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From the Department of Clinical Chemistry, College of Veterinary Medicine, Swedish University of Agricultural Sciences, and National Veterinary Institute, Uppsala, Sweden.

EFFECTS OF 2-MERCAPTOETHANOL ON THE SOLUBILITY OF COPPER AND ZINC CONTAINING PROTEINS IN LIVER SAMPLES FROM NORMAL AND CHRONIC COPPER POISONED SHEEP

By

Kamal S. M. Hussein, A. Frank, B.-E. V. Jones and *L.-E. Edqvist*

HUSSEIN, K. S. M., A. FRANK, B.-E. V. JONES and L.-E. EDQVIST: *Effects of 2-mercaptoethanol on the solubility of copper and zinc containing proteins in liver samples from normal and chronic copper poisoned sheep.* Acta vet. scand. 1984, *25*, 10—20. — The solubility of Cu and Zn binding proteins was studied in liver samples from clinically healthy and chronic copper poisoned sheep (CCP). Homogenized liver was divided by ultracentrifugation into cytosol and pellet. The cytosol was gelfiltrated, Cu and Zn contents were determined in the eluates as well as in the pellet. Liver homogenate, cytosol and pellet were treated with 2-mercaptoethanol (ME). The resulting clear solutions were fractionated by gel filtration followed by determination of the contents of the two metals in the eluates.

In CCP sheep the solubility of Cu containing proteins from the liver homogenate and pellet increased after incubation with ME.

The results suggest that in CCP sheep a considerable amount of Cu containing proteins are present in an insoluble form, which to some extent is resolubilized by ME.

chronic copper poisoning; sheep; Cu and Zn metallothionine; mercaptoethanol; gel-filtration.

Chronic copper poisoning is a wellknown problem in sheep production throughout the world. The morbidity in affected herds may approach 5 %, but the mortality is high and can exceed 75 % (*Bostwich* 1982). During a first phase the animal accumulates copper in the liver without showing clinical signs of toxicity and without a detectable increase of Cu in blood.

Later an acute phase, the hemolytic crisis, occurs characterized by release of high amounts of copper from the liver into

the blood causing hemolysis and clinical manifestations of CCP. The hemolytic crisis is considered to be induced by some stress, e.g. parturition, starvation, heat or disease.

In CCP sheep, large amounts of Cu have been found attached to metallothionein (MT) (*Norheim* 1980). MT is a low molecular weight protein, first isolated from equine renal cortex (*Margoshes et al.* 1957) and later also from liver and kidney of a wide variety of animal species. It contains about 30 % cysteine residue while aromatic amino acids are absent (*Margoshes et al.* 1957). The synthesis of MT in the liver can be induced by heavy metals, e.g. Cd, Zn, Cu and Hg. A role for MT in detoxification of these metals has been suggested (*Foulkes* 1982). This protein also plays a fundamental role in Zn homeostatis (*Bremner & Davies* 1974).

Isolation and purification of MT from rat liver and horse kidney result to some extent in oxidation and a subsequent alteration of metal distribution pattern as shown by *Minkel et al.* (1980). These authors also demonstrated that the addition of ME to the liver homogenate prevented oxidation or the altered metal distribution of an oxidized sample could be restored.

The present study was undertaken to investigate the effect of ME on the content and relative distribution of Cu and Zn in both cytosol and pellet fractions of livers from normal and CCP sheep.

MATERIAL AND METHODS

Samples of livers were obtained from 4 (nos. 1—4) apparently normal slaughtered sheep and from 3 sheep (nos. 5—7) which died in a hemolytic crisis due to CCP. All liver samples were kept at —70°C until analysed. Copper and zinc contents in the livers were analysed by atomic absorption spectrophotometry (AAS) on a model 303 Perkin-Elmer (Perkin-Elmer, USA) after automated wet ashing using concentrated nitric acid and perchloric acid (7:3 v/v) in accordance with a method described previously (*Frank* 1976).

Fifteen g of each liver was homogenized in 30 ml of tris-buffer (0.01 mol/l tris-HCl 1 % butanol-1 buffer, pH 8.0) using an Ultra Turrax® type 18/10 homogenizer (Jankel & Kunkel, W. Germany) for 1 min. A second homogenization was performed using a B. Braun type 853 202 homogenizer (B. Braun AG. W. Germany) at 1400 r.p.m. for 1 min. The preparations were made

at room temperature (≈20°C). The homogenate was divided into 3 portions of 10 g each. Separation of subcellular particles from the cytosol was carried out in a Beckman ultracentrifuge model L8-85 (Beckman Instruments Inc., USA) at 85 000 g ($r_{av.} = 61$) for 70 min. at 4°C using rotor type 70.1 Ti. Five ml of the recovered cytosol was fractionated by gel filtration as described by *Bremner & Marshall* (1974) with slight modifications. A column (720×26 mm) was packed with Sephadex G 75 fine (Pharmacia Fine Chemicals, Sweden) in tris-buffer. This buffer was also used to elute the columns at a flow rate of 10 ml/h. Eluates of 5 ml volume were collected using an LKB Ultrorac® 7000 fraction collector (LKB-Produkter AB, Sweden). The ultraviolet absorption of the eluate was recorded continuously at 280 nm with an LKB UVI-Cord II Ultraviolet Absorptiometer type 8303 A. Each of these 5 ml eluates were directly aspirated into AAS for determination of Cu and Zn. The contents of these two metals in each eluate were added to obtain the total soluble amount of the respective metals.

Copper and zinc contents of the pellet were determined by AAS after wet digestion.

The second portion of the homogenate was ultracentrifuged as above. Five ml of the cytosol was treated with 2-mercaptoethanol (Carl Roth, W. Germany) at a concentration of 0.1 mol/l for 72 h at ≈5°C. Thereafter, the cytosol was processed as described above for non-ME-treated cytosol. The pellet was resuspended in tris-buffer to the original volume and treated with ME as above. After the incubation the mixture was ultracentrifuged and 5 ml of the supernatant was gel-filtrated and Cu and Zn were determined in the eluates as described for the cytosol.

The third portion of the homogenate was treated with ME. After separation of the sub-cellular particles, Cu and Zn were determined both in the cytosol (after gel-filtration) and in the pellet.

The column was calibrated with blue dextran (MW 200000), ovalbumin (MW 43000), chymotrypsinogen A (MW 25000), ribonuclease A (MW 13700) and insulin (MW 6200) for determination of the molecular weight of the eluated proteins.

A paired t-test was performed to compare the obtained results.

RESULTS

The molecular weight of fraction III was found to be approximately 8000 daltons. This fraction had low absorbance at 280 nm and contained high amounts of Cu and Zn.

Total Cu and Zn contents of livers from normal sheep are shown in Table 1 and for CCP sheep in Table 2. Contents of Cu and Zn in the eluates were combined as described by *Bremner & Marshall* (1974) to make 4 pooled fractions. The fractions were separated from each other by cutting the combined eluates at fixed elution volumes. The tables also show the relative distribution of soluble Cu and Zn containing proteins expressed as a percentage distribution of soluble metal among the four fractions. The soluble Cu containing proteins of the cytosol from normal sheep were distributed in fractions I, II and III independently of ME treatment. Similarly, regardless of ME treatment, Zn containing proteins were present in all 4 cytosol fractions (I—IV), in 2 of the liver samples (nos. 1 and 2), while the other 2 (nos. 3 and 4) had no Zn in fraction III.

Figures 1 (a-d) and 2 (a-d) show the distribution pattern of soluble Cu and Zn containing protein in cytosol (a), ME-treated cytosol (b), supernatant from ME-treated pellet (c) and cytosol from ME-treated homogenate (d) of liver samples from animals no. 1 and 7, respectively. No appreciable difference in the pattern between ME-treated and non-ME-treated cytosol was found (a and b in Figs. 1 and 2).

As shown in Table 2, soluble Cu containing proteins in the ME-treated cytosol samples from CCP sheep were eluted in all 4 fractions. In the non-ME-treated cytosol, only 1 of the animals (no. 7) had Cu containing proteins in all 4 fractions. In 2 of the animals (nos. 6 and 7), most of the Cu was found in fraction III. Zinc was distributed in fractions I, II and IV, with no or only small amounts in fraction III.

When the pellet or homogenate from liver specimens of both normal and CCP sheep were incubated with ME, the amount of extractable Cu containing proteins increased in the supernatant, especially in the CCP sheep ($P<0.05$). When pellets were treated with ME, the yield of Cu in the supernatant increased from a mean of 16 % soluble of the total amount to 30 % in normal sheep. In CCP sheep, the soluble amount increase from 30 % of the total to 60 % after ME treatment. The increase in soluble Cu

Table 1. The relative distribution of soluble copper and zinc in sample from normal sheep. The Cu and Zn contents in the four fractions are given as a percentage of the content for the sum of eluates.

Sheep no.		Total µg/g liver	Cytosol					Cytosol + ME					Pellet + ME supernatant					Homogenate + ME cytosol				
			Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV
1	Cu	28.8	15.6	8	18	74	0	22.0	30	24	46	0	4.6	54	0	46	0	23.0	27	20	53	0
	Zn	39.6	21.4	45	25	13	17	23.8	43	24	12	21	6.3	31	18	0	51	31.7	43	23	7	27
2	Cu	65.9	40.2	43	15	42	0	46.8	49	15	36	0	9.9	88	12	0	0	44.8	40	20	40	0
	Zn	30.8	15.4	45	25	13	17	16.0	43	24	12	21	5.2	31	18	0	51	23.1	43	23	7	27
3	Cu	82.1	34.5	57	33	10	0	25.5	71	29	0	0	26.3	49	29	14	8	36.1	61	26	14	0
	Zn	37.0	25.9	44	37	0	19	25.9	51	33	0	16	5.9	52	3	0	45	25.9	44	32	0	24
4	Cu	44.6	34.8	44	36	20	0	26.8	50	26	24	0	9.8	79	16	5	0	31.2	42	31	27	0
	Zn	31.8	16.5	53	31	0	16	19.1	54	31	0	15	5.7	53	3	0	44	22.9	51	29	0	20

Table 2. The relative distribution of soluble copper and zinc in samples from chronic copper poisoned sheep. The Cu and Zn contents in the four fractions are given as a percentage of the content for the sum of eluates.

Sheep no.		Total µg/g liver	Cytosol					Cytosol + ME					Pellet + ME supernatant					Homogenate + ME cytosol				
			Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV	Sum of eluates µg	Fractions % I	II	III	IV
5	Cu	212	61.5	54	11	35	0	61.5	43	18	33	6	140	25	12	38	25	204	28	20	37	15
	Zn	15.8	4.7	66	32	1	2	4.7	36	20	5	38	5.5	31	1	0	69	12.6	24	16	17	42
6	Cu	217	126	35	6	58	1	132	31	13	47	9	65.1	19	7	54	20	211	27	15	50	8
	Zn	26.6	9.3	65	14	1	20	11.2	49	14	2	35	3.7	35	4	1	59	23.9	28	10	30	31
7	Cu	485	180	27	9	43	20	184	25	14	43	18	291	13	5	52	30	407	18	13	49	20
	Zn	19.3	5.8	41	30	0	29	6.2	26	21	1	52	3.9	19	1	0	80	13.5	18	15	3	64

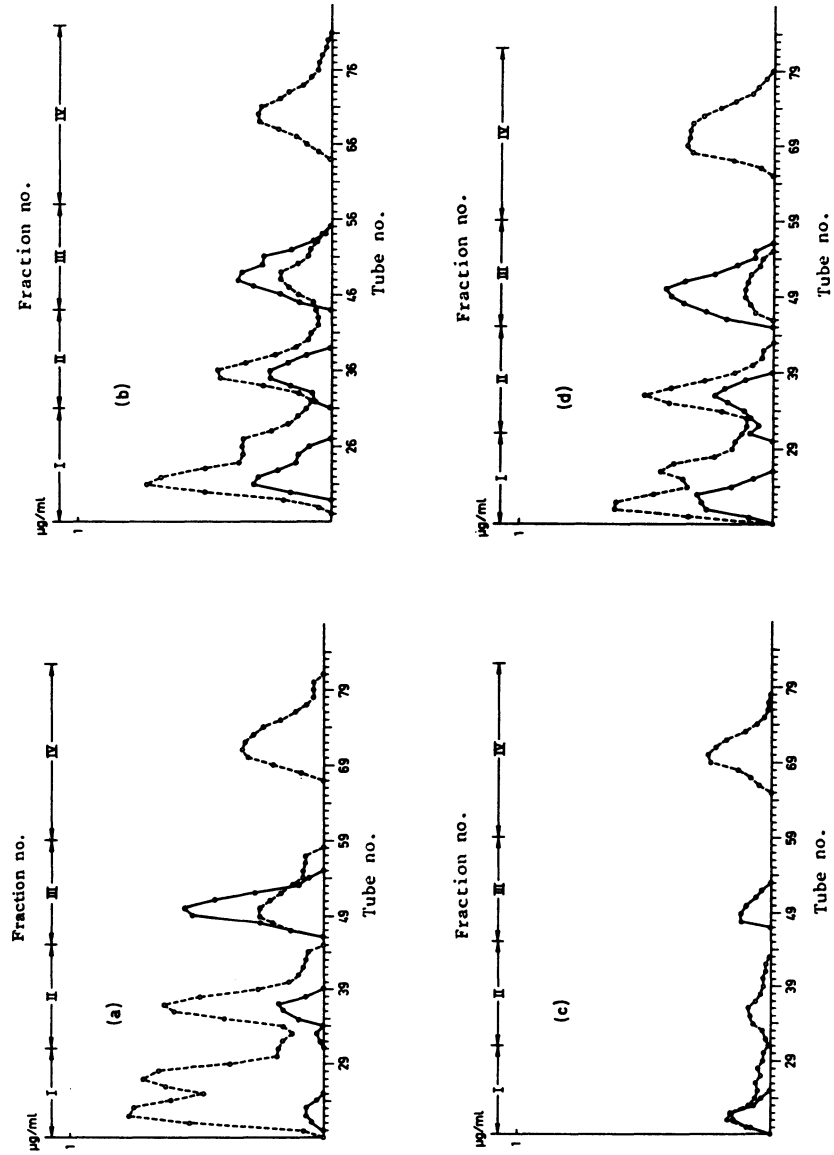

Figure 1. The distribution pattern of soluble Cu and Zn containing protein from liver sample of normal sheep.

Cu —●—●— Zn -o--o-

a) Cytosol
b) ME treated cytosol
c) Supernatant from ME treated pellet
d) Cytosol from ME treated homogenate

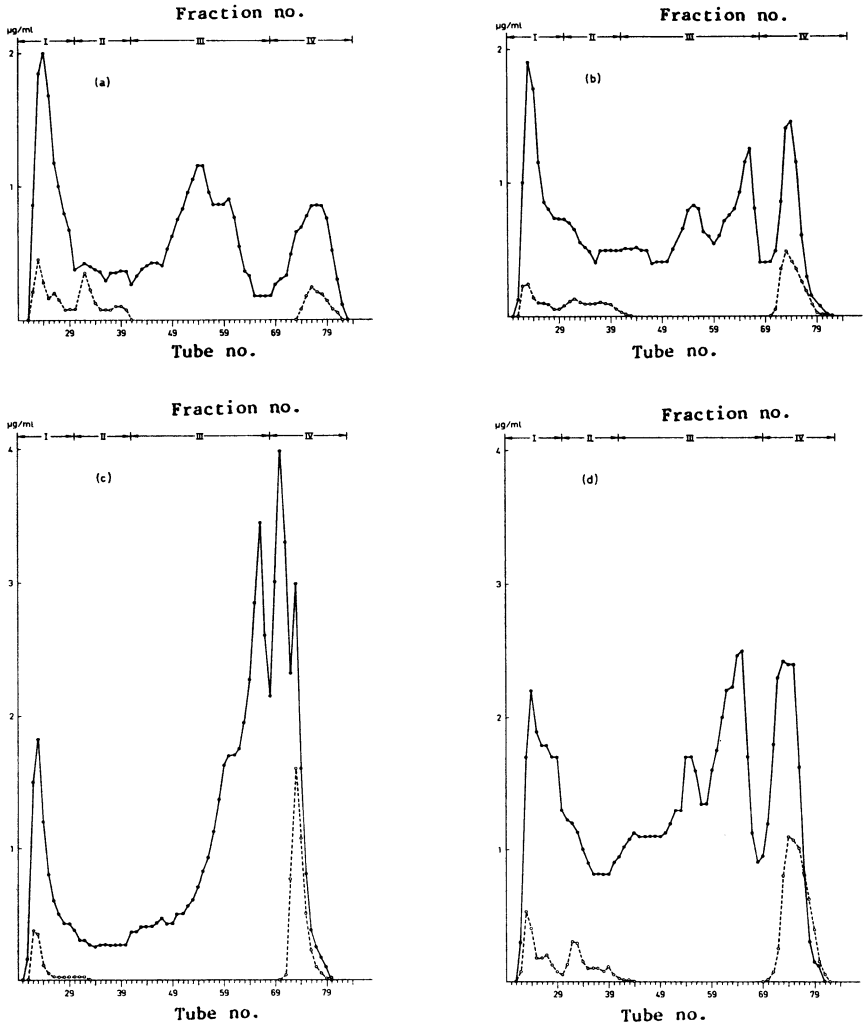

Figure 2. The distribution pattern of soluble Cu and Zn containing protein from liver sample of chronic copper poisoned sheep.

Cu —●—●— Zn -o--o-

a) Cytosol
b) ME treated cytosol
c) Supernatant from ME treated pellet
d) Cytosol from ME treated homogenate

containing proteins as the result of ME treatment of the pellet or homogenate in normal and CCP sheep is visualized in Figs. 1c, d and 2c, d, respectively.

DISCUSSION

The low absorbance at 280 nm of fraction III, its molecular weight of about 8000 daltons and the high Cu and Zn content are characteristics of metallothioneins (*Nordberg et al.* 1978). The low absorbance at 280 nm is due to absence of aromatic amino-acids in these proteins. Aminoacid analysis was not performed in this case, but we consider the observed characteristics for metallothioneins sufficient to term fraction III metallothioneins.

The presence of soluble Cu containing proteins in fraction IV of cytosol from livers of CCP sheep but not in normal animals agrees with previous observations (*Norheim* 1980). According to *Norheim* (1980), the low Zn content in fraction III of CCP sheep could be due to a competition with copper for the same binding sites on the soluble proteins in this fraction. However, in the present study only 2 of the normal sheep (nos. 1 and 2) had appreciable amounts of Zn in fraction III. The 2 animals with no Zn in fraction III had low total Cu content in their livers and no Cu could be detected in fraction IV in these animals.

The present results show that the addition of ME to the pellet or the homogenate of liver samples from CCP sheep increases the amount of Cu containing soluble proteins which can be recovered in the supernatant or the cytosol. Such an effect was also observed by *Porter* (1968), who incubated samples from livers of newborn calves with ME. Since ME is a reducing agent, its effect could be attributed to an increase in the amount of monovalent Cu with a subsequent decrease in divalent Cu. This is supported by the observation by *Suzuki & Maitani* (1981) that bivalent Cu has to be reduced to monovalent in order to be incorporated into the soluble metallothionein. It has also been shown (*Geller et al.* 1982) that oxidation of cystein in Cu-thionein from rat liver reduces its affinity for Cu. Another possibility is the ME acts by converting a structurally changed and insoluble MT to its soluble form, e.g. by depolarization of polymerized MT. According to *Corbett e al.* (1978), the highest proportion of Cu is stored in the liver cytosol fraction of pre-hemolytic copper-loaded sheep, but *Gooneratne et al.* (1979) found the highest proportion of Cu stored in the nuclear fraction. The present results agree with the

findings of *Gooneratne et al.* and, according to our results, the relative increase in Cu recovered from the soluble fraction following addition of ME, represents Cu released from the subcellular fraction after reduction by ME and conversion of structurally changed MT to a soluble form.

However, ME treatment of cytosol from livers of normal or CCP sheep did not appreciably change the relative distribution of Cu or Zn containing proteins. *Minkel et al.* (1980) have shown that in oxidized liver cytosol from rats, Cd, Cu and Zn moved from the metallothionein to higher and lower molecular weight proteins and that the addition of ME to the oxidized cytosol restored the original distribution pattern. In the present study, changes in the distribution pattern of Cu containing proteins were observed when the subcellular particles, either as resuspended pellet or as liver homogenate, were incubated with ME. The changes were more pronounced in CCP than in normal sheep. Treatment with ME seems to restore the solubility of Cu containing proteins changed by the altered redox potential in the hepatocytes during the hemolytic crisis.

An alteration of redox potential has been observed as a decrease in the amount of glutathion in blood just prior to and during the hemolytic crisis (*Todd & Thompson* 1963, *Todd* 1969, *Søli et al.* 1977).

Copper associated with the lysosome fraction of rat liver hepatocytes induces membrane changes and subsequent cell damage (*Lindquist* 1968). In CCP sheep, liver cell damage is evidenced by e.g. elevated blood plasma levels of ASAT and Cu, which are known manifestations of the hemolytic crisis (*Buckley et al.* 1981).

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SAMMANFATTNING

Effekten av 2-merkaptoetanol på lösligheten hos koppar- och zinkhaltiga proteiner i leverprover från normala och kroniskt kopparförgiftade får.

Lösligheten hos Cu -och Zn-bindande proteiner undersöktes i leverprover från kliniskt friska får och får som dött i kronisk kopparförgiftning (KKF). Homogeniserade leverprover delades med ultracentrifugering i cytosol och pellet. Cu och Zn bestämdes i cytosolen efter gelfiltrering och i pelletten. Leverhomogenat, cytosol och pellet behandlades var för sig med 2-merkaptoetanol (ME). Den klara supernatanten gelfiltrerades, och de två metallerna bestämdes i eluatet.

Vid KKF ökade lösligheten hos Cu-haltiga proteiner i homogenat och pellet efter behandling med ME.

Resultaten visar att hos får med KKF befinner sig en betydande del av de normalt lösliga Cu-haltiga proteinerna i en olöslig form, men de blir åter lösliga vid behandling med ME.

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Reprints may be requested from: B.-E. V. Jones, the Department of Clinical Chemistry, Box 7038, S-750 07 Uppsala, Sweden.