit than in the rat. Further, a perusal of the literature suggests that in general there may not be a marked species difference in the biliary excretion of compounds of molecular weight greater than about 500 and that such compounds might be extensively excreted in the bile in all species (see Millburn, 1970; Smith, 1970).

The extent of biliary excretion of 16 organic anions with molecular weights ranging from 355 to 752 has therefore been investigated in the female rat, guinea pig and rabbit. The results obtained support the view that, for anions, molecular weight has an important bearing on biliary excretion and that there is a threshold molecular weight for appreciable biliary excretion, which varies with species. That the threshold molecular weight for appreciable biliary excretion in the rat is in the region of 325 ± 50 has been confirmed. It will also be shown that for the guinea pig the threshold is about 400 ± 50 and for the rabbit about 475 ± 50 . The results also support the suggestion that anionic compounds with molecular weights of about 500 or more are extensively excreted in the bile of most species. Some implications of this interspecies variation in the molecular-weight requirements for biliary

excretion are discussed inasmuch as it affects the pattern of elimination of compounds both of endogenous and exogenous origin.

Materials and Methods

Chemicals

The following *N*⁴-carboxyacetylsulphathiazoles were either purchased or synthesized in this laboratory and purified as described by Hirom *et al.* (1972): succinyl-, glutaryl-, adipyl-, phthalyl-, 1,2,3,6-tetrahydrophthalyl-, hexahydrophthalyl-, 2-carboxy-3-naphthoyl- and tetrachlorophthalyl-sulphathiazole. Phenolphthalein glucuronide sodium salt and phenolphthalein disulphate tripotassium salt (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.), Bromochlorophenol Blue, Bromophenol Blue and Bromocresol Green (Ralph N. Emmanuel Ltd., London, U.K.), Indocyanine Green (Cardio-Green; Hynson, Westcott and Dunning Inc., Baltimore, Md., U.S.A.), Tartrazine (Williams Ltd., Hounslow, Middx., U.K.) and Lissamine Fast Yellow (Edward Gurr Ltd., London S.W.14, U.K.) were purchased. All these compounds ran as single spots when chromatographed as described below.

Animals

Female Wistar albino rats (180–350g body wt.), Duncan Hartley albino guinea pigs (300–800g) and Dutch or New Zealand White rabbits (2–3kg) were used. The animals were anaesthetized with intraperitoneal thiopentone sodium (Pentothal; Abbott Laboratories Ltd., Queenborough, Kent, U.K.; 70mg/kg for rats and 50mg/kg for guinea pigs) or intravenous pentobarbitone sodium (Nembutal; Abbott Laboratories Ltd.; 30mg/kg for rabbits). The common bile duct was cannulated as described by Abou-El-Makarem *et al.* (1967b) and the cystic duct ligated in the guinea pig and rabbit to ensure that only hepatic bile was collected. The animals were kept anaesthetized for the duration (3h) of the experiment by the injection of small amounts of anaesthetic when necessary.

Estimation and chromatography of compounds in bile

The following compounds were estimated by methods described previously: *N*⁴-carboxyacetylsulphathiazoles (Hirom *et al.*, 1972), phenolphthalein glucuronide (Millburn *et al.*, 1967a), Tartrazine (Gregson *et al.*, 1972), Lissamine Fast Yellow (Bertagni *et al.*, 1972) and Indocyanine Green (Levine *et al.*, 1970). The chromatographic properties of these compounds are also described in the above papers.

Phenolphthalein disulphate was measured by the same procedure as for phenolphthalein glucuronide except that the hydrolysis was performed by heating

equal volumes of the bile and 2M-HCl in a boiling-water bath for 5min. The recovery of phenolphthalein disulphate (0.78–1.15mg/ml) added to bile was 90–99%.

For the assay of the sulphonphthalein dyes, the bile samples were suitably diluted with 0.01 M-NaOH and the extinction was measured in a Unicam SP. 600 spectrophotometer at 592nm for Bromochlorophenol Blue, at 590nm for Bromophenol Blue and at 610nm for Bromocresol Green. The recovery of these dyes when added to bile (0.5–50μmol/ml) was 95–100%. The *R_F* values of phenolphthalein disulphate and the sulphonphthalein dyes are shown in Table 1.

Results

Table 2 shows the extent of excretion in the bile and urine by bile-duct-cannulated rats, guinea pigs and rabbits of 16 compounds having molecular weights in the range 355–752. The compounds were administered intravenously at a dose of either 50 or 100μmol/kg [except for Indocyanine Green, where the dose was 3.2μmol/kg (see below)]. These compounds were of four types, namely, (a) *N*⁴-carboxyacetylsulphathiazole derivatives, (b) triarylmethane derivatives such as phenolphthalein glucuronide and Bromophenol Blue, (c) the pyrazolone azo dyes, Tartrazine and Lissamine Fast Yellow, and (d) the tricarbocyanine derivative, Indocyanine Green. They are strongly polar and exist largely as anions at physiological pH. Further, all the compounds studied were excreted unchanged in the bile and urine with the

Table 1. *R_F* values of some triarylmethane derivatives

Bile samples were chromatographed on thin-layer (0.25mm) plates of silica gel G. Solvent systems were: A, propan-1-ol–aq. NH₃ (sp.gr. 0.88) (7:3, v/v); B, butan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (10:1:1, by vol.); C, butan-1-ol–acetic acid–water (4:1:2, by vol.). The sulphonphthalein dyes appeared as blue spots when the plate was sprayed with 0.1 M-NaOH. Phenolphthalein disulphate was made visible by spraying with 2M-HCl and heating at 100°C for 2–3min, followed by spraying with aq. NH₃ soln. (sp.gr. 0.88), when it appeared as a red spot.

Compound	Solvent system ...	<i>R_F</i>		
		A	B	C
Phenolphthalein disulphate		0.60	—	0.45
Bromophenol Blue		0.72	0.25	0.85
Bromochlorophenol Blue		0.70	0.30	0.90
Bromocresol Green		0.67	0.33	0.90

exception of phthalyl-, 2-carboxy-3-naphthoyl- and tetrachlorophthalyl-sulphathiazole, which, although excreted in the bile mainly unchanged, also formed small amounts of metabolites in rat and guinea pig (<7 and 3% respectively) (Hirom *et al.*, 1972). In the rabbit all the compounds were excreted unchanged except tetrachlorophthalylsulphathiazole, of which some 3% of the dose was found in the bile as polar metabolites.

In the rat all 16 compounds were extensively excreted (22–90% of dose) in the bile, the extent of excretion tending to be greatest (63–90% of dose) for those compounds of higher molecular weight (range 479–752), and lowest (22–54%) for those having a lower molecular weight (range 355–465), apart from hexahydrophthalylsulphathiazole (mol.wt. 409), which showed the high value of 80%.

In the guinea pig the extent of biliary excretion again tended to increase with molecular weight, compounds having molecular weights above about 500 being excreted in the bile to the extent of 53–100% of the dose. Compounds of lower molecular weight were, apart from phenolphthalein disulphate (mol.wt. 479), less extensively excreted (7–44%) in the bile. With these lower-molecular-weight compounds, low values (7–11%) were found in the guinea pig for succinyl-, glutaryl- and adipyl-sulphathiazole compared with the rat (36–42%).

The pattern of biliary excretion in the rabbit of the compounds studied is significantly different from that found in the rat and guinea pig. The extent of biliary elimination in the rabbit of the four compounds, succinyl-, phthalyl- and hexahydrophthalyl-sulphathiazole and tartrazine, which have molecular weights less than 500, is low (1–8% of dose). Biliary excretion in the rabbit, however, becomes appreciable with phenolphthalein disulphate (mol.wt. 479) and phenolphthalein glucuronide (mol.wt. 495), which show values of 24 and 22% respectively. For the remaining compounds, which have a higher molecular weight (505–752), biliary excretion was extensive (31–94%).

The values (Table 2) for the urinary excretion of the above compounds show, with few exceptions, that the urine contains most of the material not accounted for in the bile. In all three species the bile and urine are complementary excretory pathways in that the extent of urinary excretion is in general greatest for the compounds of lowest molecular weight and tends to decrease as the molecular weight increases and biliary excretion becomes more extensive. The total recovery of compounds in bile and urine was high in most cases. Of the 39 results given for total recovery in Table 2, 31 are in the range 73–100%; of the remaining eight, five are between 57 and 66%. Low total recoveries were found in three instances: succinylsulphathiazole in the guinea pig, where the total recovery was 35%, the urine containing about five times that in the bile; 2-carboxy-3-naphthoylsulphathiazole in the guinea

pig, where the total recovery was 32%, 30% being in the bile and 2% in the urine; phenolphthalein disulphate in the rabbit, where the total recovery was 48%, the bile and urine containing equal amounts.

Discussion

Molecular-weight threshold for biliary excretion

Abou-El-Makarem *et al.* (1967*b*) showed that a species variation in the extent of the biliary excretion of certain compounds can occur and Millburn *et al.* (1967*a,b*) showed that in the rat significant biliary excretion occurs when the compound or its metabolites are polar anionic substances and have a molecular weight of not less than 325 ± 50 . The present work confirms this threshold value for the rat and suggests that for the guinea pig the value is about 400 and for the rabbit 500. To get a better definition of these threshold values, the extent of biliary excretion of the compounds in Table 2 and of compounds examined in other investigations in this laboratory (Abou-El-Makarem, 1967; Abou-El-Makarem *et al.*, 1967*a,b*; Millburn *et al.*, 1967*a,b*; Millburn, 1970; Caldwell *et al.*, 1971; Hirom *et al.*, 1972) has been plotted against their molecular weights. For the rat 75 compounds (Fig. 1*a*), for the guinea pig 26 compounds (Fig. 1*b*) and for the rabbit 25 compounds (Fig. 1*c*) have been examined. The molecular weights used for constructing these Figures are those of the compound administered when it was excreted in the bile unchanged and of the metabolite where the compound was excreted in the bile mainly (>70–90% of the biliary material) as a polar conjugate.

If we define 'significant' or 'appreciable' biliary excretion as 10% or more of the dose, then Fig. 1 shows that there are differences between the female rat, guinea pig and rabbit in terms of the minimum molecular weight for this extent of biliary excretion to occur. [The sex of the animals used may be of importance since there appears to be a sex difference in the extent of biliary excretion of some compounds in rats but not in guinea pigs or rabbits; see Gregson *et al.* (1972) and Bertagni *et al.* (1972).] There is some latitude in the definition of this threshold molecular weight for biliary excretion >10% of the dose, but it appears to be about 325 ± 50 in the rat, 400 ± 50 in the guinea pig and 475 ± 50 in the rabbit.

Fig. 1(*a*), which shows results for the female Wistar albino rat, was constructed from values obtained with 53 compounds for which the bile was collected for 24 h and with 23 compounds with bile collected for 3 h. There are 18 compounds with molecular weights below 275 and their biliary excretion is less than 10% of the dose; in fact 17 of these compounds have a biliary excretion of less than 5% of the dose. Within the region of the proposed threshold molecular weight of 325 ± 50 , there are 24 compounds

Fig. 1. Threshold molecular weight for appreciable biliary excretion (>10% of dose) in the female rat, guinea pig and rabbit

The extent of biliary excretion of 75 compounds (rat), 26 compounds (guinea pig) and 25 compounds (rabbit) is plotted against molecular weight. The vertical boxes show the regions of the molecular-weight thresholds, which are 325 ± 50 for the rat (a), 440 ± 50 for the guinea pig (b) and 475 ± 50 for the rabbit (c).

whose biliary excretions range from <1% (4-amino-phenyl glucuronide; mol.wt. 285) to 92% (4-glucuronosido-4'-hydroxybiphenyl; mol.wt. 362). Of these compounds 13 have a biliary excretion greater than 10%, five between 5 and 10% and six less than 5% of the dose. There are 34 compounds in Fig. 1(a) with molecular weights greater than 375, i.e. greater than the threshold range of 325 ± 50 , and 30 of these have a biliary excretion of 40% or more of the dose and four between 10 and 30%.

Fig. 1(b) shows similar results for the guinea pig, the results being obtained with female Duncan Hartley strain guinea pigs for 17 compounds, female English strain for eight compounds and male Abyssinian guinea pigs for one compound. Fig. 1(b) shows that the threshold molecular weight for the guinea pig is about 400 ± 50 . There are six compounds with molecular weights below 350 and whose biliary excretion is less than 10%, eight compounds within the threshold region with excretion values ranging from 0.9% (succinylsulphathiazole; mol.wt. 355) to 44% (hexahydrophthalylsulphathiazole; mol.wt. 409) and 12 compounds with molecular weights above 450, with excretion values ranging from 12 to 100%.

Fig. 1(c) shows the results for female rabbits, New Zealand white rabbits being used for 18 compounds and Dutch rabbits for seven compounds. The threshold range for rabbits appears to be 475 ± 50 . Of

the compounds studied 12 had molecular weights below 425 and had a biliary excretion less than 10% of the dose; in fact, 11 compounds were below 5%. In the threshold region there are eight compounds with biliary excretion ranging from 6% (Tartrazine; mol.wt. 465) to 85% (Lissamine Fast Yellow; mol.wt. 505). There are five compounds with molecular weights greater than 525 and all of them are excreted in the bile to an extent of 30% or more.

Threshold molecular weight and urinary excretion

Compounds whose molecular weights are below the threshold value as defined above are excreted mainly in the urine and do not appear in the bile in large amounts even in the absence of kidney function. Such compounds are still poorly excreted in the bile, i.e. less than 10% of the dose, in rats and rabbits whose renal pedicles have been tied to prevent clearance by the kidney (Abou-El-Makarem *et al.*, 1967a; Hirom, 1970).

Compounds within the range of the threshold molecular weight can be excreted in both the bile and urine in appreciable amounts. The amounts appearing in the bile can often be increased markedly by ligating the kidneys. Thus in the rat succinyl- and glutaryl-sulphathiazole are excreted in the bile to the extent of 36 and 42% of the dose respectively, the rest appearing in the urine, but in the rat with ligated renal

pedicles these values are increased to 65 and 85% respectively.

Compounds with molecular weights above the threshold values are mainly excreted in the bile and it would appear that compounds with molecular weights of about 500 or more are extensively excreted in the bile in all three species examined and possibly this applies to other species (see below).

Indocyanine Green

This dye (mol.wt. of anion 752) has been used in man to measure cardiac output, hepatic function and liver blood flow (Fox *et al.*, 1957; Fox & Wood, 1957; Cherrick *et al.*, 1960; Caesar *et al.*, 1961). In the rat, guinea pig and rabbit, 80–100% of the dose is excreted unchanged in the bile in 3 h [see Table 2; for the rat see also Hunton *et al.* (1960) and Caesar *et al.* (1961)]. It is also almost quantitatively excreted unchanged in the bile by the dog (Wheeler *et al.*, 1958; Hunton *et al.*, 1960). In humans with T-tube drainage after cholecystectomy Indocyanine Green is extensively (43–88%) excreted unchanged in the bile but none is detected in the urine (P. C. Hirom, P. Millburn, P. S. Sever & R. L. Smith, unpublished work). These observations appear to support the view that anions of molecular weight greater than 500 are extensively excreted in the bile in most species.

Species differences in the pattern of elimination of endogenous and exogenous compounds

The finding that the threshold molecular weight for appreciable biliary excretion varies with species has important implications in the pattern of elimination in different species of compounds of both endogenous and exogenous origin. Thus the bile is an important route of elimination of steroid hormones and the biliary excretion of these differs from one species to another, e.g. oestrone and progesterone (see Millburn, 1970; Sandberg *et al.*, 1970; Taylor, 1971). These two compounds, which have molecular weights of 270 and 315 respectively, are converted in the body into polar conjugates (mainly glucuronides and sulphates) whose molecular weights are in the range 350–500. Compounds of this molecular weight should be extensively excreted in the bile in the rat and guinea pig but not in the rabbit. In fact it has been reported by Sandberg *et al.* (1967) that the rat and guinea pig excrete about 60% of an injected dose of oestrone in the bile whereas the rabbit excretes only 17%. Similarly, Grady *et al.* (1952) found that rats excreted about 75% of an injected dose of progesterone in the bile whereas Taylor & Scratcherd (1965) found rabbits excreted only 20–40%.

Differences in the extent of biliary excretion of various drugs and their metabolites in these species have also been observed. Thus morphine (mol.wt.

285), which is largely transformed *in vivo* into its 3-glucuronide (mol.wt. 461), shows marked species differences in the extent of its elimination in the bile as this conjugate. The rat and guinea pig excrete about 60 and 50% respectively of an injected dose of [¹⁴C]morphine in the bile whereas the rabbit excretes only 8% (P. C. Hirom, P. Millburn & R. L. Smith, unpublished work). This difference is not surprising since the molecular weight of the conjugate is appropriate for extensive biliary excretion in the rat and guinea pig but is within the region of the molecular-weight threshold for the rabbit. A marked species difference in the biliary excretion of griseofulvin (mol.wt. 353) has been also observed, for rats excrete 77% of an injected dose in the bile whereas rabbits excrete only 11% (Symchowicz *et al.*, 1967).

The implications of such species variations in biliary excretion have been discussed by Smith (1970). In the rabbit, the urine is the main channel for the elimination of polar compounds having molecular weights up to about 500, but in the rat and guinea pig excretion in the bile becomes important for anionic compounds when their molecular weights reach values of about 300 and 400 respectively. Consequently elimination in the faeces may become the main route of excretion in the last two species. In the rat high faecal excretion of many compounds is frequently observed even when they are given by injection, and this may be attributed to biliary excretion. Further, for compounds with molecular weights between about 300 and 500, enterohepatic circulation as a result of biliary excretion may occur in the rat and guinea pig but not in the rabbit and this could lead to delayed elimination and persistence of the compound in the body. With anions of molecular weights greater than about 500–600, i.e. above the threshold molecular weight for the rabbit, there are no major species differences in the extent of biliary excretion and the bile is the dominant channel of elimination (see Millburn, 1970; Smith, 1970).

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