High Molecular Weight Zinc- and Copper-binding Proteins in the Liver of the Rat; Effect of Centrifugation

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Abstract

After intravenous injection of ^{65}Zn or ^{64}Cu in the rat, the presence of high molecular weight Zn- and Cu-binding proteins in the cytosol of the liver was investigated, using gel chromatography over Sephacryl S-300. About 10% of the ^{65}Zn was found in the void volume of the column, *viz.*, in a fraction with a molecular weight of >500 000 D. A similar percentage of the cytoplasmic ^{64}Cu turned out to be associated with these proteins. Little change was seen in this distribution during the time interval of 0.25 to 8 h post injection. About the same percentage of non-radioactive Zn and Cu was found in this fraction. This high molecular weight fraction was sedimented when the liver homogenate was centrifuged for a prolonged time and/or at high *g*-value. It is different from the high molecular weight artifacts produced by, e.g., oxidation. The results stress the need for clearly specifying the conditions of centrifugation in descriptions of experiments.

Introduction

The study of Cu-binding proteins has concentrated on metallothionein and superoxide dismutase, particularly in the liver. The former is a protein with a molecular weight of about 6000 D, which can bind various trace metals [1]; the latter is an enzyme involved in the detoxification of the superoxide radical and has a molecular weight of about 30 000 D [2]. Both proteins are commonly observed in cytosol by gel chromatography, mostly using Sephadex G-75. Usually, still another Cu-binding fraction is observed, *viz.*, one in the void volume of the column. This fraction is generally ignored or only mentioned in passing. Minkel *et al.* [3] believed this to be an artifact, produced by oxidation of Cu-containing metallothionein. However, Fischer *et al.* [4] studied this protein in the wall of the gut of the rat as function of time and suggested that it has a physiological role.

A similar situation exists for Zn. Both proteins mentioned above also bind Zn. However, in contrast to the case of Cu, gel chromatography usually reveals the presence of several other Zn-binding proteins [e.g., 5] which are, nevertheless, largely ignored. But, as in the case of Cu, a Zn-binding fraction is also found in the void volume.

The presence of such a high molecular weight Zn- and Cu-binding fraction in the cytosol of the liver of the rat was investigated and the influence of the conditions during centrifugation on the results of the gel chromatography studied.

Experimental

Materials and Methods

Animals

Random-bred male Wistar rats of 200 ± 25 g were obtained from the Central Institute for the Breeding of Laboratory Animals-TNO (Zeist, The Netherlands). They were housed in Macrolon(R) cages, equipped with stainless steel lids and drinking nipples, and were fed a commercial diet (SMRA; Hope Farms, Woerden, The Netherlands) and demineralized water *ad libitum*.

Radioactive compounds

Carrier-free ^{65}Zn was obtained from the Radiochemical Centre (Amersham, UK). ^{64}Cu was prepared in the reactor of the Interuniversity Reactor Institute (Delft, The Netherlands) by irradiating copper metal with thermal neutron flux of 10^{13} s^{-1} cm^{-2} for 12 h. The specific activity at the end of irradiation was about 4 mCi/mg Cu).

Analytical procedures

Total Zn and Cu were determined with graphite furnace-AAS and flame-AAS, respectively (Perkin

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Elmer; model 2380) Radioactivity was determined with a well-type NaI scintillation crystal with automatic sample changer.

Design of the experiments

After an overnight fast the animals were injected intravenously with 10 μg Zn (containing 20 μCi ^{65}Zn) or 500 μg Cu (containing 1.5 mCi ^{64}Cu) in 0.5 ml 50 mM Na–acetate buffer (pH 5.6, containing 120 mM NaCl). The animals were decapitated at various intervals post injection. Their livers were homogenized at 0 °C with three parts (v/w) of 10 mM TRIS–acetate buffer (pH 7.4, containing 100 mM NaCl) using a Potter-Elvehjem apparatus. The homogenates were centrifuged (Beckman model L3-40; rotor #40) during the periods and at the *g*-values indicated.

After centrifugation, samples of about 2 ml of the particle-free supernatant were passed over a Sephacryl S-300 column (1.4 × 52 cm; Pharmacia), in combination with a Chelex-100 pre-column (1.4 × 2.5 cm; Bio-Rad), both equilibrated and eluted with 10 mM TRIS–acetate buffer (pH 7.4, containing 100 mM NaCl). The eluate was collected in fractions of 1.5 ml. Absorption of the eluted material was measured at 254 nm, using a Uvicord UV-monitor (LKB, Bromma, Sweden).

A sample of the particle-free supernatant was tested for reducing substances as follows: in a spectrophotometer cell (1 cm light path) were pipetted 2.0 ml biquinoline (0.025% in 25% acetic acid), 0.05 ml Cu-solution (0.1% $CuSO_4 \cdot 5H_2O$ in water) and 0.05 ml sample. After mixing, the A_{540} (= absorbance at 540 nm) was read as function of time. A cell containing 25% acetic acid instead of biquinoline solution served as a blanc. The ΔA_{540} of the reaction product of Cu(I) and biquinoline is caused by reduction of Cu(II) to Cu(I) by reducing substances in the sample and to a small extent by Cu(I), already present in the sample. A final ΔA_{540} = 0.1 corresponds to an amount of reducing substances per ml sample capable of reducing 0.066 μmol Cu(II) under the conditions employed.

Results

After intravenous injection of ^{65}Zn in rats, a substantial portion of the dose was found to be accumulated in the liver (Table I). This percentage showed a maximum at about 30 min post injection. The percentage of the liver- ^{65}Zn recovered in the cytosol, *i.e.*, in the particle-free supernatant after centrifugation, depended on the time and *g*-value employed. When spun for 2 h at 120 000 × *g* about 75% of the ^{65}Zn was found in the cytosol. By selecting a shorter time and a lower *g*-value (15 min; 85 000 × *g*) and taking care that only clear supernatant was withdrawn, about 10% more was recovered in the cytosol.

TABLE I. Percentage of Radioactive and Stable Zn and Cu in the Cytosol of the Liver of Rats as Function of Time after Intravenous Injection of the Copper or Zinc

Time post injection (min)	% of dose in total liver	(cytosol/homogenate) × 100			
		15 min; 85 000 × *g*		2 h; 120 000 × *g*	
	^{65}Zn	^{65}Zn	Zn	^{65}Zn	Zn
15	26	87	92	76	77
30	29	84	–	–	–
60	30	83	–	–	–
120	27	83	–	–	–
240	23	82	–	–	–
480	24	82	81	73	73
	^{64}Cu	^{64}Cu	Cu	^{64}Cu	Cu
15	–	94	–	91	–
240	55	84	–	–	–
480	14	89	85	88	76

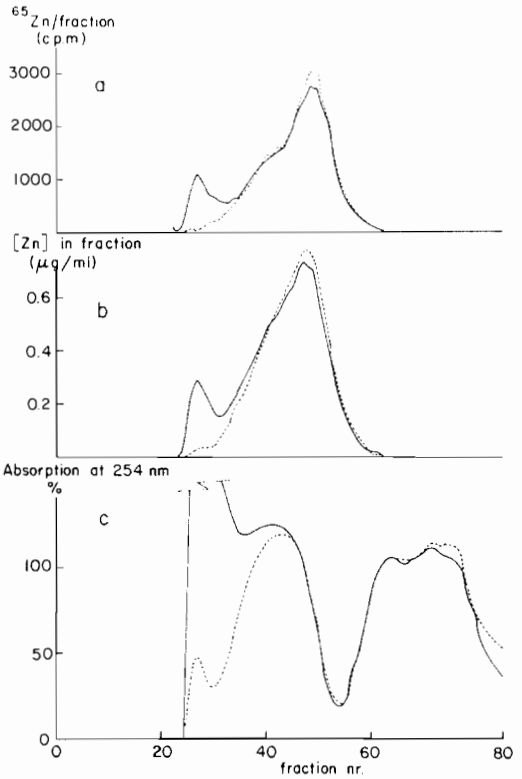

Fig. 1. Distribution over the eluate of a Sephacryl S-300 column of ^{65}Zn (a), Zn (b) and material absorbing at 254 nm (c). The sample was 2 ml of the liver cytosol of a rat, sacrificed 8 h after injection of ^{65}Zn. Full line: cytosol obtained by centrifugation for 15 min at 85 000 × *g*; broken line: cytosol obtained by centrifugation for 2 h at 120 000 × *g*.

When instead of ^{65}Zn the total (stable) Zn was measured in the homogenate, similar results were obtained (Table I).

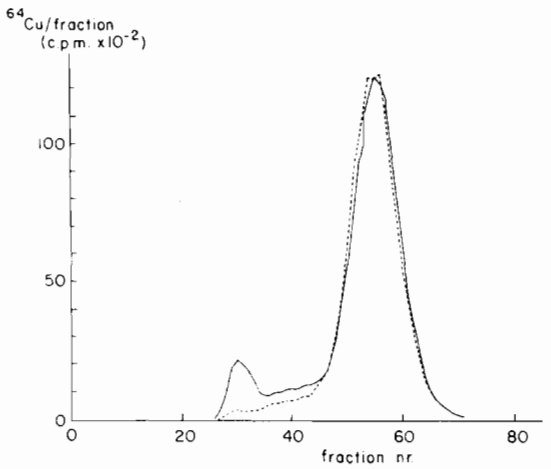

Fig. 2. Distribution over the eluate of a Sephacryl S-300 column of ^{64}Cu. Rat sacrificed after injection of ^{64}Cu. Other details as in Fig. 1.

The influence of the conditions of centrifugation was further illustrated by the change in distribution over the eluate of ^{65}Zn, total Zn and material absorbing at 254 nm, when the supernatant was passed over a Sephacryl S-300 column (Fig. 1a, 1b and 1c, respectively). After centrifugation at 85 000 $\times g$ for 15 min, a fraction containing both ^{65}Zn and total Zn was found in the 'void volume'. This fraction, which contained about 10% of the ^{65}Zn of the cytosol, was virtually absent after more extensive centrifugation. Its molecular weight was estimated to be at least 500 000 D.

Similar results were seen after intravenous injection of ^{64}Cu (Fig. 2).

The presence of reducing substances in the cytosol was independent of the conditions of centrifugation and amounted to an equivalent of $\geqslant 3.2$ μmol Cu(II) per g liver.

Discussion

At present two well-defined Zn-containing proteins, *viz.*, metallothionein and superoxide dismutase, have been reported in the literature, as well as several others which have been recognized but not further investigated [e.g., 5]. Our results stress the presence of one or more Zn-containing materials of high (>500 000 D) molecular weight. Because this fraction was eluted in the void volume of the column, it may be comprised of more than one Zn-binder, despite its well-defined, narrow shape. Because this fraction exhibits its maximum ^{65}Zn content as early as 15 min post injection, it probably acquires its Zn through binding rather than through incorporation during the biosynthesis of this material. At present, nothing can be said about its chemical composition: it could be a protein, a nucleic acid or still another cellular material.

Although the Cu- and Zn-binding fraction in the void volume is probably a mixture of species, it deserves a closer look, particularly because Fischer *et al.* [4] showed that *in vitro* more Cu was located in a similar void volume fraction of the intestinal mucosa when the rat had been fed a ratio low in Zn and duodenal segments were then exposed *in vitro* to elevated levels, e.g., 4 μg/ml, of Cu.

The high molecular weight Zn- and Cu-binding material must exist before being passed over the column, since it can be removed by ultracentrifugation. It is unlikely that it is an artifact produced by polymerization or aggregation of, e.g., metallothionein, because: (a) testing with biquinoline (see Experimental) showed that after centrifugation the sample still contains substantial amounts of reducing substances, believed to be mainly cysteine and glutathione, which certainly would have protected metallothionein and other SH-containing proteins from oxidation: (b) the ionic strength of the TRIS–buffer used excludes electrostatic interaction between proteins; and finally (c) the absence of a fixed ratio between the amount of Cu found in the void volume and in the metallothionein, as observed by Fischer *et al.* [4], also argues against the possibility that in their experiments metallothionein was polymerized.

Minkel *et al.* [3] described a shift of Zn and Cu from low (appr. 10 000 D) to high molecular weight chromatographic fractions. However, this shift was brought about by deliberate use of oxidizing conditions. Since this situation does not apply to our experiments, we must conclude that, apart from the danger of producing high molecular weight artifacts by oxidation, true ligands of Cu and Zn do exist in the void volume fractions. The high molecular weight Zn- and Cu-containing material is therefore almost certainly a true physiological entity which deserves more attention than it has attracted until now.

As shown in Fig. 1 (a and b), the high molecular weight material, containing both Zn and Cu, can be sedimented when the homogenate is centrifuged for a long time and/or at high *g*-values. The described results therefore also stress the need for clearly specifying and, when possible, standardizing the conditions of centrifugation.

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