also shown that the relative decrease in the mass coefficient of the thymus gland in five rats of the experimental group, expressed as a percentage of the control level, correlated closely with the P-450/GT_c ratio ($r = 0.971$, $p < 0.01$) but did not correlate with the P-450/GT_m ratio or levels of induction by P-450, GT_m, or GT_c, if these were taken separately (Fig. 3).

Thus percutaneous application of HPR, repeated 20 times, causes significant induction of the P-450 level and GT_m and GT_c activity, and has a marked toxic action on the immune and endocrine systems. Individual differences between rats of the experimental group as regards the toxic action of HPR on the thyroid gland and thumus did not correlate with levels of induction of P-450, GT_c, and GT_m, but did correlate closely with the ratio between levels of induction of these parameters in the liver.

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COMPARISON OF THE PROTECTIVE ACTION OF CERULOPLASMIN FROM HEALTHY HUMAN BLOOD AND PATIENTS WITH HEPATOCEREBRAL DEGENERATION ON ERYTHROCYTES

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Ceruloplasmin (CP) is a highly important antioxidant in human blood [5]. The protective action of CP on erythrocytes (ER), whose membranes contain large quantities of polyunsaturated fatty acids, and which are therefore very sensitive to destruction by radicals, is particularly important.

The aim of this investigation was to compare the protective action of CP from healthy human blood and ceruloplasmin-like protein (p-CP), isolated from the blood of patients with

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TABLE 1. Parameters Describing Interaction of CP and p-CP with ER and p-ER

Type of ER	CP	p-CP
ER	K_1=1.15 nm	K_1^p = 11.1 nm
	Q_1=3 pM	Q_1^p = 10 pM
p-ER	K_2=1.37 nM	K_2^p = 11.8 mM
	Q_2=21.2 pM	Q_2^p = 35.4 pM

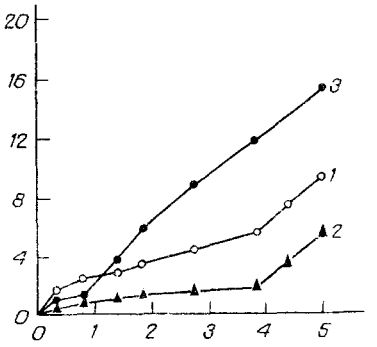

Fig. 1. Hemolysis of ER ($1.15 \cdot 10^8$ ER in 1 ml) at 37°C. Here and in Fig. 2, conditions: 1) 0.01 M Tris-HCl, 158 mM NaCl, 1 mM $CaCl_2$, pH 7.4, 0.1 mM Cu Cl_2 2) 0.01 M Tris-HCl, 158 mM NaCl, 1 mM $CaCl_2$, pH 7.4, 0.2 mg/ml CP, 0.1 mM $CuCl_2$; 3) 0.01 M Tris-HCl, 158 mM NaCl, 1 mM $CaCl_2$, pH 7.4, 0.2 mg/ml of p-CP, 0.1 mM $CuCl_2$. Abscissa, time (in h); ordinate, hemolysis (in per cent).

hepatocerebral degeneration (Wilson's disease). Since the protective action of CP includes its reception on ER [1], we studied binding of CP and p-CP with healthy human ER and ER from patients with Wilson's disease (p-ER).

EXPERIMENTAL METHOD

Commercial CP, isolated from donated blood (Bacterial Preparations Enterprise, Pasteur Research Institute of Epidemiology and Microbiology, Ministry of Health of the RSFSR) was purified by chromatography on a 20 × 2 cm column with DEAE Toyopearl ("Toyo Soda," Japan); A_{610}/A_{280} nm of the purified CP was 0.42 ± 0.44. Healthy human ER were obtained from group II blood and p-ER from freshly obtained blood from patients with Wilson's disease (WD).

The isolation of p-CP from blood plasma of patients with WD was carried out by a method of our own design, including the following stages: sedimentation with polyethylene-glycol 6000, chromatography on a column with DEAE Toyopearl (1.6 × 30 cm, NaCl gradient 0 to 0.5 M), precipitation with ethanol, chromatography on a column with Affi-Blue (1 × 6 cm, Biorad, USA), gradient pH 8.3-7.0, high performance liquid chromatography on a Mono Q 5/5 column (Pharmacia, Sweden), within the range of average pressures (NaCl gradient 0 to 0.5 M). The molecular mass of p-CP was 60 kD and electrophoresis of the labeled p-CP revealed a high degree of purity of the preparation. Immunodiffusion in 1% agar gel, using antibodies to CP, revealed identity of the CP and p-CP as regards their antigenic determinant. Labeled CP and p-CP were obtained by the method in [4]. Preparations of (^{125}I) CP and (^{125}I) p-CP, homogeneous on electrophoresis, were used. To study equilibrium binding of the ER were incubated with 0.15-3.5 mM (^{125}I) CP or with (^{125}I) p-CP. The incubation mixture contained also 1 mM $CaCl_2$. The volume of the incubated mixture was 1 ml. Nonspecific binding of the labeled preparations was determined by the addition of a 200-fold excess of unlabeled CP or p-CP. Incubation continued for 60 min until equilibrium of binding was reached. At the end of incubation the ER were sedimented by centrifugation at 8000 g for 2 min and the radio-

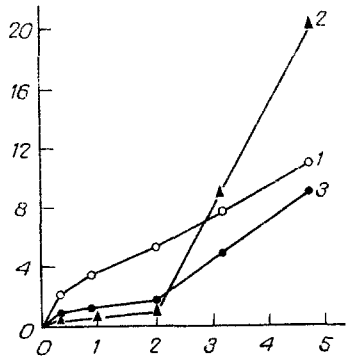

Fig. 2. Hemolysis of p-ER (1.15×10^8 p-ER in 1 ml) at 37°C.

activity of the supernatant, and of the residue washed 4 times with 1 ml of isotonic buffer solution at 4°C, was determined. The kinetics of enzymic oxidation was studied at 37°C on a Hitachi-557 spectrophotometer (Japan) in 0.05 M MES by measuring absorption at 480 nm for adrenalin, at 410 nm for pyrocatechol, and at 530 nm for p-phenylenediamine.

The kinetics of enzymic oxidation of Fe^{2+}, used in the form of Mohr's salt, was studied on an OH-105 polarograph, using a Clark's electrode (Radelkis, Hungary) at 2 pH values: 5.5 (0.5 MES) and 7.4 (0.01 M Tris-HCl, 158 mM NaCl). The process of hemolysis of ER was monitored spectrophotometrically, by measuring the optical density of the test sample at 540 nm (A 540 [3]). The percentabe of ER destroyed (percentage hemolysis) was determined as the ratio of A_{540} of the supernatant to A_{540} of the sample of completely hemolyzed ER.

EXPERIMENTAL RESULTS

We measured the kinetics of binding labeled CP and p-CP with ER and p-ER at 37°C and, having presented the data on binding as a Scatchard plot, we determined the dissociation constant of the complexes ^{125}I CP–ER (K_1), (^{125}I) p-CP–ER (K_1^p), (^{125}I) CP–p-ER (K_2), and (^{125}I) p-CP-p-ER (K_2^p).

We also determined the maximal numbers of binding sites of CP and p-CP on both types of ER (Table 1). The number of binding sites of CP on the two types of ER was appreciably less than for p-CP. Values of the binding constants of CP with ER and CP with p-ER were virtually the same.

The same rule also was observed for p-CP. This fact is evidence of the absence of changes in specific receptors on ER and p-ER which might affect interaction with CP and p-CP. However, as will be clear from Table 1, closely similar values of the dissociation constants of complexes of p-CP with both types of ER were roughly 10 times greater than the dissociation constants of the corresponding complexes (K_1 and K_2), which coincided with one another. With physiological concentrations of CP, more than three orders of magnitude greater than values of the dissociation constants found in vitro, saturation of the specific receptors of ER must be expected. In that case, the amount of p-CP bound with both types of ER must be considerably greater than the amount of bound CP (Table 1). Having determined specific binding of CP and p-CP with ER and p-ER, we went on to compare the protective action of CP and p-CP during copper-induced lysis of ER. As will be clear from Fig. 1, kinetic curves of lysis of ER in the presence of CP and p-CP (curves 2 and 3) are characterized by the presence of two regions. The first corresponds to the slower phase of lysis of ER, by the end of which less than 2% of ER are destroyed. The second region describes the significantly faster destruction of ER, in the course of which the protective action of CP and p-CP is virtually no longer exhibited. It will be clear from Fig. 1 that the duration of the slow phase of lysis in the presence of CP is 3.5 h, whereas in the presence of p-CP it was only 40 min. The degree of inhibition during the slow phase was 78 and 41% for CP and p-CP respectively.

In copper-induced lysis of p-ER (Fig. 2) the duration of the slow phase of lysis in the presence of CP was significantly shorter than during lysis of ER (curves 2 and 3), which is evidently linked with reduced activity of the protective enzymes of p-ER [2]. Just as during lysis of ER, the degree of inhibition of copper-induced destruction of p-ER in the presence of CP was somewhat higher than for p-CP (84 and 68% respectively). Thus the ability

of CP to prevent copper-induced destruction of human ER is almost three times greater than that of p-CP. It is therefore very probable that CP may be an effective means of preventing the hemolytic anemia arising in WD. To study differences in oxidase and ferroxidase activity, we studied the oxidation kinetics of adrenalin, pyrocatechol, and p-phenylenediamine, and also of Fe^{2+} in the presence of CP and of p-CP. It was found that the maximal rates of oxidation of all organic substrates in the presence of CP were much greater than in the presence of p-CP, and that the oxidation kinetics of Fe^{2+} in the presence of CP is described by a Michaelis–Menten equation with the value of $K_m = 40 \pm 4$ μM and with a high catalytic constant $K_{cat} = 32 \pm 3$ sec^{-1}, whereas p-CP does not exhibit ferroxidase activity at all. Comparative analysis of the kinetic parameters of oxidase activity of CP and p-CP, and the absence of ferroxidase activity in p-CP, and its molecular mass (60 kD) about equal to half that of CP (132 kD) suggests that p-CP is possibly half of a CP molecule, not containing high-affinity centers of catalysis of oxidation of organic substrates and of Fe^{2+}.

Despite the absence of peroxidase activity in p-CP, during lysis of ER induced by Fe^{2+} ions, the degree of inhibition for CP and p-CP was 33 and 83% respectively, i.e., p-CP was 4 times more effective than CP. During lysis of ER, induced by superoxide radicals (in a xanthine + xanthine oxidase system) the degree of inhibition for CP and for p-CP was 68 and 64% respectively, i.e., the protective agent p-CP was twice as effective as CP in this system. The degree of inhibition for CP and p-CP during lysis of ER induced by a system containing Fe^{3+} ions and superoxide radicals was 61 and 70% respectively. The greater protective effect of p-CP than of CP can perhaps be explained on the grounds that the amount of p-CP bound on the ER membrane, as has already been pointed out, was greater than the amount of CP. The results show that the protective action of CP in iron-induced lysis of ER does not correlate with ferroxidase activity. Moreover, p-CP, which exhibits much weaker oxidase activity during oxidation of organic substrates than CP, and which in general possesses no ferroxidase activity at all, has a stronger protective action in the systems tested than CP. Thus our results, obtained by direct comparison of oxidase activity and the protective action of CP, confirm the absence of direct correlation between these two factors.

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