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The Decrease of Acute Toxicity of Ethanol by Means of Zn-Metallothionein Preparation

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Metallothioneins (MT) are low-molecular cytoplasmic proteins which are able to bind selectively anions of heavy metals and contain up to 30% cysteine. MT synthesis induction results from the effect of toxic factors [3, 8, 11]. These substances have been proved to increase the organism's resistance to metals and to in the metabolic regulation of Zn and Cu levels [8]. The known increase of endogenous MT content in the liver of rodents influenced by low-molecular alcohols [3, 11] is considered to be a nonspecific response which leads to a decrease of the toxic effect of these compounds. There are few data about the effect of exogenous MT on the toxicity of both inorganic and organic substances. Thus, it was demonstrated that the preliminary injection of Zn-MT reduces the harmful influence of Cd-MT in rats [14].

In the present study it was demonstrated that exogenous Zn-MT is capable of reducing the acute toxicity of organic compounds, for example, that of ethanol.

MATERIAL AND METHODS

Mice ($(CBAxC_{57}Bl)F_1$ (25-30 g weight) and nonpedigree albino rats (300-400 g weight) were used in the experiments. All solutions were injected intraperitoneally. To induce MT synthesis 20 rats were injected with $ZnCl_2$ solution (10 mg of Zn ions per kg) and killed after 24 hours; the liver was extracted and washed with physiological solution. The tissue mass was mixed with 2.5 volumes of 10 mM Tris-HCl buffer at pH 7.4 ("standard buffer"), made homogeneous and centrifuged twice for 30 min at 15 000 g. The supernatant was heated for two minutes at 80°C and centrifuged under the same conditions. Cytoplasmic extract was concentrated by the ultrafiltration method up to 40 ml of volume and loaded onto a Sephadex G-75 column (3.2x95 cm) (Pharmacia, Sweden). The column was equilibrated and eluted with standard buffer. Protein concentration [10] and MT content using the cadmium-Hb method (from the quantity of labeled Cd bound) [5] were determined in the fractions. ^{109}Cd with a specific radioactivity of 11.3 GBq per mg (Izotop) was used. Absorption spectra of Zn-MT were registered in standard buffer. Electrophoresis under denaturation conditions was performed as previously described [9] on Pharmacia-LKB-2011 apparatus (LKB, Sweden) on polyacrylamide gel (1 mm thickness of layer) in a concentration gradient from 4 to 20%. Protein molecular mass standards

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(Pharmacia, Sweden) were used. The acute toxicity ED_{50} of ethanol was determined according to Kerber [1]. Mice were injected with Zn-MT solutions in 0.2-0.3 ml of standard buffer and, after 10 minutes, with ethanol (32% rectificed spirit in physiological solution). One control group of animals was injected with the appropriate volume of standard buffer before the ethanol injection, and the other group received a mixture of human serum albumin solution (Reanal, Hungary), HCl-cysteine (Reanal), and $ZnCl_2$. The pH of the mixture was brought to 7-8.

RESULTS

In the gel-filtration experiments four protein peaks were observed, two of which were in the range of relatively high molecular weights (more than 40 kD). As shown from the results of labeled-Cd binding experiments MT were located in a narrow range which corresponded to a molecular weight interval of 6.5-8 kD. Other eluted proteins did not bind Cd. In fact, metal-binding proteins that are not MT have been proved by other authors [6] to be thermolabile and denature during the thermopurification of the extract [6]. From the electrophoresis data (Fig. 1) the MT preparation obtained was found to be satisfactorily homogeneous. MT molecular weight was 7.2 kD, which is in accordance with published data [1, 6]. Therefore, besides its thermostability, the preparation obtained meets the requirements for MT [6, 8]. This protein is really Zn-MT, as it was extracted after Zn-salt injection, and the UV spectrum (Fig. 2) was analogous to that of Zn-MT described earlier [7].

The mean ED_{50} of ethanol was 5.79±0.15 g/kg. As shown from Fig. 3, the preliminary Zn-MT injection reduces the acute toxicity of ethanol. Although the relative ED_{50} increment was not large (about 20%, Fig. 3,b), it was reproduced in various experiments, and, moreover, a directly proportional dependence of the effect on the MT dose was observed up to 2 mg/kg; after which a certain saturation was detected (Fig. 3,a). Since Zn and cysteine from the MT composition (MT contains up to 30% cysteine) are biologically active compounds themselves, we performed a series of control experiments. Mice were injected with a mixture of 70% albumin, 30% cysteine, and 5,5% $ZnCl_2$ (from the total quantity of the first two). Since MT are able to bind up to 7 g-atoms of metal per g-mol [5, 7, 8] the calculations indicated that at complete saturation Zn-MT contains precisely 5.5% Zn [14]. No discernible changes in ED_{50} of ethanol influenced by the injection of the mixture described above at a dose corresponding to the MT dose of 3 mg/kg (2.1, 0.9, and 0.165 mg/kg for albumin, cysteine, and Zn, respectively) were detected. Therefore, the Zn-MT effect cannot be explained only by the influence of its constituents. The mechanism of this action is not yet clear. Only three propositions can be made, which we shall consider in the order of probability:

1) Zn from the Zn-MT activates alcohol dehydrogenase, which is a Zn-dependent enzyme [13] more than does free metal. Indeed, during *in vitro* experiments purified Zn-MT was found to be a Zn-donor for a variety of enzymes but not alcohol

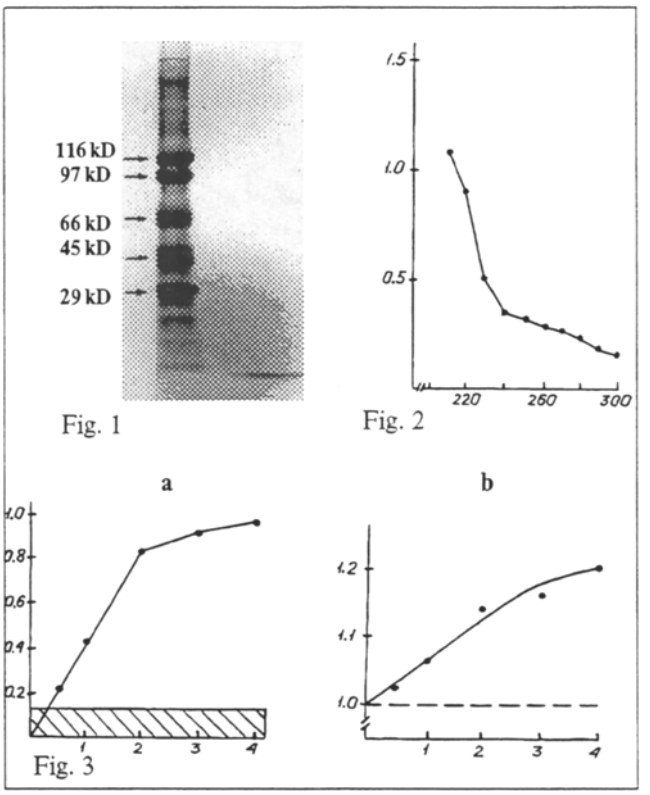

Fig. 1. Electrophoresis under denaturation conditions of protein standards (1) and Zn-MT preparation (2). Molecular weight values of protein standards are indicated by arrows.

Fig. 2. UV absorption spectrum of Zn-MT preparation.
Abscissa: wavelength (nm); ordinate: absorption (relative units).

Fig. 3. Increment of ED_{50} of ethanol after preliminary Zn-MT injection in mice (mg/kg).
Abscissa: Zn-MT dose (mg/kg); ordinate: a) absolute increment of ED_{50} (g/kg); b) relative increment of ED_{50} (ratio of ED_{50} after preliminary Zn-MT injection to ED_{50} without injection); each point plotted corresponds to the result of an independent experiment.

dehydrogenase. Maybe, however, *in vivo* the alcohol dehydrogenase activation in tissues due to MT injection nevertheless occurs.

2) A number of chemical substances (among them alcohols) are known to reduce the Zn level in tissues and blood plasma by means of the utilization of the metal in the liver, giving rise to MT synthesis [3, 11]. Exogenous Zn-MT can be expected to make up for the insufficiency in Zn level, leading to a certain stabilization of its metabolism and, eventually, to an increase in the resistance to toxic influences. The calculations show that

the injection of Zn-MT at a dose of 2 mg/kg corresponds to that of the metal at a dose of 0.11 mg/kg. The Zn basal level in mouse plasma was found to be 1-1.1 μg/ml [11]. If the plasma content per mouse does not exceed 2 ml, the Zn basal level in mouse plasma has been estimated to be 0.08-0.088 mg/kg for a 25 g mouse. This is comparable to the amount of Zn injected with MT. However, the free Zn injection in the control mixture caused no changes in ED_{50} of ethanol. Probably, the Zn from the MT is more biologically active than in the free state (for instance, owing to differences in clearance).

3) It was shown that in alcohols intoxication changes in the antioxidant protection enzyme activity and a drop in the reduced glutathione content result in an increase in the level of lipid peroxidation [2, 4]. Since MT are able to reduce the latter process [12] and contain up to 30% cysteine [8], the protective effect of these proteins can be associated both with the stabilization of lipid peroxidation in the liver and with the replenishment of the pool of thiol groups. The thiol groups in the MT molecule appear to be far more biologically active than in free cysteine.

The investigation of changes in the toxicity of chemical substances brought about by the Zn-MT preparation may be of certain practical interest. Indeed, the Zn-MT active dose (2 mg/kg) is many times lower than its toxic dose (in the experiments in rats Zn-MT was nontoxic up to the extreme dose tested of 43 mg/kg) [14].

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PHARMACOLOGY

Chronobiological Effects of Verapamil on Arterial Blood Pressure and Some Indices of Cardiac Contractility in Rabbits with Vasorenal Hypertension

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The accumulated experimental data and clinical observations clearly demonstrate [1,4,5,9] that the dynamics of various functional indices of the cardiovascular system, including arterial blood pressure, is dependent on diurnal as well as on seasonal chronobiological factors. At the same time, it is

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