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EFFECT OF THE GLUTATHIONE-DEPLETING AGENTS DIETHYLMALEATE, PHORONE AND BUTHIONINE SULFOXIMINE ON BILIARY COPPER EXCRETION IN RATS

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Abstract—The involvement of glutathione (GSH) in the biliary excretion of Cu was investigated in bile-cannulated inbred WAG/Rij and BN rats, pretreated with diethylmaleate (DEM), phorone or buthionine sulfoximine (BSO) and injected with Cu doses of 10 or 30 μg/100 g body wt. DEM reduced liver GSH to 27–56% and biliary GSH excretion to 18–38%; phorone reduced GSH in the liver to 55% and increased it in the bile (113%) followed by a slight decrease (79%); BSO reduced liver GSH to 50% and bile GSH to 20%. After injection of Cu to control rats a profile of biliary Cu excretion was found, composed of a slowly (SCuE) and a rapidly (RCuE) disappearing component, the latter only present after the dose of 30 μg Cu. DEM had no effect on SCuE after a 10 μg dose and a temporary effect on SCuE after a 30 μg dose in both WAG/Rij and BN rats. Phorone reduced SCuE after both Cu doses to 50%. Both agents abolished RCuE and reduced endogenous biliary Cu excretion to less than 50%. Release of injected Cu from plasma and uptake by the liver was inhibited by DEM and phorone in both rat strains; in BN rats basal plasma Cu level of DEM-treated rats was increased as well. BSO reduced SCuE after both Cu doses but had no influence on RCuE. Endogenous Cu excretion was reduced by BSO in BN rats but not in WAG/Rij rats. The results show that biliary Cu excretion proceeds by a pattern, the components of which can be affected differently by the various drugs. They also indicate that GSH is not directly involved in biliary Cu excretion but suggest that it may play a role in the metabolism of Cu in the liver.

Bile is the main route by which copper (Cu) leaves the body [1] and this biliary Cu excretion is thought to play an important role in maintaining Cu homeostasis. It has been suggested that inefficient biliary Cu excretion in sheep [2, 3] is one of the causes of their susceptibility to Cu intoxication.

Notwithstanding its assumed importance the process of biliary Cu excretion is poorly understood. Excretion of intravenously injected Cu was shown to be temperature dependent and increased by increasing the Cu dose [4]. Recently, we published the results of studies on the biliary Cu excretion after i.v. Cu administration to rats of different strains [5]. Our results suggested that the pattern of biliary Cu excretion contained a slow and a rapid component; the latter occurred after higher Cu doses only and was superimposed on the slow component. In addition, the amount of Cu excreted in both components after similar i.v. Cu doses seemed to be strain-dependent.

Alexander and Aaseth [6] treated bile-cannulated rats with the glutathione (GSH)-depleting agent diethylmaleate (DEM) and found that the concentrations of Cu and zinc in bile decreased. They suggested that GSH is involved in biliary Cu excretion. This is a hypothesis that is in accordance with the evidence for the involvement of GSH in the biliary excretion of cadmium [7] and mercury [8, 9] but needs further confirmation.

Therefore, it was decided to investigate in more detail the excretion of i.v. administered Cu in bile-cannulated rats and the effects on it of the GSH-depleting agents DEM and phorone and the GSH-synthesis inhibitor buthionine sulfoximine (BSO). For this study inbred WAG/Rij and BN rats were used since, of the rat strains studied by us, they had the most efficient Cu excretion as far as the slow component is concerned [5].

MATERIALS AND METHODS

Animals. Male inbred WAG/Rij rats, weighing 170–210 g were obtained from Repgo-TNO (Rijswijk, The Netherlands). Male inbred BN rats, weighing 180–200 g were obtained from CPB-TNO (Zeist, The Netherlands).

Rats were housed in plastic cages and had free access to tap water and a commercial food (Hope Farms, Woerden, The Netherlands) containing approximately 15 mg Cu/kg. The rats were maintained at 24°, using a light–dark cycle of 12 hr.

Materials. All chemicals were at least reagent grade. Diethylmaleate (DEM) was from Merck (Darmstadt, F.R.G.), buthionine sulfoxime (BSO) from Sigma Chemical Co. (St Louis, MO), and phorone from Aldrich Chemicals (Steinheim, F.R.G.).

Bile cannulation experiments. Bile cannulation was performed on rats anaesthetized with sodium pentobarbital (70–75 mg/kg body wt). PE-10 tubing (Intramedic) was inserted into the bile duct via a

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small incision and ligated. The abdominal wall was closed with a suture to prevent dehydration. Body temperature was maintained by placing the rats on an electric heating pad. Anaesthesia was maintained by injection of small doses of pentobarbital when the rats showed signs of recovery. BSO was administered intraperitoneally before surgery in a dose of 0.4 mmol/100 g body wt dissolved in physiological saline and Cu was injected 4 hr later. DEM or phorone were administered intraperitoneally within 1 hr after starting the cannulation. DEM was given in a dose of 0.13 mmol/100 g body wt, dissolved in 3 vol. of corn oil. Phorone was given in a dose of 18 μmol/100 g body wt, dissolved in corn oil. Control rats for the DEM and phorone experiments received corn oil only. Cu was given 30 min (or 1 hr) later. Cu was always given as $CuSO_4$ in 0.9% NaCl intravenously in the tail vein. Cu doses were 10 or 30 μg/100 g body wt. In one experiment DEM and phorone were given after Cu administration but the same doses were used as indicated above. All concentrations of administered solutions were chosen such that 0.2 ml/100 g body wt was injected except for BSO which was given in a volume of 1 ml per 100 g body wt. Bile samples of 30 min were collected in preweighed vials up to 4 hr after Cu injection. The volume of the bile samples was determined by weighing, assuming the specific gravity of bile to be 1. At the end of the experiment the rats were killed by exsanguination and the liver was excised, blotted dry and weighed.

The effect of the three compounds on biliary GSH excretion was studied in bile-cannulated rats under the same conditions as described for the study of Cu excretion. Two samples of each of the rats to be treated with DEM and phorone and collected before administration of the agents served as controls. Bile was collected as 30 min samples in 0.5 ml metaphosphoric acid (10% in distilled water) and frozen at −20° until further use. After thawing and centrifugation (10 min; 20,000 *g*) the supernatant was used immediately for GSH determination. Data were corrected for dilution of the bile samples with acid. Three rats were used for DEM- and phorone- and two for BSO-treatment.

Other experiments. For the determination of the effects of DEM, phorone and BSO on liver GSH concentrations, rats were injected between 9.00 and 11.00 a.m. with the doses of the three agents mentioned above. After indicated time intervals they were exsanguinated by aorta puncture under heavy ether anaesthesia. The liver was excised, blotted dry and immediately frozen in liquid nitrogen. Subsequently the livers were stored at −70° until further use.

For the determination of the effects of DEM and phorone on Cu concentrations in liver and plasma rats were injected intraperitoneally with either of the agents dissolved in corn oil between 9.00 and 11.00 a.m. After 30 min Cu was injected intravenously under light ether anaesthesia. Control rats received corn oil and/or 0.9% NaCl only. After 0, 1 and 2 hr following Cu injection rats were killed by exsanguination under ether anaesthesia. Blood was centrifuged to obtain plasma; its Cu concentration was determined the same day. Livers were excised and blotted dry and stored at −70° until further use. To study the effect of BSO, Cu was injected 4 hr after BSO administration and plasma Cu was determined 1 hr after Cu injection.

Analytical procedures. For the determination of hepatic and biliary GSH the fluorometric method of Hissin and Hilf [10] was used. For the determination of Cu in bile and plasma, samples of both were diluted ten times with butanol (6%), together with samples of blank and standard solutions. Liver samples were digested with nitric acid under high pressure as described before [11]. Digested samples were diluted with deionized water to a known volume. To blanks and standards nitric acid was added to the same final concentrations. Cu concentrations in diluted samples of bile, plasma and liver and in blanks and standards were determined with an atomic absorption spectrophotometer (Perkin Elmer, model 400), using an air-acetylene flame. Biliary Cu and GSH excretion is expressed as μg/30 min/g liver.

Statistics. The data of pretreated rats were compared to those of untreated rats at the same time points. Statistical evaluation was performed using Student's *t*-test.

RESULTS

Effects of diethylmaleate (DEM)

With a dose of 0.13 mmol/100 g body wt, DEM decreased liver GSH concentration of WAG/Rij rats to 27, 33 and 33% of control values (6.03 ± 0.14 μmol/g) at 0.5, 1 and 2 hr respectively and in a second experiment to 47 and 56% of control values (7.20 ± 0.36 μmol/g) at 2 and 4 hr. Biliary GSH excretion was reduced to 26 (1 hr), 18 (2 hr), 24 (3 hr) and 38% (4 hr) of the control value (0.50 ± 0.04 μmol GSH/30 min/g liver).

The effect of DEM pretreatment on the excretion of intravenously injected Cu in those rats is shown in Fig. 1. Intravenous injection to control rats of 10 μg Cu/100 g (Fig. 1A) and 30 μg Cu/100 g (Fig. 1B) caused rapid increases in biliary Cu excretion.

The patterns of Cu excretion were similar to those described before [5]: both a slowly decreasing component and a rapidly decreasing component in the excretion of Cu could be found, the former (SCuE) present after both doses, the latter (RCuE) only present after the higher dose and superimposed on SCuE.

DEM had a choleretic effect (Fig. 1, inset). It had no effect on SCuE after the 10 μg dose and a temporary effect on SCuE after the 30 μg Cu dose, which lasted 1.5 hr. The RCuE component, representing the high peak of Cu excretion and visible only in control rats after the high dose, totally disappeared after DEM pretreatment.

DEM also caused a reduction in endogenous Cu excretion, i.e. the level of Cu in bile before Cu injection. When control rats were compared to DEM-treated rats this reduction was significant in the experiment of Fig. 1B only. When the endogenous Cu excretion in DEM-treated rats was compared to Cu excretion in the same rats before DEM was administered, it appeared to be reduced from 0.088 ± 0.007 to 0.051 ± 0.008 μg/30 min/g liver

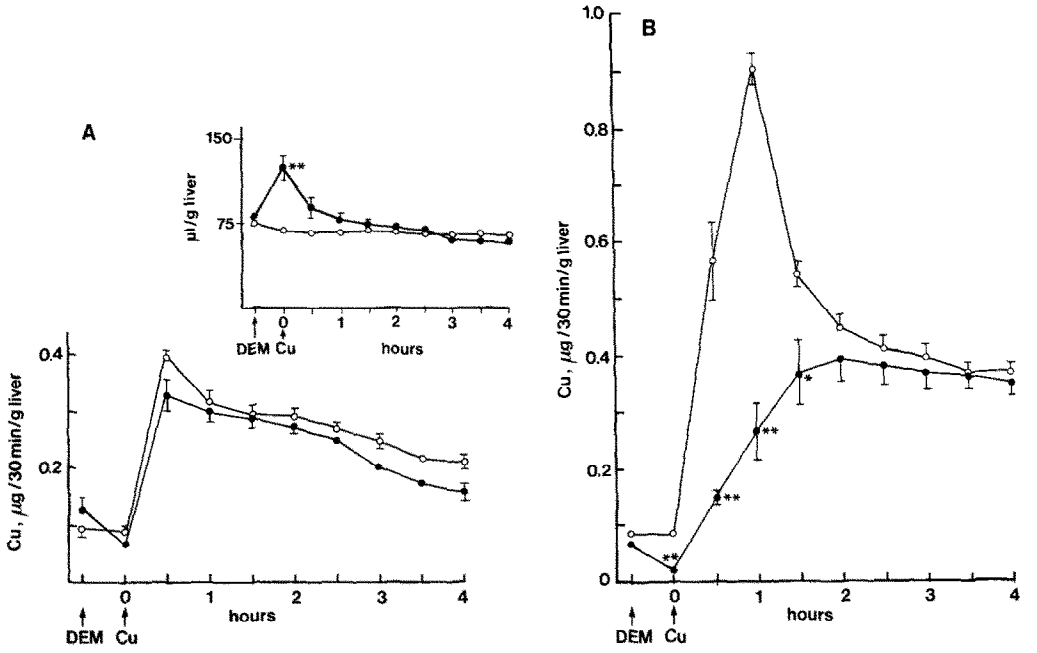

Fig. 1. Effect of DEM on biliary Cu excretion in WAG/Rij rats. To bile-cannulated rats DEM (0.13 mmol/100 g body wt in corn oil) was given i.p. and Cu was given i.v. 30 min later in doses of 10 µg (A) or 30 µg (B) per 100 g body wt. Control rats received corn oil only. Bile samples of 30 min were collected. Inset: effect of DEM on bile volume. ○: control rats, N = 4; ●: DEM-treated rats, N = 3 (A) or 5 (B). Points represent mean values with SE indicated by vertical bars. * $P \leq 0.05$; ** $P \leq 0.01$.

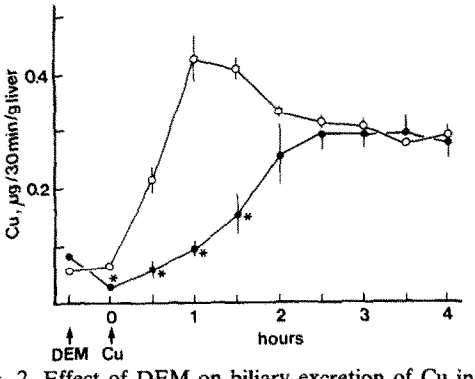

Fig. 2. Effect of DEM on biliary excretion of Cu in BN rats. To bile-cannulated rats DEM (0.13 mmol/100 g body wt in corn oil was given i.p. and Cu (30 µg/100 g body wt) was given i.v. 30 min later. Control rats received corn oil only. Bile samples of 30 min were collected. ○: control rats; N = 3; ●: DEM-treated rats, N = 4. Points represent means with SE indicated by vertical bars. * $P \leq 0.05$.

(58%; $P < 0.01$) in both experiments of Fig. 1 taken together.

It may be argued that the differences in the effect of DEM on Cu excretion after the 10 and the 30 µg Cu doses are not related to different effects on SCuE and RCuE but are related to differences in the absolute amount of Cu to be excreted in the bile after both doses. Therefore, the experiments were repeated with BN rats of which we have shown that their biliary Cu excretion is less efficient than that of WAG/Rij rats [5]. BN rats injected i.v. with 30 µg Cu/100 g body wt excreted approximately the same amount of Cu as WAG/Rij rats injected with 10 µg Cu (c.f. Figs 1A and 2; see also Ref. 5). Nevertheless, the effect of DEM pretreatment was qualitatively comparable to that in WAG/Rij rats, resulting in a temporary reduction in SCuE (Fig. 2). In addition DEM caused a reduction in endogenous Cu excretion in BN rats to 31%, from 0.086 ± 0.002 before to 0.027 ± 0.006 µg Cu/30 min/g liver ($P < 0.01$) after DEM administration.

Since the amount of Cu to be excreted in the bile directly depends on the amount of Cu taken up by the liver immediately after its administration, experiments were performed to investigate whether DEM had an effect on hepatic Cu uptake. Rats were pretreated with DEM as in the bile cannulation experiments but were killed at 1 and 2 hr after injection of 30 µg Cu/100 g body wt. Cu was determined in total liver and in plasma (Table 1). The effects of DEM were similar in WAG/Rij and BN rats, demonstrating a DEM-induced delay in the uptake of Cu by the liver, which can be concluded from the significantly lower hepatic Cu concentration 1 hr after Cu injection in DEM rats. The concentration of Cu in the plasma was significantly higher 1 and 2 hr after Cu injection in WAG/Rij rats but only 1 hr after Cu injection in BN rats; in addition DEM caused an increase in basal Cu levels of plasma in BN rats that was not observed in WAG/Rij rats (Table 1). However, plasma Cu levels are in accordance with liver Cu levels in that they point to an inhibition of Cu uptake by the liver caused by DEM.

Effects of phorone

The effects of phorone on Cu excretion were investigated in WAG/Rij rats only. A phorone dose of 18 µmol/100 g body wt decreased the liver GSH concentration to 49, 53 and 57% of the control value

Table 1. Effect of DEM and phorone pretreatment on liver and plasma Cu concentrations after intravenous Cu injection to rats

Strain			Time after Cu injection (hr) 0	1	2
WAG/Rij	Liver	Control	4.56 ± 0.14	10.15 ± 0.30	10.29 ± 0.43
		DEM	4.57 ± 0.21	8.67* ± 0.23	11.02 ± 0.49
	Plasma	Control	1.20 ± 0.03	1.19 ± 0.01	1.19 ± 0.01
		DEM	1.20 ± 0.07	1.98** ± 0.10	1.41** ± 0.03
BN	Liver	Control	3.56 ± 0.10	9.86 ± 0.33	10.10 ± 0.18
		DEM	4.04 ± 0.16	6.65** ± 0.49	9.44 ± 0.28
	Plasma	Control	0.76 ± 0.03	1.05 ± 0.18	0.70 ± 0.01
		DEM	0.90** ± 0.02	1.95** ± 0.25	0.91** ± 0.04
WAG/Rij	Liver	Control	5.16 ± 0.13	10.43 ± 0.05	10.48 ± 0.02
		Phorone	5.15 ± 0.10	9.26* ± 0.37	10.04 ± 0.08
	Plasma	Control	1.11 ± 0.05	1.13 ± 0.02	1.07 ± 0.02
		Phorone	1.11 ± 0.04	1.99** ± 0.04	1.63** ± 0.04

WAG/Rij or BN rats were pretreated by i.p. injection of DEM (0.13 mmol/100 g body wt in corn oil) or phorone (18 μmol/100 g body wt in corn oil) 30 min before Cu injection at 0 hr. Control rats were injected with corn oil only, rats at 0 hr received no Cu. Cu (30 μg/100 g body wt) was injected into a tail vein. Rats were killed after 0, 1 and 2 hr. Values are means ± SE, expressed as μg Cu/g liver or μg Cu/ml plasma; N = 4. * $P \leq 0.05$; ** $P \leq 0.01$.

(6.05 ± 0.23 μmol/g) at 0.5, 2 and 4 hr after administration. Biliary GSH excretion was increased by phorone to 113 and 109% at 1 and 2 hr respectively, but decreased thereafter at 3 and 4 hr to 93 and 79% of control values (0.36 ± 0.04 μmol GSH/30 min/g liver). Phorone had a choleretic effect similar to DEM (Fig. 3, inset). In contrast to DEM, phorone caused a significant reduction in SCuE, after both the 10 μg (Fig. 3A) and the 30 μg Cu dose (Fig. 3B), that amounted to 50% of the control value and lasted throughout the experiment. Similarly to the effect of DEM the RCuE was totally absent after phorone pretreatment. A reduction of endogenous biliary Cu excretion was also observed after phorone administration which was significant ($P < 0.01$) when in both experiments Cu in bile was determined before and after phorone administration: Cu excretion decreased from 0.092 ± 0.011 to 0.044 ± 0.009 μg/30 min/g liver, i.e. to 48%.

Although the effects of DEM and phorone on Cu excretion were different, their effects on liver and plasma Cu (Table 1) were similar; phorone caused a delay in the release of Cu from the plasma and in the uptake of Cu by the liver.

Effects of DEM and phorone administration following Cu injection

To study the effect of DEM and phorone on the biliary excretion of Cu that is already taken up by the liver, both agents were administered to bile-cannulated WAG/Rij rats 30 min after 30 μg Cu had been injected per 100 g body wt. Approximately 50% of injected Cu will be taken up by the liver within 30 min after injection [12, 13]. The results are given in Fig. 4 and show that when biliary Cu excretion reached its peak level, the amount of Cu excreted in the bile of DEM- or phorone-treated rats was significantly reduced compared to control rats. As in the former experiments DEM only temporarily reduced biliary Cu excretion whereas by phorone it was reduced to a larger extent (50%) up to the end of the experimental period.

Effects of buthionine sulfoximine (BSO)

The GSH-depleting potential of BSO (0.4 mmol/100 g body wt) was tested in rats of both strains. It reduced liver GSH of BN rats to 45% of the control value (4.02 ± 0.20 μmol/g) at 4 hr and in WAG/Rij rats to 58 and 56% of control value (7.20 ± 0.36 μmol/g) at 4 and 8 hr after administration. The excretion of GSH in the bile of WAG/Rij rats was reduced from 0.43 to 0.09, 0.08 and 0.08 μmol/30 min/g liver at 4, 6 and 8 hr after injection which is approximately 20% of the control value.

The effects of BSO on biliary Cu excretion in WAG/Rij rats is shown in Fig. 5 and on Cu excretion in BN rats in Fig. 6. Contrary to DEM and phorone BSO had no influence on RCuE which is the peak of Cu excretion which can be seen after a 30 μg Cu dose. Although this RCuE is much smaller in BN rats (Fig. 6B) than in WAG/Rij rats (Fig. 5B) it remains visible after BSO pretreatment in both strains. On the other hand, SCuE after both 10 and 30 μg Cu doses was reduced in both strains; this

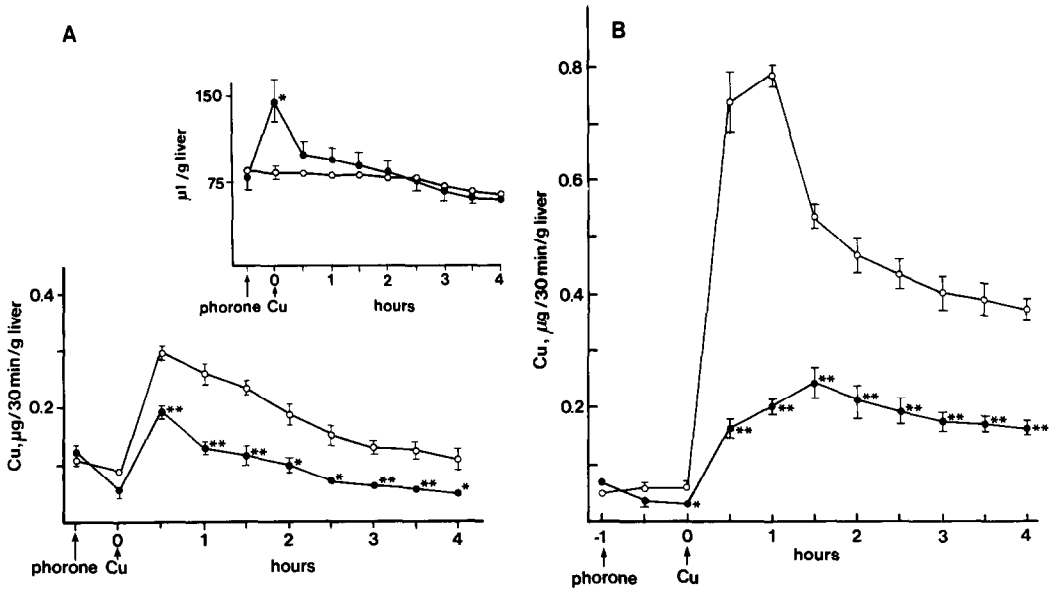

Fig. 3. Effect of phorone on biliary excretion of Cu in WAG/Rij rats. To bile-cannulated rats phorone (18 µmol/100 g body wt in corn oil) was given i.p. and Cu was given i.v. 30 or 60 min later in doses of 10 µg (A) or 30 µg (B) per 100 g body wt. Control rats received corn oil only. Bile samples of 30 min were collected. ○: control rats, N = 4; ●: phorone-treated rats, N = 4. Points represent mean values with SE indicated by vertical bars. * $P \leq 0.05$; ** $P \leq 0.01$.

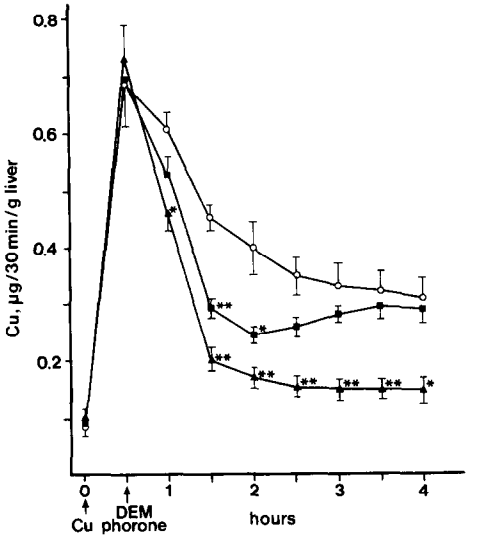

Fig. 4. Effect of DEM and phorone on biliary excretion of Cu in WAG/Rij rats when given after Cu administration. To bile-cannulated rats DEM (0.13 mmol/100 g body wt) or phorone (18 µmol/100 g body wt), both in corn oil, were given 30 min after an i.v. injection of 30 µg Cu/100 g body wt. Control rats received corn oil only after the Cu injection. Bile samples of 30 min were collected. ○: control rats, N = 3; ■: DEM-treated rats, N = 5; ▲ phorone-treated rats, N = 6. Points represent mean values with SE indicated by vertical bars. * $P \leq 0.05$; ** $P \leq 0.01$.

reduction reached immediate statistical significance only in WAG/Rij rats after the 10 µg Cu dose (Fig. 5A). In the other three experiments significant reduction of SCuE was reached 2.5–3.0 hr after Cu injection.

In the experiments with BSO endogenous Cu excretion was compared to that in untreated controls. It was reduced to 88% in WAG/Rij rats, from 0.066 ± 0.008 to 0.058 ± 0.005 µg Cu/30 min/g liver which was not significant. This was contrary to the effect of BSO in BN rats in which endogenous Cu excretion decreased significantly ($P < 0.01$) from 0.087 ± 0.008 to 0.056 ± 0.002 µg Cu/30 min/g liver (64%).

The effect of BSO on uptake by the liver was much less than that of DEM and phorone: 1 hr after Cu injection of 30 µg Cu/100 g body wt in BSO-pretreated rats the plasma Cu concentration was 1.24 ± 0.03 µg/ml whereas control rats had a concentration of 0.99 ± 0.03 µg Cu/ml.

DISCUSSION

The results of the investigations presented here suggest that GSH is not directly involved in biliary Cu excretion but has a role in hepatic Cu metabolism, consequently affecting Cu excretion in an indirect way. This can be concluded from the different effects of the agents used in this study on liver and bile GSH and on biliary Cu excretion respectively.

The first question to be discussed is that of the components of the pattern of biliary Cu excretion. In a previous paper [5] it was shown that upon intravenous Cu administration two components could be distinguished in this pattern, the RCuE being a peak of Cu excretion that rapidly disappeared and could be found after higher Cu doses and the SCuE as a slowly disappearing component. It is now necessary to add a third component, i.e. the endogenous Cu excretion which is the excretion of Cu without a previous Cu injection.

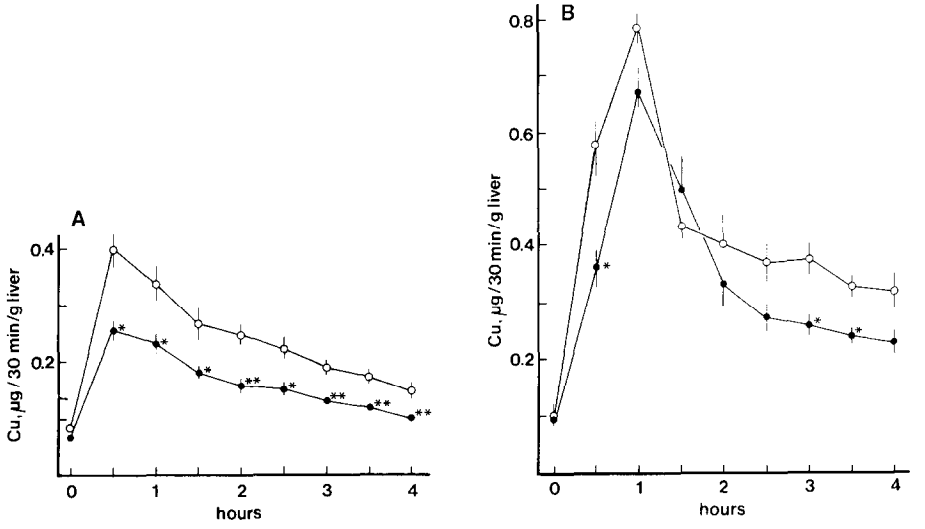

Fig. 5. Effect of BSO on biliary excretion of Cu in WAG/Rij rats. BSO (4 mmol/100 g body wt) was given i.p. to rats in which the bile duct was cannulated between 2.5–3.5 hr afterwards. Four hours after BSO administration Cu was injected i.v. in doses of 10 (A) and 30 (B) µg per 100 g body wt. Bile samples of 30 min were collected. ○: control rats, N = 5; ●: BSO-pretreated rats, N = 5 (A) or 4 (B). Points represent mean values with SE indicated by vertical bars. * $P \leq 0.05$, ** $P \leq 0.01$.

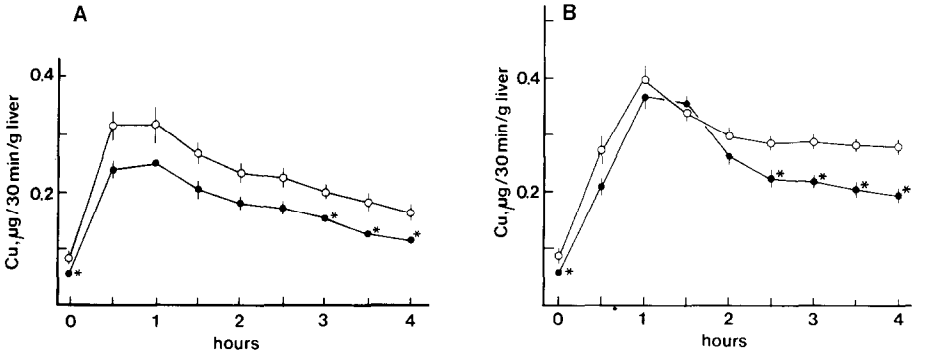

Fig. 6. Effect of BSO on biliary excretion of Cu in BN rats. BSO (4 mmol/100 g body wt) was given i.p. to rats in which the bile duct was cannulated between 2.5–3.5 hr afterwards. Four hours after BSO administration Cu was injected i.v. in doses of 10 (A) and 30 (B) µg per 100 g body wt. Bile samples of 30 min were collected. ○: control rats, N = 4 (A) or 6 (B); ●: BSO-pretreated rats, N = 4 (A) or 9 (B). Points represent mean values with SE indicated by vertical bars. * $P \leq 0.05$; ** $P \leq 0.01$.

The interpretation of the biliary Cu excretion pattern as consisting of three components has to be considered as tentative; at this moment they can not be related to different biochemical processes or mechanisms. In addition they can be explained as pharmacokinetic phases, the rate of which can be influenced by the rate of Cu uptake by the liver (see below). However, the finding of strain-related differences in both RCuE and SCuE independently [5] and the finding presented here that they can be manipulated differently by various agents point to the possibility that different processes may be involved.

The results of the effects of the agents on the three components of biliary Cu excretion can be summarized as follows: (i) RCuE is abolished by DEM and phorone but not by BSO; (ii) SCuE is reduced by phorone and BSO after both Cu doses; it can also be reduced by DEM after a 30 µg Cu dose but then the reduction in SCuE is temporary; DEM has no effect in SCuE after a Cu dose of 10 µg/100 g body wt.; (iii) endogenous Cu excretion is reduced by all three agents, except in WAG/Rij rats in which it is not affected by BSO.

At least one of the components of biliary Cu excretion may be related directly to the uptake of Cu by the liver, as is illustrated by the data of Table 1. It is likely, but not definitely sure, that retardation or inhibition of Cu uptake by the liver may cause the RCuE peak to disappear. When hepatic Cu uptake is only slightly affected such as after BSO pretreatment the top of the RCuE peak seems to be shifted to a later timepoint (Figs 5 and 6); such a phenomenon can also be observed after Cu injections

in fasted rats (unpublished). A shift of the RCuE peak to later timepoints in combination with a reduction of biliary Cu excretion may have caused the patterns found after DEM and phorone pretreatment (Figs 1B, 2B and 3B). In fact, a pharmacokinetic approach of the data in which a decreased initial rate of Cu uptake by the liver is taken into consideration can explain a decreased initial rate of Cu excretion in the bile of these rats.

Even endogenous Cu excretion may be affected by uptake of Cu by the liver; this is suggested by the increase of endogenous plasma Cu by DEM in BN rats (Table 1) in combination with the reducing effect of DEM on endogenous Cu excretion in those rats which is more pronounced than in any of the other experiments.

However, inhibition of Cu uptake by the liver can not be the sole mechanism responsible for reduced biliary excretion of injected Cu since DEM and phorone inhibited the excretion of Cu that was already taken up by the liver (Fig. 4).

The second question to be discussed is the involvement of GSH in biliary Cu excretion. The method used in this study to determine GSH is that of Hissin and Hilf [10] and although it is not very specific it is considered valid when GSSH is the major thiol present in the sample [14]. Our results regarding the effect of DEM and phorone on biliary GSH excretion confirm those of Siegers *et al.* [15] in which GSH in the bile is decreased by DEM and increased by phorone; a reduction of biliary GSH by BSO has been described before as well [16].

The only evidence for involvement of GSH in biliary Cu excretion to be found in the literature is the publication of Alexander and Aaseth [6]. Their results were obtained from DEM-treated rats in which endogenous Cu excretion was studied. They did not take into account the choleretic effect of DEM and only measured Cu excretion as Cu concentration of the bile. Our own results lead to the conclusion that, in general, the three agents used here cause a reduction in biliary Cu excretion but that their effect cannot be explained simply by GSH depletion of the liver or reduced biliary GSH excretion. This follows from the following considerations: (i) DEM had no influence on SCuE, or only temporarily, although liver and bile GSH were both reduced; RCuE totally disappeared; (ii) Phorone was the most potent inhibitor of Cu excretion in the bile and although its administration caused liver GSH to be reduced, it increased bile GSH; (iii) The effect of BSO on liver and bile GSH was comparable to that of DEM but it affected SCuE in all experiments and RCuE was not affected; endogenous Cu excretion was reduced in WAG/Rij rats only.

It is unlikely that the doses of Cu used in these experiments have influenced liver and bile GSH concentrations: preliminary experiments had shown that a significant decrease (18%) of liver GSH could be induced by a Cu dose of 300 μg/100 g body wt and that a dose of 50 μg Cu had no effect on biliary GSH excretion.

It has been shown that after intravenous administration to rats both Cd [7] and Hg [8] are found in the bile as a metal–GSH complex. However, Martin *et al.* [17] were unable to detect GSH-bound Cu in human bile. The explanation for this difference may be that Cd and Hg are supposed to bind to the sulphydryl-group of GSH whereas Cu merely oxidizes this SH-group, at least *in vitro* [18]. One of the possible explanations of the results presented here is that the reduction in biliary Cu excretion by the three agents may be mediated by other effects than those on GSH. Among the effects, known of DEM, are inhibition of several pathways of mixed function oxidases *in vitro* [19], changes in the Golgi apparatus in rat hepatocytes [20], induction of cholestasis in rabbits [21] and inhibition of protein synthesis in mice [22]. Phorone has no effect on mixed function oxidases [23] and on protein synthesis [22] and, as far as is known up to now, only shares with DEM effects on acetaldehyde oxidation and aldehyde dehydrogenase in rat hepatocytes [24] and on bile flow and GSH concentrations of the liver [15]. No other effects of BSO than on GSH have been described.

Another probable explanation for the effects of the agents on biliary Cu excretion may be that one or more of its components are regulated in processes in which GSH is indirectly involved. Cu uptake by the liver may be one of these. Other possibilities in this respect are that Cu metabolism in the liver may depend on the reduced state of the hepatocyte or on the sulphydryl status of one or more proteins, both of which will be affected by GSH depletion.

The situation in the GSH-depleted rat bears some similarity to that in patients with Wilson's disease in which low hepatic GSH-concentrations have been found [25], next to a decreased biliary Cu excretion [26] and a delayed uptake of intravenously injected Cu by the liver [27].

Further studies are necessary to give more insight in the relation between Cu metabolism and GSH.

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