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BILIARY EXCRETION OF COPPER AND ZINC IN THE RAT AS INFLUENCED BY DIETHYLMALEATE, SELENITE AND DIETHYLDITHIOCARBAMATE

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Abstract—An i.p. diethylmaleate injection (dose: 3.9 mmoles/kg) decreased the endogenous Cu and Zn excretion in rat bile to 20 and 50 per cent, respectively. A corresponding decrease of the glutathione levels in liver and bile suggests that copper, at least partly, is excreted in the bile as a glutathione complex or by other glutathione-dependent mechanisms. Similar mechanisms may also be active for Zn. Selenite (5 μmoles/kg) or diethyldithiocarbamate (67 μmoles/kg) treatment also decreased the copper excretion in bile, but the glutathione levels were not significantly affected. These agents may act either by forming metal complexes which are not excreted, or by blocking the transfer of the metal from proteins to glutathione in liver cells. Selenite and diethyldithiocarbamate did not significantly influence the excretion of zinc in the bile. Orally given selenite (5.7 or 57 μmoles/100 g food) increased the kidney levels of copper and zinc and decreased liver levels of zinc, whereas diethyldithiocarbamate (6.7 mmoles/100 g food) caused increased liver content of copper and decreased kidney levels of copper and zinc. The differences observed between copper and zinc distribution when using selenite or diethyldithiocarbamate treatment may be due to different organ retention of the metal complexes formed.

Bile is known to be the main excretory route of copper and zinc [1]. The mechanisms of transport of copper and zinc from liver cells to bile are unknown [2], although several copper-binding substances in bile have been suggested [3–5]. Copper and zinc resemble methyl mercury and cadmium in their complexing abilities, as they form stable mercaptides with thiols [6]. Both methyl mercury and cadmium occur in rat bile as glutathione complexes [7, 8]. Administration of substances which bind glutathione in the liver cells, i.e. diethylmaleate (DEM) and bromsulphtaleïn, leads to a depressed biliary excretion of methyl mercury and cadmium [8, 9]. It is therefore presumed that the secretion of these toxic metals across the canalicular membrane of the liver cell into bile involves glutathione as the carrier molecule. A question is if the same metal-complexing carrier is active in the physiological biliary excretion of copper and/or zinc.

Previous studies have shown that selenium [10] and diethyldithiocarbamate (DDC) [11] affect methyl mercury metabolism by inhibiting the biliary excretion and influencing the organ distribution in rats.

In the present work, we have injected rats with diethylmaleate, selenite or DDC in order to study if the mechanism of biliary copper and zinc excretion may be similar to that of methyl mercury and cadmium.

MATERIALS AND METHODS

Female Wistar rats of our own breed as well as from Møllegaard, Copenhagen, Denmark, weighing about 200 g, were used for the experiments on biliary excretion. The organ distribution was studied in male and female Wistar rats from Møllegaard, Denmark. The rats were fed on a standard diet (Institute of Occupational Health, Oslo, Norway). Diethyldithiocarbamate and diethylmaleate were obtained from Koch Light Ltd., U.K. Selenite was obtained from Merck, Darmstadt, Germany.

Studies on biliary excretion of copper, zinc and thiols. The bile duct was canulated during barbiturate anasthesia (pentobarbitone sodium, Mebumalum NFN). A tracheal tube was inserted to ensure free airways during the collection period, and the body temperature was kept constant. The bile flow was 0.5–1.0 ml/hr. The bile was collected for control purpose for 1 hr prior to the i.p. injection of either selenite or DDC. Diethylmaleate was injected 30 min after starting bile collection. Subsequently, the bile was collected over periods of 30 min. The total period of bile sampling was 4 hr. The biliary concentration and total content of copper and zinc was determined by atomic absorption spectrophotometry after weighing each sample. The rats were killed by blood collection from the abdominal aorta and serum samples were prepared for copper and zinc determinations.

Experiments with thiol excretions into bile were also done in female rats and bile was sampled over periods of 30 min into vials containing ice-cold EDTA (ethylenediamine tetraacetic acid). Total thiol (SH) concentration in bile was determined spectrophotometrically with Ellman's reagent [12]. Specific determination of glutathione was done by the use of glutathione reductase in combination with Ellman's reagent [9] as well as by ion exchange chromatography method (Joel, automatic amino acid analyzer).

Organ distribution studies. Since the effects of

selenite and DDC on biliary metal excretion were difficult to interpret, organ distribution studies on copper and zinc were undertaken in rats given oral treatment with these agents. After one day of fasting, the rats were fed either standard diet (controls) or diet containing appropriate amounts of selenite or DDC. The rats had free access to water. After one week of treatment, the animals were killed by blood collection from the aorta, and organs were removed for copper and zinc determinations.

Analysis of copper and zinc in bile and tissues. Duplicate specimens of 50 μl serum or bile were diluted with 200 μl 1% HNO_3. This dilution liberates copper and zinc from insoluble proteins (unpublished results). The solutions were analyzed by atomic absorption spectrophotometry with flame atomization technique (instrument: Perkin Elmer 403).

Standard solutions for the analyses were prepared by adding different amounts of copper and zinc to bile or serum samples. The obtained standard curves for absorbance vs added metal were found to be linear for both copper and zinc in these body fluids. The metal contents in the standard sample before addition of copper and zinc could be obtained by extrapolation. By comparing sets of standard series from different serum samples, it was found that the matrix effect did not vary between the samples. Hence, the metal concentration in serum samples were found from their absorbance by using the obtained standard curve. To control the precision of our analyses, aliquots from a human serum sample with predetermined copper and zinc concentration were included in each series. The matrix effect in bile was found to be negligible. All test samples were analysed as duplicates and averages were used in calculations and figures.

The trace elements in organs were determined after digestion with Lumatom (H. Kürner, Neyberg, F.R.G.) with atomic absorption spectrophotometry. Aliquots were diluted with ethanol. A parallel sample was prepared for each aliquot by adding standard solutions of copper and zinc. A standardized bovine liver sample (National Bureau of Standards, U.S.A.) was analysed for copper and zinc using the above mentioned method. The results obtained on the latter standard sample were within 5–10 per cent of the given values.

Statistical treatment. Differences in organ content or bile concentration of copper, zinc or thiols were considered to be significant when $P < 0.05$, using Wilcoxon two-sample test, two-tailed.

For the biliary excretion experiments, each rat was used as its own control by recording biliary excretion for a 30 or 60 min period before treatment. Since the various treatments did not influence the bile flow significantly, the concentration of copper and zinc was used as a parameter for excretion. The mean biliary copper concentration (range: 14.2–42.5 μmoles/l) and mean zinc concentration (range: 4.6–24.5 μmoles/l) during the first 30 min after starting collection of bile, were taken as control value (100 per cent) for each rat.

RESULTS

The bile flow and colour were not affected significantly by the treatment with DEM, selenite or DDC in these experiments. Cannulation of the bile duct for 4 hr did not influence the serum levels of copper or zinc (results not shown). Following the injection of DEM (3.9 μmoles/kg), there was a rapid and parallel depression of both the copper and the thiol concentration in the bile (Figs. 1 and 2). The glutathione concentration in the bile from 4 rats was 1.2–1.4 mmoles/l. Cysteine accounted for only 0.06–0.1 mmoles/l of the biliary thiols. The initial biliary thiol concentration in eight rats was 1.0–2.4 mmoles/l. After the DEM treatment, the total biliary thiol level was reduced to about 0.1 mmoles/l whereas the level did not change in the control rats (Fig. 2). The biliary copper level was reduced to 20–50 per cent of the control levels.

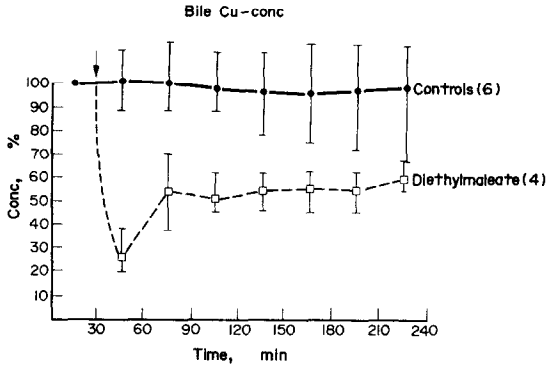

Fig. 1. Effect of diethylmaleate on biliary copper concentration. Bile samples were collected from anesthesized rats as described in the text, and copper was analysed by the atomic absorption method. Each value and vertical bar represents the mean and experimental range for copper concentration in the samples collected during the time indicated. The mean concentration during the first 30 min was set to 100 per cent for each animal. The number of animals is given in parentheses. The arrow indicated the time for the intraperitoneal injection of diethylmaleate (3.9 mmoles/kg).

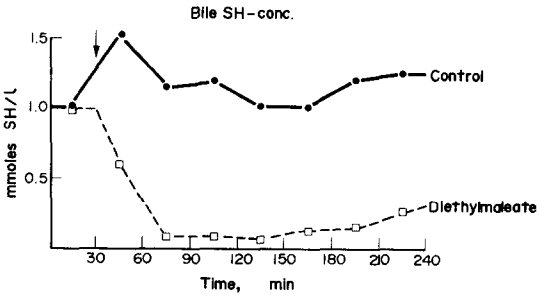

Fig. 2. Effect of diethylmaleate on biliary thiol concentration. The total concentration of thiols in the collected samples of bile was determined spectrophotometrically by Ellman's method [12]. This SH-concentration varied from 1.0 to 2.4 mmoles/l in the 8 rats before treatment. Diethylmaleate (3.9 mmoles/kg) was injected to 4 rats at the time indicated by the arrow, and 4 untreated rats served as controls. The results for one control and one treated rat which are given in the figure are characteristic for the two groups of animals.

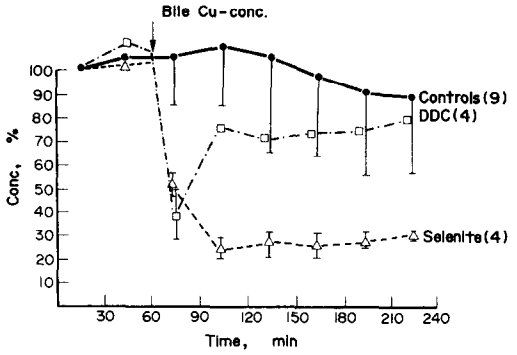

Fig. 3. Effect of selenite (3.0 μmoles/kg) and diethyldithiocarbamate (67 μmoles/kg) on biliary copper concentration. Each value and vertical bar represents the mean and experimental range for copper concentrations in the samples collected during the time indicated. The mean concentration during the first 30 min was set to 100 per cent for each animal. The number of animals is given in parentheses. The arrow indicates the time for treatment.

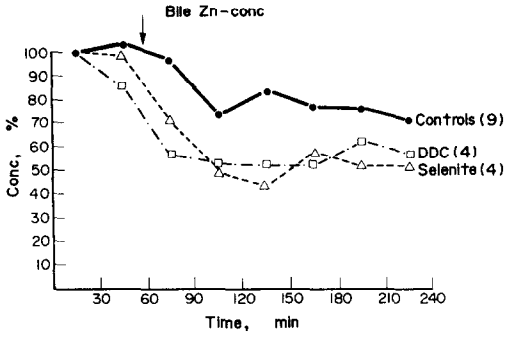

Fig. 4. Effect of selenite and diethyldithiocarbamate on biliary zinc concentration. Each value represents the mean of the samples collected during the time indicated. The mean concentration during the first 30 min was set to 100 per cent for each animal. The number of the animals is given in parentheses. The arrow indicates the time for treatment.

Injection of DDC (67 μmoles/kg) or selenite (5 μmoles/kg) were followed by a decreased biliary excretion of copper (Fig. 3). The biliary copper concentration remained low for 3 hr when using diethylmaleate or selenite, whereas DDC caused a concentration decrease of shorter duration. DDC and selenite did not influence significantly the biliary excretion of zinc (Fig. 4). DEM, however, caused a reduction of zinc levels in bile to about 50 per cent of control values (not shown).

Oral treatment with selenite (5.7 μmoles/100 g food) for 7 days increased considerably the kidney copper as compared to the controls (Table 1). The same treatment did not cause any significant changes in the content of copper in the serum, liver, brain or spleen. A higher dose of selenite (57 μmoles/100 g food) yielded similar results, i.e. raised kidney levels, but unchanged copper levels in the other organs tested. Oral DDC treatment (dose: 6.7 mmoles/100 g food) caused a moderate increase in the liver and spleen copper, and a slight decrease in the kidney content of copper, whereas the contents in serum and brain were unaffected (Table 1).

As far as zinc was concerned, the treatment with selenite lowered its liver concentration and increased kidney concentration. Neither the serum, brain nor spleen levels of zinc were altered by selenite treatment (Table 2). DDC treatment caused a slight decrease in serum and kidney content of zinc. The levels of zinc in any of the other organs measured did not change (Table 2).

DISCUSSION

The effect of diethylmaleate injected to rats is characterized by an immediate decrease of the transport of unconjugated glutathione into bile, reflecting a depletion of glutathione in liver cells [9, 13]. The demonstrated depression of biliary excretion of cop-

Table 1. Effect of selenite or diethyldithiocarbamate (DDC) on copper concentration (nmoles/g wet tissue, mean and range)

	Controls (6 ♀)*	Selenite† (6 ♀)	DDC‡ (6 ♀)	Controls (5 ♂)	Selenite§ (5 ♂)
Serum	20.7 (18.9–26.8)	22.0 (18.9–26.8)	23.6 (20.5–26.8)	15.4 (14.2–17.3)	15.7 (15.7–17.3)
Liver	62.6 (58.2–66.1)	56.7 (47.2–63.0)	74.0‖ (66.1–88.1)	55.4 (48.8–61.4)	47.2 (40.9–55.1)
Kidney	111.0 (67.7–230.0)	266‖ (214–312)	78.7‖ (66.1–81.8)	116.0 (67.7–146.0)	301.0‖ (216.0–362.0)
Spleen	19.9 (17.3–26.8)	18.9 (17.3–20.5)	23.6‖ (23.6–25.2)	19.8 (17.3–22.0)	17.3 (17.3–18.9)
Brain	38.9 (34.6–44.1)	37.8 (36.2–41.0)	39.3 (34.6–44.1)	39.0 (33.0–42.5)	40.9 (39.3–44.1)

* Number of animals.
† 5.7 μmoles selenite/100 g food.
‡ 6.7 mmoles DDC/100 g food.
§ 57 μmoles selenite/100 g food.
‖ Different from control, $P < 0.05$.

Table 2. Effect of selenite or diethyldithiocarbamate (DDC) on zinc concentration (nmoles/g wet tissue, mean and range)

	Controls (6 ♀)*	Selenite† (6 ♀)	DDC‡ (6 ♀)	Controls (5 ♂)	Selenite§ (5 ♂)
Serum	17.9 (15.3–19.9)	15.3 (13.8–16.8)	15.3‖ (15.3)	20.2 (16.8–24.5)	19.9 (18.4–21.4)
Liver	382 (271–548)	249‖ (236–265)	343 (294–401)	337 (318–353)	278‖ (252–300)
Kidney	227 (199–239)	288‖ (269–311)	188‖ (176–205)	266 (205–292)	294‖ (251–344)
Spleen	225 (207–259)	240 (229–248)	252 (242–277)	223 (165–283)	223 (176–242)
Brain	145 (124–167)	155 (147–159)	151 (139–173)	168 (153–193)	162 (159–167)

* Number of animals.
† 5.7 μmoles selenite/100 g food.
‡ 6.7 mmoles DDC/100 g food.
§ 57 μmoles selenite/100 g food.
‖ Different from control, $P < 0.05$.

per parallel to this binding of glutathione by diethylmaleate is similar to the previously shown inhibition of the methyl mercury excretion [9]. The most probable interpretation of these results is that copper compounds are excreted into bile, at least partly, by glutathione-dependent biochemical processes. Hepatic detoxification of copper and methyl mercury by glutathione conjugation, i.e. by the formation of transportable metal–glutathione complexes within the liver cells, appears to be an attractive hypothesis [9]. This hypothesis is consistent with the reported findings of Evans and Cornatzer [14], who suggested that copper in the bile is bound to a small molecular peptide or an amino acid. Such binding of copper to glutathione seems to depend upon the activity of transferring enzymes (transferases), since increased copper and methyl mercury excretion have been observed in bile following injection of microsomal enzyme inducers, i.e. spironolactone [15, 16]. A glutathione-dependent mechanism also seems to contribute to the biliary zinc excretion.

From a chemical point of view, it seems reasonable that thiol groups are the most important complexing ligands of copper in vivo, owing to the 'soft' character of the copper ions [6]. The concentration of glutathione in rat bile (1–2.5 mmoles/l) [17] accounts for more than 90 per cent of the total SH-content [9], and is about 50 times higher than the biliary concentration of copper (≃ 30 μmoles/l). *In vitro* studies of complexes between copper and glutathione have revealed the existence of both a stable 1:1-species and an even more stable diligandic (1:2) species of Cu(I)/glutathione complex at the pH of bile [18]. The stability constant of the 1:1-species is reported to be about 10^{25}. The 'overall' stability constants of copper–amino acid complexes are reported to be much lower, viz. around 10^{15} [19]. Furthermore, the concentration of most of the essential amino acids in rat bile are lower than 0.1 mmole/l (J. Alexander and J. Aaseth, to be published). Accordingly, it seems most likely that the main fraction of biliary copper is bound to glutathione rather than to L-amino acids. To the authors' knowledge, however, direct identification of Cu–glutathione complexes in bile has not been reported. This may be explained as follows: The thiol group of glutathione is not stable in sampled bile (pH 8.0–8.5). The presence of copper, iron and also the enzyme λ-glutamyl transpeptidase will catalyse oxidation of glutathione to the corresponding disulphide and to 'mixed disulphides' with other thiols [20–22]. The λ-glutamyl transpeptidase may also catalyse hydrolytic cleavage of the tripeptide [21]. The end result of these reactions which can proceed in collected bile is a reduced concentration of free glutathione. The consequent shift in chemical equilibria leads to transfer of methyl mercury from glutathione to proteins, even in bile stored frozen overnight [23]. A similar transfer of copper to proteins, and also to bile pigments or bile salts (taurochenodeoxycholate), may explain the findings of previous authors on copper-binding substances in bile [3–5, 24, 25]. The findings of these authors may, however, also suggest the existence of other mechanisms for the biliary excretion of copper.

Our proposed hypothesis for the biliary copper excretion, involves several steps: firstly, the intracellular formation of a transportable Cu–glutathione complex which requires glutathione and available copper compounds, and secondly, an active [2] efflux into bile of the formed copper complexes. In contrast to DEM, neither DDC (T. Norseth, personal communication) nor selenite [10] have been found to influence the glutathione efflux into bile to a significant degree. The inhibited transfer of methyl mercury [10, 11] and copper from liver cells into bile after treatment with DDC or selenite may be due to occupation of intracellular copper or inhibition of the necessary enzymes. It is known that DDC [26], as well as selenide [27], can form very stable copper complexes *in vitro* and DDC can even extract copper bound to ceruloplasmin [26]. It is reasonable to assume that a similar complex formation can take place in liver cells and perhaps also in other tissues.

Previous studies [28] have reported increased copper levels in the brain, following intravenous DDC-treatment after ^{64}Cu-exposure, and intravascular formation of lipophilic complexes have been suggested as an explanation. Orally-given DDC may, however, be partially broken down by stomach acid [29], and

thus the effect of DDC in our distribution studies might have been limited as compared to other reports.

The increased renal levels of copper in selenium treated rats may be attributed to copper–selenide complex formation, presumably not only in the liver, but also intravascularly. Studies in silver-exposed individuals have shown that silver–selenide complexes are deposited in the kidney [30]. Furthermore, it is known that selenite treatment is able to decrease the binding of heavy metals (e.g. Hg^{2+}, Cd^{2+}) to the sulphur-rich protein metallothioneine [31, 32] and change the organ distribution of the same heavy metals [33]. A similar mechanism may be active in the selenite induced redistribution of copper and zinc, since the liver uptake of the metal ions, particularly of zinc, is known to be associated with complexation to metallothioneine [34].

Several investigators have reported retention of copper in later stages of liver cirrhoses, including primary biliary cirrhosis and Wilson's disease [35, 36]. Their findings are consistent with our hypothesis of Cu–glutathione conjugation as a necessary (and perhaps rate-limiting) step for the excretion of the metal into bile. The existence of an impaired glutathione conjugation of some foreign substances, e.g. bromsulphtaleïn and acetaminophen, in similar liver diseases, is in accordance with our hypothesis of the involvement of glutathione in biliary copper excretion.

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