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Mechanism of Mercurial Perturbation in Proteins

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Abstract

Kinetics of the S-mercurized horse oxyhemoglobin coagulation has been turbidimetrically studied in the presence of ligands with different affinity for the mercury(II) atom. Coordination of the mercaptide mercury with ligands results in a marked inhibition of the coagulation process, which supports the previously suggested mechanism according to which the mercaptide mercury affects the protein conformation via its additional coordination with one of the neighboring mercury-binding sites, supposedly carboxylate group of an aspartic acid residue. A new insight into the mechanism of the currently practiced mercury-intoxication therapy is discussed.

Index Entries: Hemoglobin; oxyhemoglobin; mercury; aggregation; coagulation; higher oligomers.

Introduction

Mercury and other heavy metals, especially lead and cadmium, are sadly known as protein poisons because of their specificity to bind the protein thiol sulfur *(1–3)*. Naturally, the most vulnerable functional proteins are enzymes, because of their high sensitivity to conformational integrity. However, the most abundant protein in the human and animal organism is the blood hemoglobin, which, when "poisoned," not only undergoes structural perturbations, but acts as a heavy metal transportation means throughout the organism.

Our long-term studies on the chemistry of hemoglobin led us to the idea of investigating some aspects associated with the mechanism of denaturation effect of the mercury(II) compounds by way of turbidimetrically measuring kinetics of mercurized oxy- or methemoglobin (HbO_2 or Hb^+) coagulation *(4,5)*. Hemoglobin, in many aspects, is an excellent object for this kind of investigation. First, it is a typical multichain globular protein with the perfectly documented tertiary and quaternary structures and, on

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the other hand, it possesses a prosthetic group (heme) that is very sensitive to the slightest structural alterations in the protein globule. The latter circumstance is very advantageous in the case of HbO_2 when one needs to rapidly estimate the extent of denaturing alternations in the protein, because all of them contribute to HbO_2 oxidation into Hb^+ accompanied by a visual change in the color of the protein solution. When a solution is brought into contact with excess mercuric salt, two reactive thiol groups (belonging to β 93 cysteine residue in most mammalian hemoglobins) are rapidly and nearly stoichiometrically substituted by the mercury residues. The rest of the mercury reagent interacts less selectively with other protein binding sites. The problem was investigated rather intensively in the 1960s *(6–8)* and appears to be regarded as virtually exhausted. However, our comparatively recent studies *(9–13)* have shown the mercurization of hemoglobin and its consequences to be quite an unexplored subject.

In the current work, we are focusing on the most exciting aspect of the problem: What occurs when the hydrogen atom of the reactive thiol group is substituted by the mercury atom? The studies have been fulfilled kinetically on the horse oxyhemoglobin using the turbidimetric technique exclusively.

Materials and Methods

Materials

Lyophilized horse methemoglobin (Reanal); tris(hydroxymethyl)-amino-methane (Tris) (Olaine Chemical Works) recrystallized from ethanol; mercuric acetate (Sigma); phenylmercury acetate synthesized in the laboratory, mp 149–151°C.

The initial HbO_2 solution was prepared as follows. A weighed portion of lyophilized Hb^+ was dissolved in water and the solution obtained was centrifuged to remove small amounts of undissolved materials. Approximately double molar excess of sodium dithionite was added to the centrifuged solution, and the resulting HbO_2 solution was passed through a 50-cm column with Sephadex G-25 superfine equilibrated by 0.025 *M* Tris-AcOH buffer, pH 7.2. Concentration of HbO_2 solution coming out of the column was evaluated spectrophotometrically at 576 nm wavelength and was in the range of 2 to 3×10^{-3} *M* (calculated for tetramer).

Measurement of Coagulation Kinetics

A buffer aliquot (2.85–2.90 mL) with a specified mercury salt concentration exceeding 2×10^{-4} *M* by 3–5% was thermostatically equilibrated (usually at 30°C) in a glass cuvet placed in the cuvet chamber of a SF-26 spectrophotometer, whereupon 0.10–0.15 mL (depending on concentration) of the initial concentrated HbO_2 solution was added and the protein coagulation rate was then measured turbidimetrically at 760 nm for 20 min. The initial HbO_2 concentration in the reaction mixture was always 1×10^{-4} *M*

(for tetramer). In the experiments with the addition of supplementary Tris at the fifth minute, the initial volume of reaction mixture was less than 3 mL by a portion depending on the volume of the concentrated Tris-AcOH solution to be added. All the experimental and approximation curves were plotted using the Easy Plot program, which was also utilized for mathematical treatment of the experimental curves.

Results and Discussion

In one of our previous papers devoted to the kinetics of human HbO_2 coagulation *(12)*, we suggested that the coagulation initiated by the mercurization of the reactive hemoglobin thiol groups was directly caused by the coordination of the Hg(II) atom with the carboxylate group of one of the neighboring aspartic acid residues, most likely Asp β 94. This suggestion was supported by the effect of the iodide ion, which, when binding to mercury atom, should affect the interaction of the latter with the aforementioned carboxylate group. Now, we have studied the effect of some efficient mercury ligands on the kinetics of coagulation of the horse HbO_2 with the mercurized reactive thiol groups. The first ligand studied was that amine component of the Tris buffer widely used in numerous biochemical work. Tris is known to form 2:1 complexes with Hg(II) compounds *(14)* and, in our own investigation, formation of a weak 3:1 complex has also been suggested *(15)*. The kinetic curves of coagulation of the HbO_2 mercurized by double molar excess of mercuric acetate in the buffers with varied Tris concentration (pH 7.2) are presented in Fig. 1. The series of curves enables evaluating the stoichiometry and the Tris-Hg binding degree when suggesting an inverse dependence of the coagulation rate on the number of Tris ligands bound to mercury. We usually approximated kinetic curves by the second-order kinetic equation, which, in our case, was expressed in the terms of Eq. 1, where D is the measured apparent optical density at 720 nm (in fact, light scattering) characterizing the coagulate concentration; D_{max} is the maximum attainable D value (computed) characterizing both the maximum amount of formed coagulate and the corresponding amount of the protein taking part in the coagulation process; k is the second-order kinetic constant in conventional units; and t is time in minutes.

$$D = (D_{max})^2 kt / (1 + D_{max} kt) \quad (1)$$

Parameters found from Eq. 1 allow the initial rate of the coagulation to be calculated in terms of Eq. 2:

$$v_o = k(D_{max})^2 \quad (2)$$

Figure 2 demonstrates the dependence of v_o value on the Tris concentration. If we assume the inhibition of coagulation to be due only to combining Tris with the mercaptide mercury, we are able to calculate the number of Tris ligands needed to completely suppress the coagulation

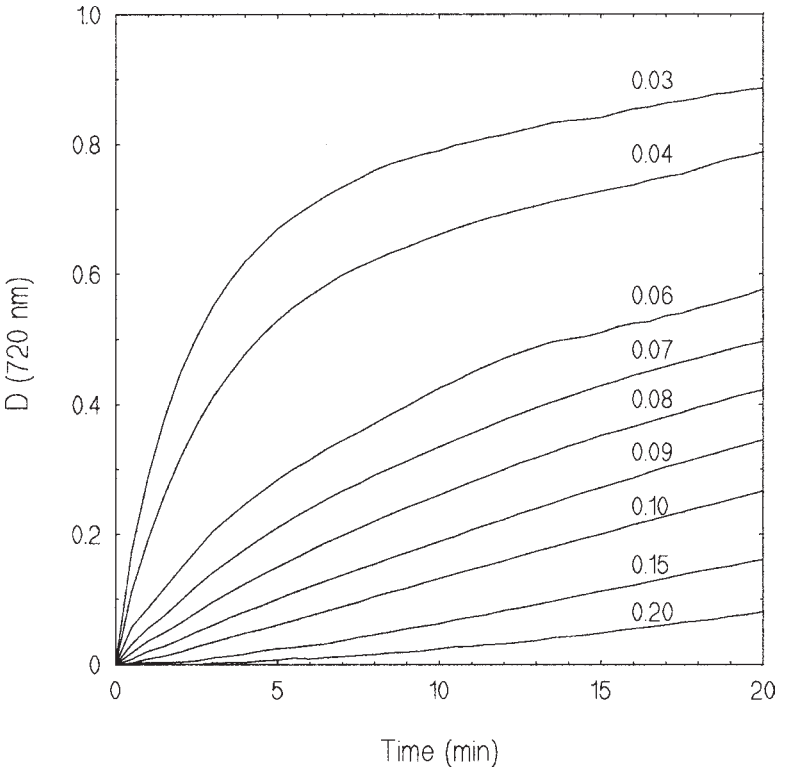

Fig. 1. Kinetic curves of coagulation of HbO_2 mercurized by mercury(II) acetate in reactive thiol groups in Tris-AcOH buffer with varied buffer concentration at 30°C. $(HbO_2) = 10^{-4}$ *M* (for tetramers), $[Hg(OAc)_2] = 2 \times 10^{-4}$ *M*. Numbers on curves correspond to Tris concentration, *M*.

process. In this case, we also have to accept the following idealized picture: protein component of the solution is composed of coagulation-active and coagulation-passive protein molecules. The former are those in which mercury atom preserves some coordination vacancies. In the latter, such vacancies are lost because of the formation of the maximum number of the Tris-mercury coordination bonds with the thiol sulfur and external ligands. We omit here all reasons, special experiments, and mathematical manipulations that allowed us to draw the conclusion that two Tris ligands bound to mercaptide mercury completely suppress the coagulation activity of the mercurized hemoglobin. When phenylmercury residue was used to initiate the protein coagulation, only one Tris ligand on mercury atom fully inhibited the coagulation process.

Much stronger mercury ligands, ethylenediaminetetraacetic acid (EDTA) and thiourea, have also been investigated. In Fig. 3, the dependence of the initial coagulation rate is plotted vs x = (EDTA)/(Hg) molar ratio. The curve (curve 1) cannot be satisfactorily approximated by Eq. 3 in which v_o depends on the n^{th} power of the portion of the active mercurized

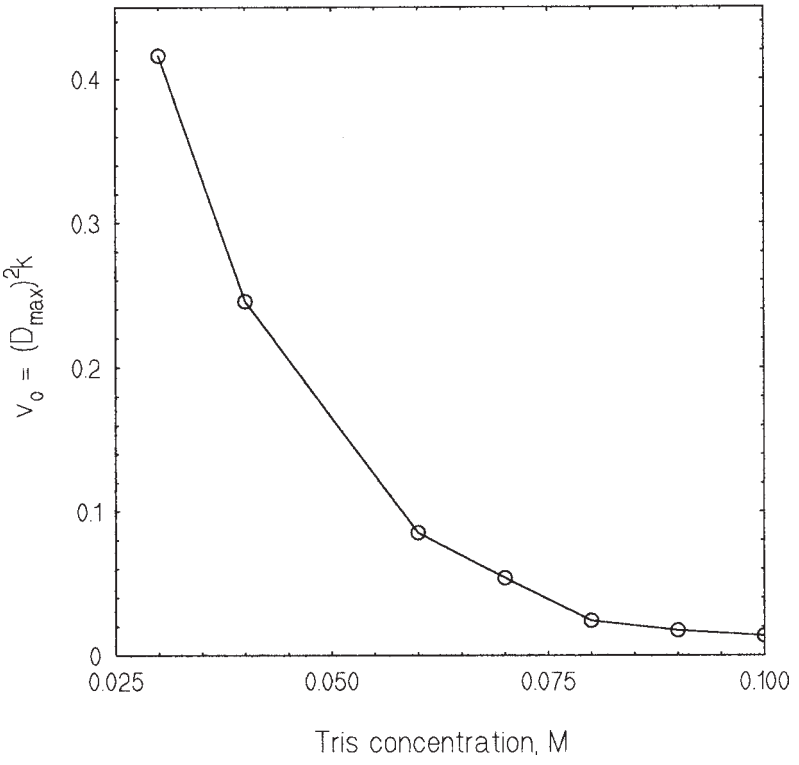

Fig. 2. Dependence of the initial mercurized HbO_2 coagulation rate, calculated for curves in Fig. 1 using Eqs. 1 and 2, on the Tris concentration.

HbO_2 dimers. However, Eq. 3 appears to be the most reasonable form to describe the process within x range from 0 to 1.

$$v_o = (v_o)_{max}(1 - x)^n \quad (3)$$

So we divided the curve into three sections, the two extreme ones being further computed in terms of Eq. 3 to give n = 6.95 and 3.87 for the initial and end sections, respectively. When comparing the second approximation curve (curve 3) with curve 4 obtained by varying the total mercurized HbO_2 concentration (100% at $x = 0$ and zero at $x = 1$), we see that binding of the last part of the mercaptide mercury by EDTA affects v_o the same way as the total loss of the mercurized protein. On the contrary, small amounts of EDTA added exhibit an abnormally large effect. For example, when only one-tenth part of mercaptide mercury is bound, the initial coagulation rate is reduced more than twice, and when each twentieth mercury atom is bound, v_o value drops by some 40%. In a word, quite a small alteration in the mercurized protein system results in a marked kinetic effect. Such an effect supports the cooperative mechanism of the mercurized hemoglobin coagulation we suggested in ref. *13*, where the similar effect of the iodide ion has been observed. A steep dependence of the HbO_2 coagulation rate on the small EDTA additives also suggests that EDTA not only

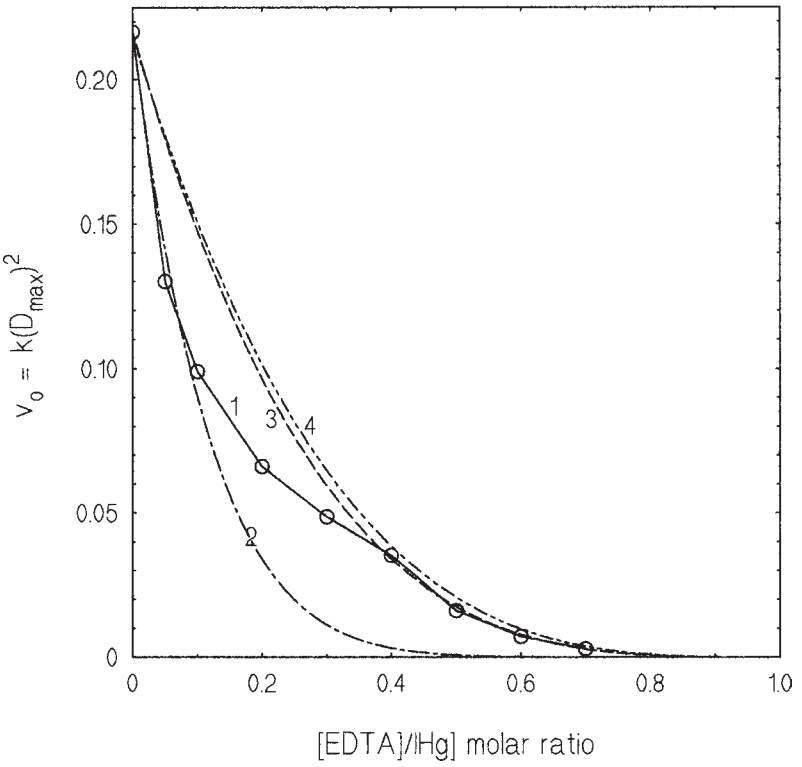

Fig. 3. Dependence of the initial mercurized HbO_2 coagulation rate on EDTA/mercury molar ratio (curve 1) and approximation curves for initial and end sections of the experimental curves (2 and 3) (more detail in the text) plus curve 4 obtained when varying the total mercurized HbO_2 concentration (100% at $x = 0$ and zero mercurized HbO_2 concentration at $x = 1$).

simply lowers the concentration of the coagulation-active protein, but gives rise to specifically modified protein molecules that they themselves inhibit the coagulation of the rest of mercurized protein molecules. According to our ideas, the reason behind this phenomenon lies in that, in the primarily formed one-dimensional higher oligomers, conformationally different dimers are incorporated that constitute a kind of "dislocation" in the regular oligomer chain thereby creating unfavorable conditions for protein aggregation. It is also interesting that up to 20 mol % of EDTA does not affect the D_{max} value, i.e., does not probably reduce the total amount of the aggregated protein. Larger amounts of EDTA, however, do so. It seems that up to 20% of the EDTA-modified mercurized HbO_2 molecules incorporated in the higher oligomers inhibit coagulation activity of these oligomers but do not suppress it completely, whereas a larger extent of the higher oligomer modification makes them incapable of aggregation.

Thiourea is a considerably weaker ligand for mercury(II) but its affinity for mercury is still high enough to consider the 1:1 Hg-thiourea interaction to be stoichiometric with a probability of the second thiourea ligand

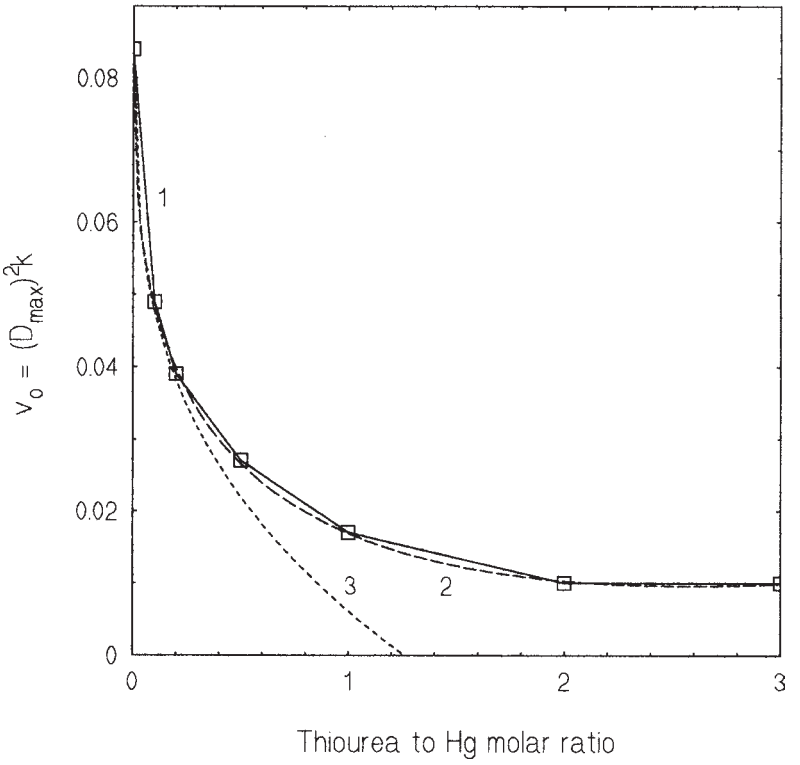

Fig. 4. Dependence of the initial mercurized HbO_2 coagulation rate on the thiourea/mercury molar ratio (curve 1) plus curve 2 approximating the experimental curve by Eq. 4 (computed $a = 0.07939$, $b = 0.32155$, $c = 0.01029$, $d = 1.1741$) and curve 3 obtained when using Eq. 4 with computed a and b parameters and cx^d term omitted.

binding *(16)*. Figure 4 demonstrates the thiourea effect in a similar way as it was for EDTA. It is obvious that small amounts of thiourea inhibit the coagulation process almost like EDTA and so we conclude that thiourea-modified mercurized HbO_2 dimers take part in the coagulation process being incorporated into the primary one-dimensional higher oligomers. However, as we can see in Fig. 4, higher thiourea concentrations are not so harmful for the protein coagulation as compared to the same EDTA concentrations. We formally approximated experimental curve in Fig. 4 by Eq. 4, where x is the thiourea to mercury molar ratio, into which we specially introduced two components depending on x to an arbitrary power.

$$v_o = v_o(D_{max}) - ax^b + cx^d \quad (4)$$

The computation enables finding optimal values of the parameters in Eq. 4 corresponding to curve 2 in Fig. 4. This curve has a minimum at $x \approx 2.5$. An additional experiment showed the type of Eq. 4 to be chosen successfully: with further augmentation of x value, the coagulation rate really increases. Thus, the pattern of the curve 1 is caused by not only the inhibitory effect of thiourea but simultaneously by a coagulation-acceler-

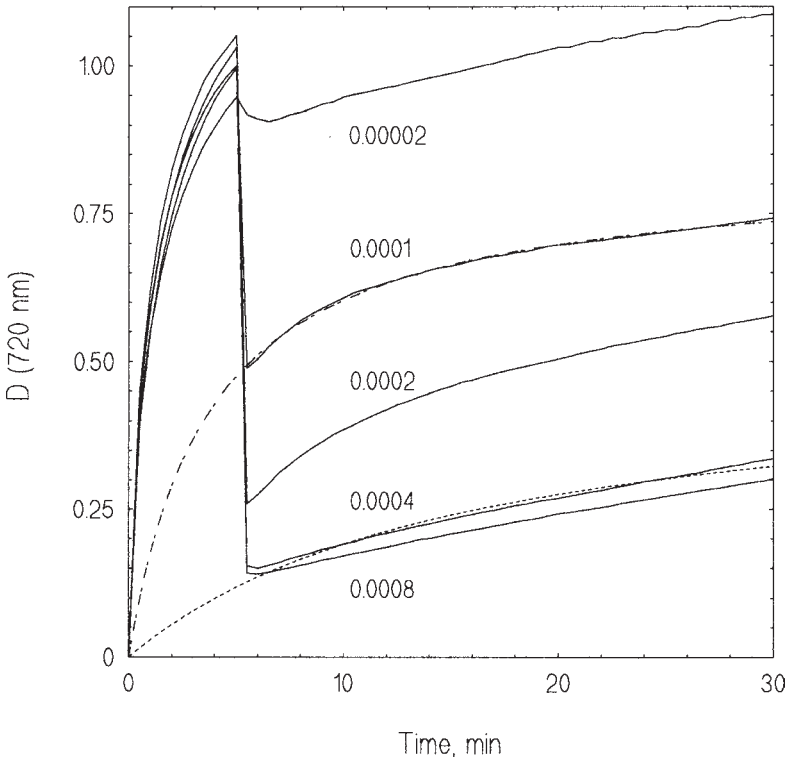

Fig. 5. Kinetic curves of phenylmercurized HbO_2 coagulation involving addition of varied thiourea amounts 5 min after the beginning of coagulation process. Numbers on the curves correspond to thiourea concentration × 10^4 *M*. Dashed sections are used to extrapolate delayed thiourea-inhibited coagulation process to zero point in accordance with Eq. 1 (second-order kinetics).

ating factor associated with the presence of thiourea. Curve 3 in Fig. 4 corresponds to Eq. 4 without cx^d component (acceleration) and it is thought to describe the pure coagulation inhibiting effect of thiourea. It is remarkable that this curve intersects the *x*-axis close to $x = 1$, i.e., to the point at which all mercury atoms stoichiometrically bind one thiourea ligand. We, therefore, may conclude that the accelerating effect of thiourea is largely associated with binding of the second thiourea ligand with mercury or with a possible interaction of thiourea with other thiourea-binding protein sites.

According to our custom experimental technique, the concentrated HbO_2 solution was added to preliminarily prepared mixtures of the mercury reagent with EDTA or thiourea in Tris buffer. Some apprehension may appear concerning possible kinetic complications in the interaction of the liganded mercury reagent with the protein thiol sulfur. To avoid this apprehension, an experimental series was carried out in which thiourea was added to the system with coagulated (for 5 min) phenylmercurized HbO_2. Figure 5 demonstrates a qualitative conformity of the delayed thiourea effect to that already described. Furthermore, we computed right-hand

branches of the three kinetic curves followed by the addition of thiourea in terms of Eq. 1 for the second-order reactions. It appears that, after the fast thiourea-induced coagulate-dissolution process, coagulation proceeds in accordance with the normal second-order kinetics. In similar experiments with EDTA, after sufficient EDTA amount was added, coagulate dissolution step is not followed by a further protein coagulation.

In conclusion, it is of major importance to underline that an essential macroeffect as the protein aggregation with the separation of isolated phase is caused by an apparently insignificant event as the binding of only two metal ions to sulfur of the two reactive thiol groups that are not buried in the protein globule *(8)*. This effect, allosteric in its nature *(17)*, may be compared, with regard to its scale, with the classical allosteric effect caused by the binding of molecular oxygen to heme iron of hemoglobin *(18)*. It is of no less importance, as we have found in this and previous works *(4,12)*, that the mercury effect is not due to a simple substitution of the thiol hydrogen by a heavy metal atom but to the presence of free coordination vacancies in mercury atom, which can be controlled by adding reagents with a sufficiently high affinity for Hg(II). Hence, we believe that the therapeutic effect of the reagents commonly used in the mercury intoxication treatment is achieved not only when a reagent takes away the mercury ion bound to enzymes and other physiologically important proteins and withdraws it from the organism, but already when a reagent only coordinates with the protein-bound mercury thereby decreasing the protein-perturbation effect of the latter. Thus, the suppression of the unfavorable effect of mercury on the proteins involving reduction of the number of coordination vacancies in mercury can be regarded as a component in the mercury intoxication therapy.

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