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THE RATE-LIMITING STEP IN THE BILIARY ELIMINATION OF SOME SUBSTRATES OF URIDINE DIPHOSPHATE GLUCURONYLTRANSFERASE IN THE RAT

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Abstract—Phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline mutually inhibit each others enzymatic conjugation by microsomal UDP glucuronyltransferase (EC 2.4.1.17; acceptor unspecific) *in vitro*. After intravenous injection these UDP glucuronyltransferase substrates are excreted in the rat in bile as β-D-glucuronides. When these glucuronides were injected i.v. they were excreted partially in the bile and also in the urine. Ligation of the kidneys caused an increased biliary excretion of the i.v. injected glucuronides. To determine if these UDP glucuronyltransferase substrates also inhibited each others glucuronidation *in vivo*, two compounds were injected i.v. simultaneously into rats and the mutual effects on the biliary excretion of these compounds were studied. Phenolphthalein inhibited the biliary excretion of 4-methylumbelliferone in the form of its glucuronide conjugate in bile. This inhibition was not due to substrate competition for UDP glucuronyltransferase but to inhibition of excretion of the formed glucuronides from the liver cell into bile. From further experiments in which the mutual effects of the i.v. injected glucuronides on each others biliary excretion were studied it could be concluded that the rate-limiting step in the biliary elimination of phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline as glucuronides in the rat was the excretion of the products of UDP glucuronyltransferase activity *in vivo* into the lumen of the bile canaliculus and not conversion by UDP glucuronyltransferase.

THE MICROSOMAL enzyme, UDP glucuronyltransferase (EC 2.4.1.17) obtained from mammalian liver, converts a large number of endogenous and exogenous substrates into β-D-glucuronides. Thereafter these products are excreted in urine or bile; in the rat the glucuronides are preferentially excreted in bile. As the unconjugated substrates often cannot be excreted (in urine or bile) the glucuronidating enzyme plays an important role in the elimination of drugs and endogenous compounds, such as bilirubin.[1]

If bilirubin is not conjugated with glucuronic acid it cannot be eliminated from the body and jaundice develops. A number of drugs causing jaundice of the unconjugated type are suspected to do so because they are also glucuronidated and thus may cause a "competitive" inhibition of bilirubin glucuronidation; possibly leading to jaundice. Hargreaves[2] has discussed some of these suspected drugs. In this tentative mechanism of inhibition two things are implied; firstly, the compounds are glucuronidated at the same enzymic active site as bilirubin, and secondly UDP glucuronyltransferase activity can become rate-limiting in the biliary excretion of its substrates as glucuronides in these inhibited conditions. However, regarding the first point, it is generally assumed that UDP glucuronyltransferase is a heterogeneous collection of enzymes, differing in

substrate specificity, although the evidence in favour of this heterogeneity is not conclusive[3] and experiments giving results contrary to this supposition have been published. Thus, in this laboratory evidence has been found to support the theory that the substrates *o*-aminophenol, *p*-nitrophenol, phenolphthalein, 4-methylumbelliferone and bilirubin appear to be conjugated at the same active site by the same enzyme.[4,5] It was found that 4-methylumbelliferone and phenolphthalein inhibit the *in vitro* glucuronidation of each other by a postnuclear supernatant from a rat liver homogenate. In the rat both these substrates are excreted as glucuronides in bile. Therefore it seemed of interest to investigate whether the mutual inhibition of 4-methylumbelliferone and phenolphthalein could also be shown *in vivo*, in effects on their biliary excretion as glucuronides. 8-Hydroxychinoline, a compound which is glucuronidated in some animals[6,7] was included in this study.

Further, from this type of study it might be possible to draw some conclusion as to the actual rate limiting step in the elimination of substrates of UDP glucuronyltransferase from the body. The results suggest that UDP glucuronyltransferase is not rate limiting in the biliary excretion of 4-methylumbelliferone, phenolphthalein and 8-hydroxychinoline in the rat. Rather the excretion of the resulting glucuronides from liver cell into the bile canaliculus might be rate limiting.

MATERIALS AND METHODS

Chemicals. Phenolphthalein and 8-hydroxychinoline from Merck, Darmstadt, Germany; phenolphthalein-β-D-glucuronide from Koch–Light, Colnbrook, England; 4-methylumbelliferone from J. T. Bakker, Deventer, Netherlands; 4-methylumbelliferyl-β-D-glucuronide from British Drug Houses, Poole; 8-hydroxychinoline-β-D-glucuronide, this was prepared biosynthetically according to the method of Robinson *et al.*[7] Six g of 8-hydroxychinoline (dissolved in water, acidified with hydrochloric acid to pH 1·0) was fed to two rabbits by stomach tube. The 24-hr urine was acidified to pH 4·0 after filtration over glass-wool and the green crystals (slowly formed during 2 days at 0–4°) were recrystallized several times from hot water (85°). Analysis with β-glucuronidase and its specific inhibitor saccharolacton ascertained the presence of only 8-hydroxychinoline-β-D-glucuronide. β-Glucuronidase from Boehringer, Mannheim, Germany; saccharo-1,4-lacton from CalBiochem, U.S.A. Brentamine Fast Blue B was obtained from Rohner, Pratteln, Switzerland, as Diazoechtblau B, kindly provided by Fa. J. H. ter Heege, Enschede, Netherlands.

Bile cannulation experiments. Male rats (Wistar, obtained from TNO, Zeist, the Netherlands) weighing 250–350 g were anesthetized with 60 mg/kg pentobarbital (Nembutal®) intraperitoneally. By way of midline incision a polyethylene cannula was introduced in the biliary duct. The body temperature of the rats, rectally measured, was kept between 37·5 and 38·5° by means of a heat lamp.

The rate of bile production was measured by fractional collection of bile during periods of 15 or 30 min. During the present experiments the bile production remained constant for at least 3 hr. Intravenous injection of alkaline (pH 10·3) or acid (pH 1·0) solutions in which the compounds were dissolved had no influence on blood pH or bile production.

Intravenous injections were applied in the vena dorsalis penalis in a volume of 0·25 ml/100 g body wt, injected within 15 sec. All compounds injected, except for

8-hydroxychinoline, were dissolved in an alkaline solution, pH 10·3. 8-Hydroxychinoline was dissolved in an acid solution, pH 1·0.

Chemical determinations. Phenolphthalein–glucuronide in bile was determined by hydrolysis in 4 N HCl after appropriate dilution of the bile.[8] After hydrolysis the solutions were neutralized with 8 N NaOH and colour was developed with 0·4 M glycine–NaOH buffer, pH 10·4. The E_{555} was measured.

4-Methylumbelliferyl–glucuronide in bile was determined by an identical hydrolysis. After neutralization and colour development with glycine buffer, the extinction at 365 nm was measured. When phenolphthalein was also present, the E_{365} had to be corrected for E_{365} due to the presence of phenolphthalein, which has an E_{365} about one-sixth of the value of its E_{555}.

8-Hydroxychinoline was determined according to the method of Robinson *et al.*[7] Solutions, containing 0–5 μg 8-hydroxychinoline/ml in 0·3 M acetate buffer, pH 4·5 were made 0·25% (w/v) in respect to Brentamine Fast Blue B. After 5 min incubation at 37° the E_{510} was measured spectrophotometrically.

8-Hydroxychinoline–glucuronide was hydrolysed in 6 N HCl during 2 hr. After neutralization with 8 N NaOH the solution was diluted with 0·3 M acetate buffer, pH 4·5 and the colour was developed as described above. With each series of determination a calibration curve was made, using standard concentrations of 8-hydroxychinoline–glucuronide because the calibration curve, though straight, was variable in height.

Blank values for the various determinations were obtained by subjecting appropriately diluted bile, collected before injection of the compound to be eliminated, to the same hydrolysis and colour development.

UDP glucuronyltransferase activity. With *p*-nitrophenol, phenolphthalein and 4-methylumbelliferone as substrates the UDP-glucuronyltransferase activity was estimated as described before[4] in 75 mM Tris–HCl buffer, pH 7·3 which was 5 mM in $MgCl_2$. The enzyme preparation used was a rat liver microsomal preparation as described before, which was activated by addition of 0·25% Triton X-100 (v/v) before further dilution with 0·154 M KCl.[5] 8-Hydroxychinoline glucuronidation was measured in the same medium, with 1·5 mM UDP-glucuronate also present. The substrate, dissolved in a HCl-solution (pH 2·8) was added to a final concentration of 1·0 mM in the incubation. At several times 50 μl of the incubation was withdrawn and the enzymatic reaction was stopped by adding this sample to 2·95 ml of 0·3 M acetate buffer, pH 4·5 on ice. After colour development as described above, the disappearance of 8-hydroxychinoline during the incubation was measured.

β-Glucuronidase hydrolysis. Hydrolysis of glucuronides by β-glucuronidase was performed in 0·075 M acetate buffer, pH 5·0. To 1 ml of the buffer 50 μl of bile were added and about 52 mU of β-glucuronidase (in a volume of 0·1 ml). After incubation for 30 min the reaction (which was complete at that time) was stopped by addition of 0·4 M glycine–NaOH buffer, pH 10·4. β-Glucuronidase activity could be completely inhibited by addition of saccharo-1,4-lacton to the incubation in a final concentration of 1 mM.

RESULTS

8-Hydroxychinoline glucuronidation in vitro. Rat liver microsomes, activated by the detergent Triton X-100,[4] were able to glucuronidate 8-hydroxychinoline *in vitro* in a

reaction that proceeded linearly in time. Typical 8-hydroxychinoline glucuronidating activity was 60 nmoles/min/mg microsomal protein. As shown in Table 1 this glucuronidation was inhibited by some other substrates of UDP-glucuronyltransferase: about equal degrees of inhibition by 4-methylumbelliferone and *p*-nitrophenol were found and much stronger inhibition by phenolphthalein. Conversely, the glucuronidation of phenolphthalein was least inhibited by 8-hydroxychinoline, whereas *p*-nitrophenol and 4-methylumbelliferone conjugation were about equally affected. Therefore it seemed of interest to include 8-hydroxychinoline in this study on a possible *in vivo* mutual inhibition of substrates of UDP-glucuronyltransferase.

TABLE 1. UDP-GLUCURONYLTRANSFERASE ACTIVITY; MUTUAL INHIBITION OF SOME OF ITS SUBSTRATES

Substrate	mM	Inhibitor	mM	Inhibition (%)
8-Hydroxychinoline	1·0	*p*-Nitrophenol	0·6	17
		p-Nitrophenol	1·2	30
	1·0	4-Methylumbelliferone	0·6	21
		4-Methylumbelliferone	1·2	36
	1·0	Phenolphthalein	0·040	37
		Phenolphthalein	0·075	57
Phenolphthalein	0·075	8-Hydroxychinoline	0·5	4
		8-Hydroxychinoline	1·0	15
4-Methylumbelliferone	0·6	8-Hydroxychinoline	0·5	29
		8-Hydroxychinoline	1·0	38
p-Nitrophenol	0·6	8-Hydroxychinoline	0·5	44
		8-Hydroxychinoline	1·0	54

All experiments were performed in 75 mM tris-HCl buffer, pH 7·3, containing 5 mM $MgCl_2$; the UDP-glucuronate concentration was 1·5 mM throughout. The microsomal enzyme preparation was activated by Triton X-100 (0·25%, v/v) and there was about 200 μg/ml microsomal protein in the incubation. With phenolphthalein and 4-methylubelliferone present there was 2·5 and 5·0% (v/v) ethanol present in the incubation media (also in the controls).

Effects of excretion of glucuronides on bile production. The i.v. injection of all compounds investigated, both in conjugated and unconjugated form, caused an increase in bile production. When two glucuronides were injected at the same time the resulting extra bile produced was the sum of the responses found when each was injected singly.

As an explanation for the choleretic effects of biliary excreted compounds it has been proposed that the increase of bile production is related to the amount of the compounds in the bile (Sperber[9]). In the present work such a correlation was found indeed when the glucuronides each apart or two in combination (see next section) were injected and the total amount of glucuronides present in bile fractions was measured (FIG. 1). A correlation coefficient of 0·97 was found between extra bile produced and number of micromoles of glucuronide excreted One μmole of glucuronide in the bile caused the production of 15–20 mg of extra bile. This value agrees with that

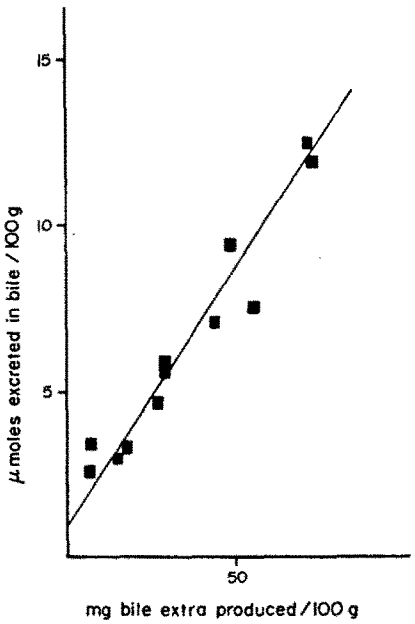

FIG. 1. Relationship between micromoles of glucuronide in bile and extra bile produced in rats with intact kidneys. The amount of micromoles of glucuronides excreted in bile per 100 g of rat in a period of 15 min is plotted against the extra amount of bile produced in the same period, also expressed per 100 g of rat, as compared with a 15-min period of bile collection before injection of the compounds. The points correspond to experiments in which the glucuronides of phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline were injected either single or two of them at the same time. The total amount of both glucuronides excreted was used in the latter cases. Two periods of 15 min after injection of the compounds were used for these data, only obtained from rats with intact kidneys.

reported by Koss *et al.*:[10] 20 mg, for a number of compounds, mainly glucuronides, excreted in bile in rats.

Biliary excretion of phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline in rats with intact kidneys. Phenolphthalein is known to be excreted in bile in rats.[8] 4-Methylumbelliferone and 8-hydroxychinoline were identified in urine of rabbits by Robinson *et al.*[7] and Mead *et al.*,[11] respectively as glucuronide conjugates.

In the present work it was found that phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline were excreted in bile in the rat, after intravenous injection, exclusively as glucuronide conjugates. This was ascertained by hydrolysis of the biliary excreted conjugates with β-glucuronidase and inhibition of this hydrolysis by the specific β-glucuronidase inhibitor saccharolactone. The total amount of excreted 4-methylumbelliferone and 8-hydroxychinoline conjugates was specifically β-glucuronidase sensitive.

Experiments with phenolphthalein have shown that the upper limit for the concentration of phenolphthalein–glucuronide in bile seems to be about 30 mM. This was attained by intravenous injection of 310 μmole/kg of both phenolphthalein and its glucuronide simultaneously, thus giving a total dose of 620 μmole phenolphthalein–equivalents/kg. Thereafter, for two 30-min periods, the same concentration of phenolphthalein–glucuronide in bile was found (~ 30 mM) After administration of

each of these compounds apart, in both cases a concentration of about 23 mM phenolphthalein–glucuronide was found in bile, thus their biliary excretion was not cumulative, saturation of the transport process being attained under these circumstances.

Effects of the compounds on each others biliary excretion in rats with intact kidneys. A distinct inhibition of 4-methylumbelliferone and phenolphthalein on each others glucuronide conjugation by microsomal UDP-glucuronyltransferase was found *in vitro*. This has been interpreted to mean, that both substrates are conjugated at the same active site.[4]

In an attempt to find out whether this inhibition was also present *in vivo*, both compounds were administered separately and together. If there were mutual inhibition at the level of the enzyme a decrease in the biliary excretion of both compounds (as the corresponding glucuronide) might be expected, provided that the enzymic conversion is a rate limiting step in the elimination process. Furthermore, similar

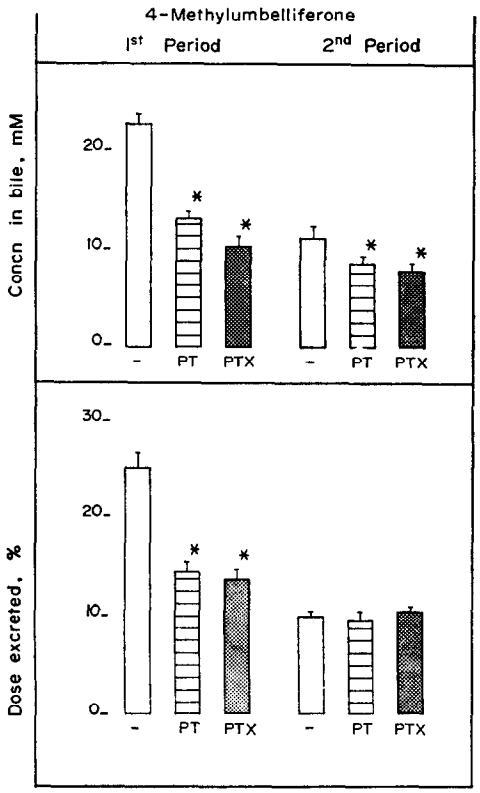

FIG. 2. Effect of phenolphthalein and its glucuronide on the biliary excretion of 4-methylumbelliferone in rats with intact kidneys. 4-Methylumbelliferone (155 μmoles/kg) was injected intravenously either alone or in combination with phenolphthalein (PT) or phenolphthalein-glucuronide (PTX), both 77·5 μmoles/kg. The bile was collected during two periods of 15-min afterwards. The concentration in bile of resulting 4-methylumbelliferyl-glucuronide (mM) and the percentage of the dose of 4-methylumbelliferone excreted during both periods are shown. Below each bar is indicated whether there was a second compound present (PT; PTX) or none (—). The S.E.M. is indicated and * indicates that the values are significantly different from the values without a second compound present ($P < 0{\cdot}05$). ($n = 5$ for each group of rats.)

experiments were performed in which the glucuronides of the compounds were injected intravenously separately and together. Eventual inhibitory effects of the glucuronide products on each others excretion into bile could be found this way.

In the first experiment groups of rats received intravenous injections of either 4-methylumbelliferone alone or 4-methylumbelliferone in combination with phenolphthalein or phenolphthalein-glucuronide. The results (Fig. 2) showed that both phenolphthalein and phenolphthalein-glucuronide inhibited the biliary excretion of 4-methylumbelliferone as glucuronide to the same extent. This suggested that the biliary excretion of 4-methylumbelliferone as glucuronide is not inhibited by phenolphthalein at the level of UDP-glucuronyltransferase (substrate competition) but that the inhibition may be due to phenolphthalein-glucuronide (eventually formed from phenolphthalein). This might interfere in some way with the transport of 4-methylumbelliferyl-glucuronide from its site of synthesis into the bile or it might inhibit 4-methylumbelliferone glucuronide conjugation by product inhibition of UDP glucuronyltransferase.[12,13]

To discriminate between these possibilities the effect of phenolphthalein-glucuronide on the excretion of intravenously injected 4-methylumbelliferyl-glucuronide was investigated (Fig. 3). It is quite clear that the excretion of 4-methylumbelliferyl-glucuronide was inhibited by phenolphthalein-glucuronide. The biliary concentration

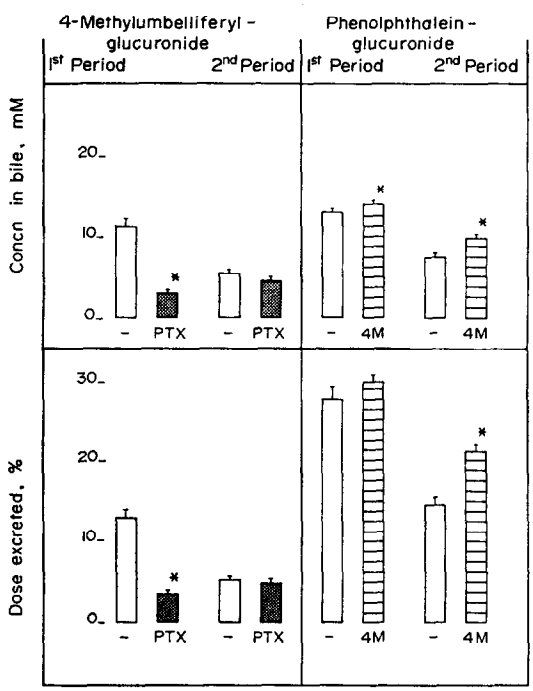

FIG. 3. Effect of phenolphthalein-glucuronide on the biliary excretion of 4-methylumbilliferyl-glucuronide in rats with intact kidneys. 4-Methylumbelliferyl-glucuronide (4 M; 155 μmoles/kg) and phenolphthalein-glucuronide (PTX; 77·5 μmoles/kg) were injected intravenously either alone or both together. The bile was collected during two periods of 15 min afterwards. The concentration in bile of the resulting glucuronides (mM) is shown; similarly the percentage of the dose excreted during both periods. For further details see the legend to Fig. 2. ($n = 6$ for each group of rats.)

of the former compound is very much depressed by the presence of the latter. Thus product inhibition of UDP glucuronyltransferase seems to be excluded as an explanation and the results seem to indicate competition of glucuronides for excretion from the liver cell into the bile canaliculus. At the same time there is a small but significant rise in the concentration of phenolphthalein–glucuronide in the bile when 4-methylumbelliferyl-glucuronide is injected at the same time; the possible explanation of this phenomenon will be discussed later.

A further investigation of the influence of glucuronides on each others biliary excretion is shown in Fig. 4. The mutual effects of phenolphthalein–glucuronide and

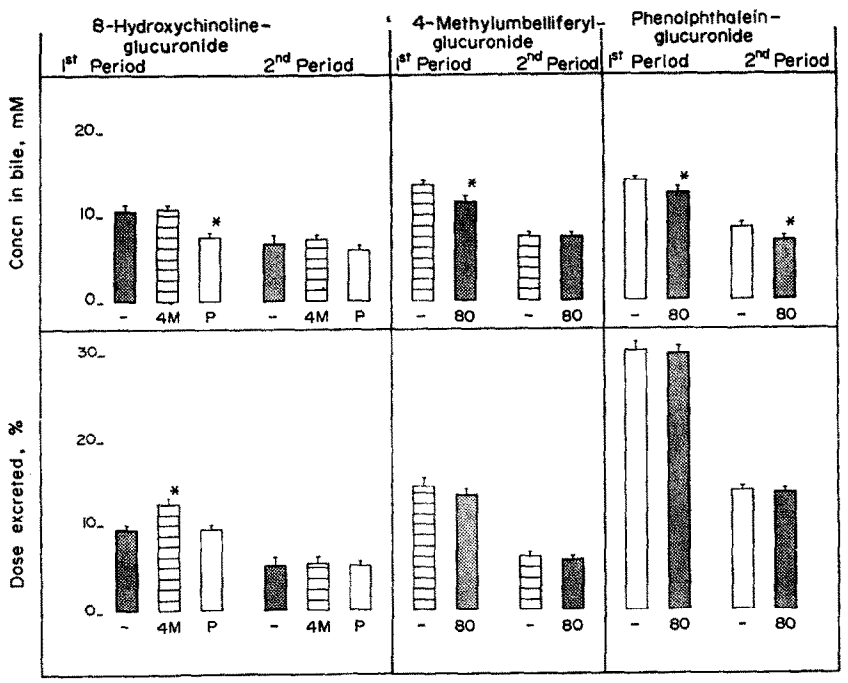

FIG. 4. Effect of phenolphthalein–glucuronide, 4-methylumbelliferyl-glucuronide and 8-hydroxychinoline-glucuronide on each others biliary excretion in rats with intact kidneys. 8-Hydroxychinoline-glucuronide (80; 155 μmoles/kg), 4-methylumbelliferyl-glucuronide (4 M; 155 μmoles/kg) and phenolphthalein–glucuronide (P; 77·5 μmoles/kg) were injected intravenously either alone or two in combination. The bile was collected during two periods of 15-min after injection of the compounds. The concentration in bile of the various glucuronides is shown (mM) and the percentage of the dose excreted during both periods. For further details see legend to Fig. 2. ($n = 6$ for each group of rats.)

4-methylumbelliferyl-glucuronide on the biliary excretion of 8-hydroxychinoline-glucuronide are shown. With most combinations of two glucuronides there was no mutual effect upon the amount of the respective glucuronides excreted in bile. Only the amount of 8-hydroxychinoline-glucuronide excreted in bile in the presence of 4-methylumbelliferyl-glucuronide was significantly increased during the first 15 min after injection, which is an effect similar to that of again 4-methylumbelliferyl-glucuronide on excretion of phenolphthalein–glucuronide in bile. Further, in most cases, the concentration of the glucuronides in bile was reduced by the presence of a second glucuronide, probably due to "dilution" of bile, caused by extra bile produced

as a consquence of the biliary excretion of a second glucuronide (Fig. 1). Only with 4-methylumbelliferyl-glucuronide present during 8-hydroxychinoline excretion the concentration of the latter in bile did not decrease, giving rise to a greater biliary excretion of 8-hydroxychinoline-glucuronide as related just above.

As there was no mutual inhibition of the biliary excretion of the glucuronides of 8-hydroxychinoline and 4-methylumbelliferone, it was interesting to find out whether the aglycons themselves showed mutual inhibition when administered at the same time. However, no mutual inhibition was found with the aglycons either. Thus, no competition for UDP-glucuronyltransferase *in vivo* could be found with these substrates.

Biliary excretion and mutual inhibition of biliary excretion in rats with ligated renal vessels. Considerable amounts of the intravenously injected glucuronides were excreted in the urine by way of the kidneys (unpublished observations). The unconjugated compounds are only sparingly soluble at pH 7·3 in aqueous solutions and will be protein-bound after their intravenous administration; thus, they will be much less excreted in urine than their water-soluble glucuronide counter-parts. To exclude this alternative route of excretion the renal vessels were ligated in a series of rats so that urinary excretion of the compounds was made impossible (Fig. 5).

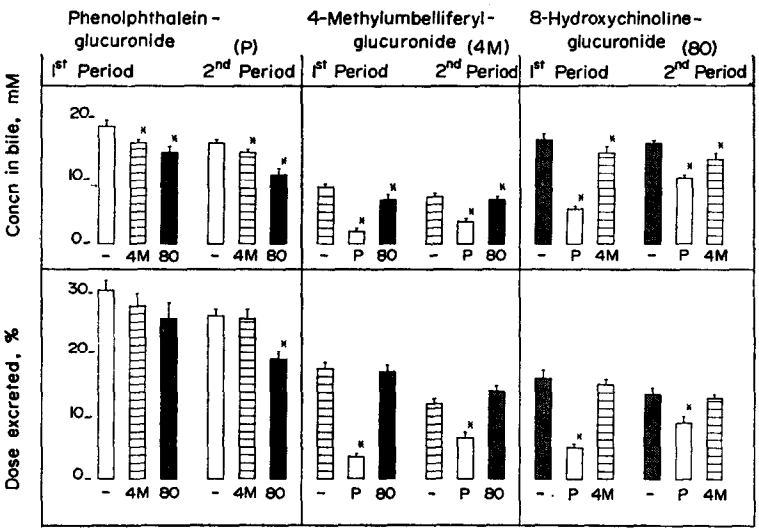

FIG. 5. Effect of phenolphthalein–glucuronide, 4-methylumbelliferyl-glucuronide and 8-hydroxychinoline-glucuronide on each others biliary excretion in rats with ligated kidneys. Phenolphthalein–glucuronide (P; 77·5 μmoles/kg), 4-methylumbelliferyl-glucuronide (4 M; 77·5 μmoles/kg) and 8-hydroxychinoline-glucuronide (80; 155 μmole kg) were injected intravenously either alone or two in combination. In the rats the renal vessels had been ligated. The bile was collected during two periods of 15 min after injection of the compounds. The concentration in bile of the various glucuronides is shown (mM) and the percentage of the dose excreted during both periods. For further details see legend to Fig. 2. ($n = 6$ for each group of rats.)

The results show that the biliary excretion of 8-hydroxychinoline-glucuronide and phenolphthalein–glucuronide was significantly enhanced by ligation of the kidneys compared with rats with intact urinary excretion. The excretion of 8-hydroxychinoline-glucuronide increased (by 100 per cent) to 29 per cent of the dose during 30 min, whereas the excretion of phenolphthalein–glucuronide increased (by 25 per cent) to

56 per cent of the dose in 30 min. In this experiment equimolar doses of 4-methylumbelliferyl-glucuronide and phenolphthalein–glucuronide were administered and it can be seen that 4-methylumbelliferyl-glucuronide was much less excreted than phenolphthalein–glucuronide: only 30 per cent of the dose during 30 min. This is due to a lower biliary concentration of 4-methylumbelliferyl-glucuronide as compared with phenolphthalein–glucuronide (9·3 mM, viz 18·3 mM in the first period).

The concentration of a glucuronide in bile was in all cases significantly decreased by the presence of a second glucuronide, injected along with the first, as shown in Fig. 5. The amount excreted in bile, however, was only in a few cases decreased by the presence of a second glucuronide. It can be seen that phenolphthalein-glucuronide inhibited the biliary excretion of both 4-methylumbelliferyl-glucuronide and 8-hydroxychinoline-glucuronide. The presence of 4-methylumbelliferyl-glucuronide, on the contrary, did not influence the biliary excretion of the other two glucuronides, whereas 8-hydroxychinoline-glucuronide only decreased the excretion of phenolphthalein–glucuronide significantly during the second 15 min after injection of both glucuronides together. 4-Methylumbelliferyl-glucuronide excretion was not affected.

DISCUSSION

In the rat phenolphthalein, 4-methylumbelliferone and 8-hydroxychinoline are extensively excreted in bile as glucuronides. The molecular weights of these glucuronides are 495 for phenolphthalein–glucuronide; 352 for 4-methylumbelliferyl-glucuronide and 321 for 8-hydroxychinoline-glucuronide. The present results show that biliary excretion of phenolphthalein–glucuronide is more extensive than biliary excretion of the two other glucuronides. This might be related to their respective molecular weights which are for 8-hydroxycholine-glucuronide and 4-methylumbelliferyl-glucuronide only just above the lower limit of 325 ± 50,[14] which in some unknown way determines, along with other factors, whether a compound can be excreted in bile in the rat. That may be the reason why they are less well excreted in bile than phenolphthalein–glucuronide, which has a molecular weight far above that lower limit and, thus, is extensively excreted in bile.[8]

The glucuronides, when intravenously injected, are also excreted in urine[1] in the rat. When the kidneys are ligated this alternative is lost and greatly increased amounts of the compounds are excreted in bile in that case. A similar, although smaller effect of renal ligation was found with some other compounds.[15] In the kidneys glucuronides are actively secreted into urine. Low molecular weight glucuronides seem to be secreted preferentially to high molecular weight glucuronides.[1] Perhaps at this level lies the explanation of the increase in biliary excretion of phenolphthalein–glucuronide and 8-hydroxychinoline-glucuronide due to concomitantly administered 4-methylumbelliferyl-glucuronide. The latter glucuronide may have a higher affinity for the active secretory process and thus cause inhibition of urinary excretion of the former glucuronides, which, at its turn, is followed by an increased biliary excretion. The fact that in rats which ligated renal vessels in which there is of course no longer urinary excretion, this enhancement by 4-methylumbelliferyl-glucuronide is no longer present (Fig. 5) supports this explanation.

The question of the rate limiting step in the elimination from the body into the bile of an aglycone which has first to be converted to a glucuronide conjugate before it can

be eliminated has not been solved so far. Moreover, it is very well conceivable that it depends on the particular substrate used and its resulting glucuronide, which step finally will be rate limiting. In the present work it was found that for the three compounds studied UDP-glucuronyltransferase seems not to be rate limiting in the biliary elimination, though the compounds are mutually inhibitory towards each others glucuronidation *in vitro*. The arguments in favour of this conclusion are:

(1) Phenolphthalein–glucuronide inhibits stongly the biliary excretion of 4-methylumbelliferyl-glucuronide and 8-hydroxychinoline-glucuronide. Thus, the aglycon phenolphthalein will be inhibitory towards the biliary excretion of the other aglycons by means of the resulting phenolphthalein–glucuronide which competes with the other glucuronides for excretion into bile. Therefore, for 4-methylumbelliferone and 8-hydroxychinoline the transport into bile of the formed glucuronides seems rate limiting because this step can be inhibited when the glucuronidating step is not yet inhibited.

(2) With 310 μmole/kg phenolphthalein (i.v.) a concentration of about 23 mM phenolphthalein–glucuronide is reached in bile; this can be increased to only 30 mM by the concomitant administration of 310 μmole/kg phenolphthalein–glucuronide (i.v.), which of its own also gives rise to 23 mM in the bile. Thus saturation of the transport process seems to be reached and the glucuronide excretion into the bile canaliculus has become rate limiting in the elimination process.

(3) When phenolphthalein is administered *in vivo* about 2 μmoles of phenolphthalein–glucuronide are excreted in bile per gram of liver per 30 min and thus, during this period, at least this amount of glucuronide was formed. When measured *in vitro*, however, the glucuronidating capacity was at least 10 μmoles/g of liver/30 min at a phenolphthalein concentration of 150 μM and UDP-glucuronate 1·5 mM.[4] In liver the UDP-glucuronate concentration is 0·1–0·2 mM if it were homogeneously distributed in all cell compartments.[16–18] Thus, 1 g of liver contains 0·1–0·2 μmole of UDP-glucuronate. When 2 μmoles of phenolphthalein can be glucuronidated during 30 min in 1 g of liver this means that UDP-glucuronate can be synthesized very actively by UDPG-dehydrogenase on demand. At least it seems not to be a rate-limiting factor, because at a dose of 310 μmole/kg (i.v.), phenolphthalein and phenolphthalein–glucuronide are excreted at equal rates (unpublished results). With 4-methylumbelliferone as substrate the difference between *in vivo* excretion rate and *in vitro* measured enzymatic activity is still larger. 1·5 μmoles are excreted per g of liver per 30 min *in vivo*. At least 100 μmoles can be converted in the same time (at 0·72 mM 4-methylumbelliferone and 1·5 mM UDP-glucuronate) *in vitro*.[4] Therefore, UDP-glucuronyltransferase activity seems not rate limiting (though one should of course be cautious with this kind of *in vivo–in vitro* comparisons).

Thus, the excretion step into bile may be rate-limiting. Inhibition of biliary excretion of glucuronides, mostly concerning bilirubin-glucuronide, has been found by others.[19–22] Several authors have shown the biliary excretion of bilirubin-glucuronide to be the rate-limiting step in the elimination of bilirubin.[23,24] If indeed UDP-glucuronyltransferase is not rate-limiting, a phenobarbital treatment, which is known to induce this enzyme activity[25–27] might not enhance the rate of biliary excretion of substrates of this enzyme (unless it would also induce in some way the transport process). The various reports on this point, however, are not yet conclusive.[28–31]

Experiments in which only the competition for biliary excretion is measured of

substrates of UDP-glucuronyltransferase[32] never can admit conclusions on competition for UDP-glucuronyltransferase *in vivo* of these substrates, because an inhibition of biliary excretion may, in fact, be (mutual) inhibition of glucuronides on each others biliary excretion. As a control the effects of the respective glucuronides on each others biliary excretion should be investigated. Measuring blood and liver concentrations of aglycons may be a better means of detecting inhibition of enzymatic conversion *in vivo* by UDP-glucuronyltransferase as did Yeh and Mitchell[33,34] for inhibition of morphine glucuronidation by monoamino-oxidase inhibitors.

The results of the experiments on mutual inhibition of the excretion of glucuronides in bile do not permit an unequivocal conclusion. Without ligation of the kidneys only biliary excretion of 4-methylumbelliferyl-glucuronide is inhibited by phenolphthalein-glucuronide; after ligation also the excretion of 8-hydroxychinoline-glucuronide is strongly inhibited. The most prominent effect of ligation of the kidneys in this last case is the very great increase in biliary excretion of 8-hydroxychinoline-glucuronide, presumably by an increased supply of this compound due to loss of the urinary excretion. Possibly as a consequence of this in these renal ligated animals 8-hydroxychinoline-glucuronide inhibits the biliary excretion of phenolphthalein-glucuronide. The inability of 4-methylumbelliferyl-glucuronide to inhibit the biliary excretion of the other glucuronides may be caused by the relatively low excretory rate for this compound which results in low bile concentrations.

A carrier model is often used to describe the transport of compounds from the liver cell to the lumen of the bile canaliculus.[19] The various glucuronides might bind at the same site of such a carrier and could competitively inhibit the binding of each other. However, the present results are not consistent with this model and can, as yet, not be fully explained.

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