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TWO PATHWAYS FOR BILIARY COPPER EXCRETION IN THE RAT

THE ROLE OF GLUTATHIONE

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Abstract—To evaluate the role of glutathione in biliary copper excretion, we studied this process in control Wistar rats and in mutant Wistar rats (GY rats), in which the secretion of glutathione into bile is deficient. For comparison, biliary zinc excretion was determined simultaneously. In spite of the markedly reduced bile flow (−45%) in GY rats, biliary output rates of endogenous copper were virtually identical in GY and control rats. In contrast, zinc output was drastically reduced in GY rats compared to controls (−80%). Biliary excretion patterns after intravenous administration of copper, in doses ranging from 65 to 2265 nmol/100 g/body wt, showed a distinct rapid and slow phase in control rats. In GY rats, on the other hand, the rapid phase in copper excretion was absent but the slow phase appeared to be unaffected. Pretreatment of rats with diethylmaleate to deplete hepatic and biliary glutathione abolished the rapid phase of copper excretion in control rats, while the slow phase remained unaffected. No significant effect of diethylmaleate on the hepatic handling of exogenous copper was observed in GY rats. The maximal capacity of the slow copper excretion pathway was 40–45 nmol/hr/100 g body wt, both in control and GY rats; the capacity of rapid excretion pathway depended on the administered copper load. Intravenous injection of copper induced the biliary excretion of a substantial amount of zinc in control rats, but not in GY rats. These results indicate the existence of at least two distinct biliary excretory pathways for copper in the rat, i.e. a slow and a rapid pathway, with a glutathione dependency of the latter only. The basal excretion of (endogenous) copper, in contrast to that of zinc, can proceed independently of glutathione excretion. However, glutathione appears to be involved in the rapid secretion of excess copper.

The biliary pathway is the main route for removal of copper from the body [1, 2]. A blockade of this pathway will cause accumulation of copper in the liver and eventually liver cell damage. Two hereditary disorders associated with a defective hepatobiliary copper transport and accumulation of copper in the liver have been described: Wilson's disease in man [3, 4] and copper toxicosis in Bedlington terriers [3, 5]. Both disorders are characterized by a decreased biliary copper excretion, but differ from each other in the hepatic distribution of the excess copper [3]. Consequently, the defective step in the copper transport cascade may be different in these two disorders. However, it is difficult to understand these disturbances in terms of a specific defect in one of the steps of copper metabolism, because the mechanism(s) of normal biliary copper excretion is still poorly understood. Several pathways may exist for the overall transport of copper from blood to bile. Using tracer amounts of radio copper in rats, Kressner *et al.* [6] have proposed the existence of at least two transcellular pathways via hepatocytes as well as a transcytotic pathway via biliary epithelium.

It has been suggested that glutathione (GSH) plays a key role in the final step in copper excretion from hepatocyte into bile, i.e. its transport across the canalicular membrane [7]. GSH has also been implicated in the transfer of zinc from liver to bile [8, 9]. To evaluate the role of GSH in the hepatic disposition of these trace elements, biliary copper and zinc excretion were compared in control Wistar rats and GY rats [10]. This latter mutant rat strain shows a defective hepatobiliary transport of several organic anions [10–13]. In spite of a normal hepatic GSH concentration [11], GSH is virtually absent in the bile of these animals [12, 13] and the concentration of its constituent amino acids is also greatly reduced [12]. These animals therefore provide an excellent tool to study GSH dependency of biliary excretory processes.

MATERIALS AND METHODS

Animals. Normal Wistar rats and GY (Groningen Yellow) Wistar rats were bred at the Central Animal Laboratory, University of Groningen. The GY rats display conjugated hyperbilirubinemia, due to an autosomal recessive defect in the hepatobiliary excretion of conjugated bilirubin and a number of other organic anions [10–13] including GSH [12, 13]. Details of the GY rat are described elsewhere [10]. Available data indicate that the genetic defect in the GY rat is similar to that of the mutant rat described by Jansen *et al.* [14].

The rats were housed in plexiglass cages with free access to food and water. The diet (RMH-B, Hope

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Table 1. Plasma and bile concentrations and biliary output rates of copper and zinc in control and GY rats

	Control	GY
Copper		
Plasma concentration (μmol/L)*	25.8 ± 1.7	31.1 ± 2.2
Bile concentration (μmol/L)†	26.4 ± 0.9	40.6 ± 1.7‡
Output rate (nmol/hr/100 g body wt)†	12.9 ± 0.5	12.8 ± 0.4
Zinc		
Plasma concentration (μmol/L)*	30.0 ± 1.5	31.6 ± 3.1
Bile concentration (μmol/L)†	7.9 ± 0.9	2.7 ± 0.1‡
Output rate (nmol/hr/100 g body wt)†	3.8 ± 0.4	0.8 ± 0.1‡

* Mean values (±SE) of plasma samples collected 30 min after creation of a bile fistula in pentobarbital-anesthetized animals. Data represent means of 15 (control) and 16 (GY) rats, respectively.

† Mean values (±SE) of two subsequent 30 min bile samples, collected immediately after creation of a bile fistula in pentobarbital-anesthetized animals. Data represent means of 15 (control) and 16 (GY) rats, respectively.

‡ Significant difference between control and GY rats.

Farms NV, Woerden, The Netherlands) contained 22.4 mg/kg copper and 63.0 mg/kg zinc. For experiments, only male rats of approximately 300 g were used. All experiments followed the institutional guidelines for the care and use of laboratory animals in research.

Experimental procedures. All experiments were started at 11.00 a.m. to exclude effects of the circadian rhythm in bile formation [15]. The rats were anaesthetized with pentobarbital (6 mg/100 g body wt) and anaesthesia was maintained by injection of small doses of the drug. During the experiments body temperature was maintained at 37.5°–38° by means of a heating pad. The rats were equipped with silastic catheters (i.d. 0.50 mm, o.d. 0.94 mm) in the common bile duct and, via the left jugular vein, in the heart. Bile samples were collected into preweighed vials for 4 hr in 30 min intervals. When indicated, 0.39 mmol/100 g body wt of diethylmaleate (DEM) was injected intraperitoneally at 15 min after the start of bile collection. One hour after the start of bile collection, copper was injected intracardially via the jugular vein catheter in doses of 65 nmol, 305 nmol, 325 nmol or 2265 nmol per 100 g body wt, respectively. Copper was given in the form of $CuSO_4$ dissolved in saline, in a final volume of 0.2 mL/100 g body wt. At the end of the experiment the rats were killed by an overdose of pentobarbital.

Analyses. To prevent contamination, all vials were washed prior to use with concentrated HNO_3 and bidistilled water and subsequently dried. Biliary concentrations of copper and zinc were determined by Proton Induced X-ray Emission [16]. Total biliary glutathione concentration (GSH+GSSG) was assayed according to Griffith [17].

Calculation and statistics. Results are expressed as mean values ± SE for each group. Statistical significance of differences was evaluated by Student's *t*-test. Differences were considered to be significant at a level of $p < 0.05$.

RESULTS

Table 1 shows the plasma and bile concentrations as well as biliary output rates of endogenous copper and zinc in bile of pentobarbital-anesthetized control and GY Wistar rats. In spite of the significantly lower bile flow in GY rats, (0.483 ± 0.011 vs 0.269 ± 0.011 mL/hr/100 g/body wt), the biliary output of endogenous copper was virtually identical in the control and GY rats, due to its 53% higher concentration in the latter. In contrast, the output rate of zinc was very markedly reduced in GY rats when compared to controls. Plasma concentrations of copper and zinc were similar in both strains of rats. As reported previously [12, 13], GSH was not detectable in bile of GY rats, while its concentration in control bile averaged 3.5 mM in these experiments. The output rates of copper, zinc and GSH remained stable during a 4 hr period after creation of the bile fistula (data not shown).

The pattern of biliary copper excretion in control and GY rats after injection of three different doses of copper (65, 325 and 2265 nmol/100 g body wt, respectively) is shown in Fig. 1a–c. After injection of the low dose, copper excretion showed a small peak and stabilized afterwards at a somewhat lower level in control rats (Fig. 1a). During this experiment, copper excretion in control rats was slightly higher than in GY rats, resulting in a higher fractional copper excretion after three hours (Table 2). After injection of 325 nmol copper/100 g body wt (Fig. 1b), copper was excreted more rapidly in control rats than in GY rats, with peak excretion rates observed within 30 min after injection and a gradual decline thereafter. In GY rats no peak excretion was observed, but copper output gradually increased and stabilized after 60 min. The absence of peak copper excretion in GY rats resulted in a significant lower recovery of copper after 3 hr (Table 2). With the highest dose of copper, the maximal excretion rate in control rats was 105 nmol/hr/100 g body wt. However, this excretion rate was only obtained after 2 hr (Fig. 1c). The fractional excretion obtained with the highest dose was much lower than with the intermediate and low dose (Table 2). In GY rats, copper excretion pattterns did not significantly differ between the high and intermediate dose. The maxi-

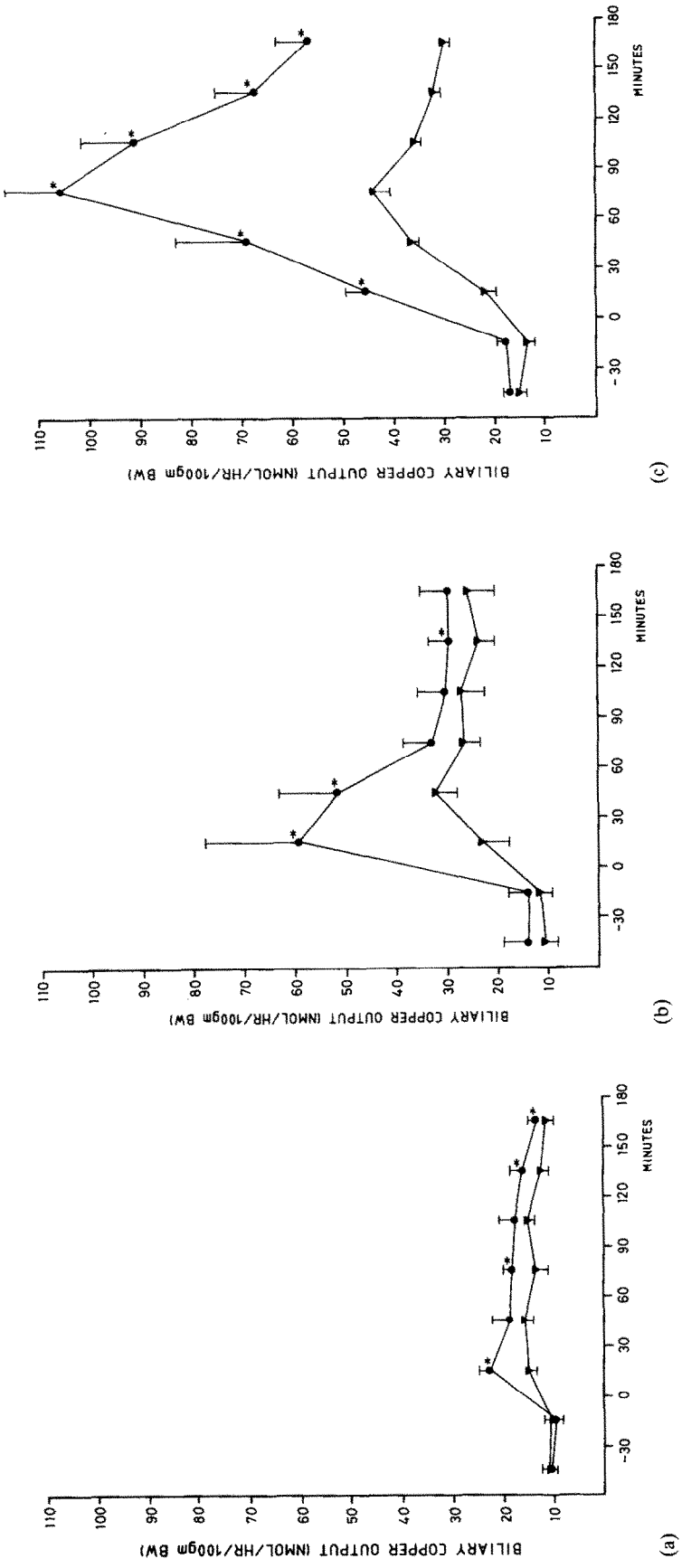

Fig. 1. Biliary excretion of copper after injection of 65 (a), 325 (b) or 2265 (c) nmol copper/100 g body wt at time = 0. Normal rats are represented by (●) and GY rats by (▼). Vertical bars indicate SD; N = 4–5 in all experiments. Significant differences between control and GY rats are indicated by an asterisk.

Table 2. Biliary copper secretion (nmol) and its fractional excretion (% dose) during 3 hr after intravenous injection of copper*

Dose (nmol/100 g body wt)	Control	GY
65	78.7 ± 10.4 (41.4)	32.9 ± 6.1 (17.3)‡
325	217.1 ± 15.8 (22.4)	138.4 ± 11.6 (14.3)‡
2265	487.9 ± 14.4 (14.4)	147.7 ± 2.5 (2.1)‡
DEM† + 305	130.3 ± 14.0 (14.5)	178.0 ± 9.3 (19.3)‡

N = 4–6 in all experimental conditions.

* Data are expressed as nmoles excreted during 3 hr after injection (mean ± SE) and as the percentage of the injected dose, calculated after subtraction of the basal copper output.

† Diethylmaleate (0.39 mmol/100 g body wt) administered 45 min prior to copper injection.

‡ Significantly different from control rats at $P < 0.05$.

mal excretion rate observed was approximately 40 nmol/hr/100 g body wt in both cases.

The GSH output in the bile of GY rats was <0.001 μmol/hr/100 g body wt, while in control rats the basal GSH output was 1.57 ± 0.21 μmol/hr/100 g body wt (N = 5). Treatment with DEM (0.39 mmol/100 g body wt) reduced the biliary GSH output to less than 0.1 μmol/hr/100 g body wt in control rats within 30 min after administration (data not shown). Treatment of rats with DEM reduced basal copper excretion by 40–50% in both strains of rats (data not shown), which is comparable to the results obtained by Alexander and Aaseth [7].

The pattern of biliary copper excretion after injection of an intermediate dose of copper was markedly changed in control rats after DEM pretreatment (Fig. 3). Peak copper excretion was absent and the recovery after 3 hr was lower than that in untreated controls and in GY rats treated with DEM (Table 2). DEM treatment did not significantly influence the copper excretion pattern in GY rats after copper injection (compare Figs 1b and 3).

A steep rise in the biliary excretion of zinc was observed within 30 min after injection of the intermediate and the high copper dose in control rats. In contrast, zinc excretion remained unchanged in GY rats (Fig. 4). In addition, biliary output of endogenous zinc fell substantially (32%) in control rats after DEM treatment. No significant effect of DEM on zinc output was observed in GY rats.

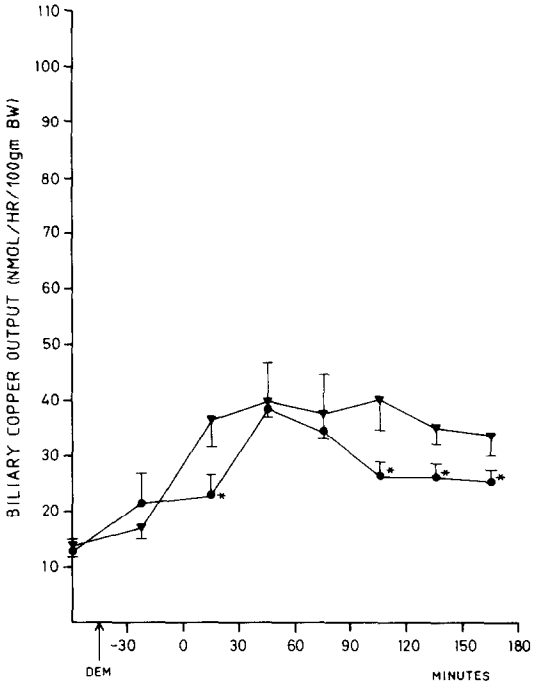

Fig. 2. Biliary excretion of copper after injection of 0.39 mmol DEM/100 g body wt at minus 45 min (arrow) and 305 nmol copper100 g body wt at time = 0. Control rats are represented by (●) and GY rats by (▼). Vertical bars indicate SD; N = 4 (GY) and 5 (control), respectively. Significant differences between control and GY rats are indicated by an asterisk.

DISCUSSION

In the present study, we have focused on the mechanism of biliary copper excretion in the rat. Alexander and Aaseth [7] have provided evidence that GSH may be involved in this process; these authors were able to demonstrate that the biliary excretion of endogenous copper was uniformly inhibited in rats after depletion of hepatic and biliary GSH with diethylmaleate (DEM). However, the excretion of copper was only reduced to 50% of its basal value in these experiments in spite of the fact that GSH excretion was almost completely inhibited, suggesting that at least a part of biliary copper excretion is GSH-independent.

To investigate the role of GSH in biliary copper excretion, we made use of a mutant rat strain (GY) in which biliary GSH concentrations are below detection limits [12, 13], whereas hepatic GSH concentrations are in the same range as those of control Wistar rats [11]. The observation that the excretion of endogenous copper was almost identical in GY and control rats clearly demonstrates that under basal conditions the presence of GSH in bile is not required to maintain adequate copper excretion. As a consequence of the lower bile flow in GY rats, when

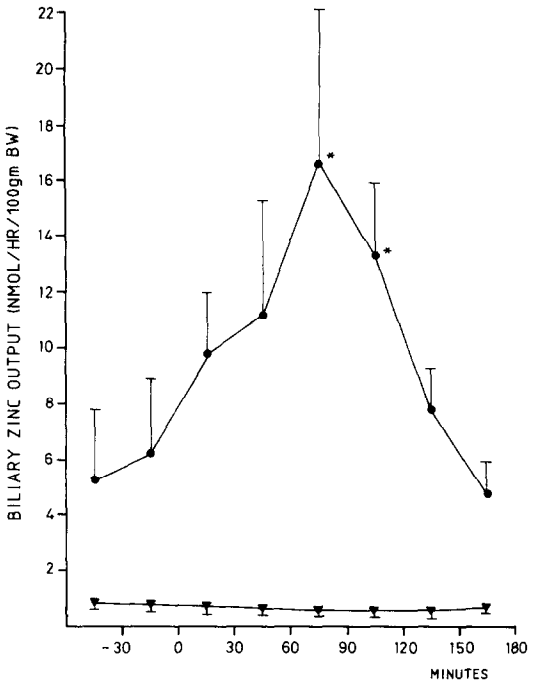

Fig. 3. Biliary excretion of zinc after injection of 2265 nmol copper/100 g body wt at time = 0. Control rats are represented by (●) and GY rats by (▼). Vertical bars indicate SD; N = 4. Significant differences from basal values are indicated by an asterisk.

compared to controls, biliary copper concentrations were significantly higher in the first group of animals. The ability of GY rats to maintain copper balance was also evident from plasma concentrations very similar to those in control rats. The reduction of endogenous copper excretion after DEM-treatment observed by Alexander and Aaseth [7], which was confirmed in the present study both in control and GY rats, may be due to interactions of DEM with other compounds involved in copper excretion, or to non-specific effects of DEM on intracellular metabolism [18]. The effect of depletion of intracellular GSH in itself has no distinct effects on biliary Cu excretion as shown by other experiments (unpublished results).

In contrast to copper excretion, the excretion of endogenous zinc was very strongly reduced in GY rats when compared to controls. This is indicative for a strong GSH-dependency for this metal, as was also suggested in a number of other studies [8, 9].

The hepatic disposition of copper after intravenous administration of varying doses of ionic copper, however, appeared to be partly dependent upon GSH. The copper excretion profiles observed in control rats in the present study were very similar to those reported by Nederbragt and Lagerwerf [19], with a distinct rapid phase and a slow phase. We were able to inhibit this rapid phase, without affecting the slow phase, by depletion of biliary GSH through administration of DEM. Similarly, the rapid phase in biliary copper excretion was absent in untreated GY rats while the slow excretion proceeded at a rate similar to that in control rats. DEM treatment of GY rats did not alter the pattern of copper excretion after injection of a copper load and even slightly increased, albeit not significantly, its fractional recovery in bile. Up to now, we have no explanation for this latter observation.

Taken together, presented data indicate that the removal of copper via bile after an intravenous load is executed via (at least) two mechanisms, giving rise to a rapid and a slow excretion phase, and that the rapid phase is mediated by a GSH-dependent process. To what extent this GSH-dependency relates to the proposed transcellular pathways for copper excretion [6] and to the subcellular localization (e.g. lysosomes) of bile-destined copper [20] remains to be established. The maximal transport rate of the GSH-dependent pathway increased when larger amounts of copper were injected. However, this increase was not proportional to the administered dose, resulting in a lower fractional excretion with the higher doses. This dose-dependent copper excretion has also been observed in previous studies [21]. The maximal capacity of the "slow pathway" was approximately 40 nmol/hr/100 g body wt both in controls and GY rats, i.e. only three to four times the value of basal copper excretion in these animals. Whether this will be sufficient to maintain copper balance in GY rats under the condition of increased dietary copper is currently under investigation.

Injection of copper resulted in a significant rise in biliary zinc excretion in control rats. We suggest that this is due to displacement of zinc by copper from hepatic metallothionein followed by its excretion into bile, since it is known that metallothionein has a higher affinity for copper than for zinc [22]. This effect was not observed in GY rats or in control rats treated with DEM, giving further support to the requirement of GSH in biliary zinc excretion. In addition to zinc, GSH has been shown to play an important role in the biliary disposition of other metals, such as cadmium and mercury (see Ref. 23 for review); the GY rat may provide a useful animal model to study the toxicity of these metals in relation to the process of their elimination from the body.

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REFERENCES

1. Klaassen CD, Biliary excretion of metals. *Drug Metab Rev* **5**: 165–196, 1976.
2. van Berge-Henegouwen GP, Tangedahl TN, Hofmann AF, Northfield TC, LaRusso NF and McCall JT, Biliary secretion of copper in healthy man. *Gastroenterology* **72**: 1228–1231, 1977.
3. Sternlieb I, Copper and the liver. *Gastroenterology* **78**: 1615–1628, 1980.

4. Danks DM, Hereditary disorders of copper metabolism in Wilson's disease and Menkes' disease. In: *The Metabolic Basis of Inherited Disease,* 5th edn. (Eds. Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS), pp. 1251–1268. McGraw-Hill, New York, 1983.
5. Johnson GF, Morell AG, Stockert RJ and Sternlieb I, Hepatic lysosomal copper protein in dogs with an inherited copper toxicosis. *Hepatology* **1**: 243–248, 1981.
6. Kressner MS, Stockert RJ, Morell AG and Sternlieb I, Origins of biliary copper. *Hepatology* **4**: 867–870, 1984.
7. Alexander J and Aaseth J, Biliary excretion of copper and zinc in the rat as influenced by diethylmaleate, selenite and diethyldithiocarbamate. *Biochem Pharmacol* **29**: 2129–2133, 1980.
8. Alexander J, Aaseth J and Refsvik T, Excretion of zinc in rat bile. A role of glutathione. *Acta Pharmacol Toxicol* **49**: 190–194, 1981.
9. Gregus Z and Varga F, Role of glutathione and hepatic glutathione *S*-transferase in the biliary excretion of methyl mercury, cadmium and zinc: a study with enzyme inducers and glutathione depletors. *Acta Pharmacol Toxicol* **56**: 398–403, 1985.
10. Kuipers F, Enserink M, Havinga R, van der Steen ABM, Hardonk MJ, Fevery J and Vonk RJ, Separate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. *J Clin Invest* **81**: 1593–1599, 1988.
11. Polhuys M, Kuipers F, Vonk RJ and Mulder GJ, Stereoselectivity of glutathione conjugation in the rat. Blood elimination of α-bromoisovalerylurea enantiomers and biliary excretion of the conjugates in unanesthetized normal or congenitally jaundiced rats. *J Pharmacol Exp Ther* **249**, 874–878, 1989.
12. Kuipers F, Enserink M, Havinga R, van der Steen ABM, Hardonk MJ, Fevery J and Vonk RJ, Separate transport systems for biliary secretion of sulphated and unsulphated bile acids in the rat. In: *Trends in Bile Acid Research* (Eds. Paumgartner G, Stiehl A and Gerok W), pp. 143–152. Kluwer Academic Publishers, Lancaster, 1989.
13. Jansen PLM and Oude Elferink RPJ, Hereditary hyperbilirubinemias: a molecular and mechanistic approach. *Sem Liver Dis* **8**: 168–178, 1988.
14. Jansen PLM, Peters WH and Lamers WH, Hereditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. *Hepatology* **5**: 573–579, 1985.
15. Vonk RJ, van Doorn ABD and Strubbe JH, Bile secretion and bile composition in the freely moving rat. *Clin Sci Mol Med* **55**: 253–259, 1978.
16. Boerma DO, Smit EP and Roosnek N, PIXE trace element determination and its accuracy in the analysis of bile. *Nuclear Instruments and Methods in Physics Research* **B36**: 60–73, 1989.
17. Griffith OW, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**: 207–212, 1980.
18. Meister A and Anderson ME, Glutathione. *Ann Rev Biochem* **52**: 711–760, 1983.
19. Nederbragt H and Lagerwerf AJ, Strain-related patterns of biliary excretion and hepatic distribution of copper in the rat. *Hepatology* **6**: 601–607, 1986.
20. Gross JB, Myers BM, Kost LJ, Kuntz SM and LaRusso NF, Biliary copper excretion by hepatocyte lysosomes in the rat. Major excretory pathway in experimental copper overload. *J Clin Invest* **83**: 30–39, 1989.
21. Gregus Z and Klaassen CD, Disposition of metals in rats: a comparative study of fecal, urinary and biliary excretion and tissue distribution of eighteen metals. *Toxicol Appl Pharmacol* **85**: 24–38, 1986.
22. Bremner I, Involvement of metallothionein in the hepatic metabolism of copper. *J Nutr* **117**: 19–29, 1987.
23. Ballatori N and Clarkson TW, Biliary secretion of glutathione and of glutathione-metal complexes. *Fundam Appl Toxicol* **5**: 816–831, 1985.