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The Influence of Low- and High-Molecular Chelating Additives upon the Reduction of Nitrotetrazolium Blue to Formazane in the Presence of *d*-Metal Cations

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Abstract—The influence of high- and low- molecular additives upon the reduction of nitrotetrazolium blue to formazane in the presence of *d*-metal cations was studied. It was shown that the introduction of chelating additives did not essentially change the earlier found regularities in the variation of anti- and prooxidant activity within the range of *d*-metal cations. In most cases the observed effects may be understood in the terms of the theory of hard and soft acids and bases.

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Ions of transition metals in contact with biological objects not only become involved into redox processes [1–6] but also undergo the complex formation [6–8] because both the main monomer units of biomolecules like monosachharides [9, 10], amino acids, nitrogen bases [11], and biopolymers themselves (nucleic acids, homo- [12] and heteropolysaccharides [13], phospholipids and proteins [14]) show the pronounced tendency to complexation with *d*-metals.

Ions of Pb^{+2}, Hg^{+2}, Co^{+2}, Cd^{+2} are known to form strong complexes with amino acids and other biomolecules [15]. Most of metal complexes with organic ligands are close in their parameters (size, charge distribution) to the traditional substrates of the main metabolic processes (amino acids, phosphorylated carbohydrates) and key regulating molecules such as hormones and neuromediators and therefore the complxes are able to bind with appropriate receptors (the effect of mimikria) [16, 17]. For example, the complex of mercury ion with a cysteine molecule imitates methionine which is necessary for the initiation of protein synthesis and adrenaline [18].

Heavy and transition metals are also able to substitute biometals in biocomplexes. Such substitution results in the loss of their biological activity [19].

For instance, because of the substitution of zinc ion in the molecules of carboanhydrase and aminolevullinate dehydratase, enzymes taking part in the synthesis of heme, for mercury or lead ions their deactivation occurs leading to drastic disorders in metabolic processes [18, 20].

The complex formation leads to pronounced deviations in redox properties of *d*-metal cations, cations in higher degrees of oxidation becoming more stable [21] which may crucially alter the nature of their influence upon the reactions with active oxygen species (AOS) resulting in the changes of redox equilibrium of the system *in vivo*. The overall redox equilibrium of the living object is characterized by the ratio of anti- and prooxidants.

The reaction of nitrotetrazolium blue reduction to formazane is one of the current test methods for the estimation of anti- and prooxiddant activity of different compounds, solutions of biomolecules, extracts of biomaterials [22]. In practice each of these complex biological solutions always contains metal cations and the understanding of changes in redox properties of metal cations in the presence of chelating additives both in the natural products and their structural and functional analogs plays a crucial role in assessing the variation in the ratio of pro- and antioxidants in the tested biological object because many pathological states are either accompanied with or directly result from the disorders in free radical oxidation processes.

In this connection we carried out an investigation of the influence of *d*-metal cations upon the reaction of

Amino acid composition of gelatin

Run no.	Amino acid forming the peptide chain	Amount of amino acid residues in gelatin molecule
1	Glycine (Gly)	347
2	Proline (Pro)	120
3	Alanine (Ala)	113
4	4-Hydroxyproline (Hyp)	96
5	Glutamic acid (Glu)	74
6	Arginine(Arg)	48
7	Aspartic acid (Asp)	44
8	Serine (Ser)	31
9	Lysine (Lys)	27
10	Leucine (Leu)	24
11	Valine (Val)	22
12	Threonine (Thr)	17
13	Phenylalanine (Phe)	12
14	Isoleucine (Ile)	12
15	Hydroxylysine (Hyl)	5
16	Histidine (His)	4
17	Methionine (Met)	4
18	Tyrosine (Tyr)	1

nitrotetrazolium blue reduction to formazane in the presence of several active chelating agents. Nitrogen-containing compounds were chosen as chelating agents because proteins are the main nitrogen-containing polymers *in vivo* performing the overwhelming majority of the functions in cells whereas low-molecular nitrogen-containing compounds act as enzyme cofactors, neuromediators, and different regulators *in vivo*. As it was mentioned above the chelation drastically changes the oxidative-reductive properties of heavy and transition metal cations. For example, the redox potential of Co^{+2} cation after chelation is so strongly decreased that complexes of Co^{+2} are readily oxidized despite the fact that salts of Co^{+2} are usually very stable in aqueous solutions, whereas Co(III) salts immediately decompose under the action of water with the evolution of molecular oxygen [23].

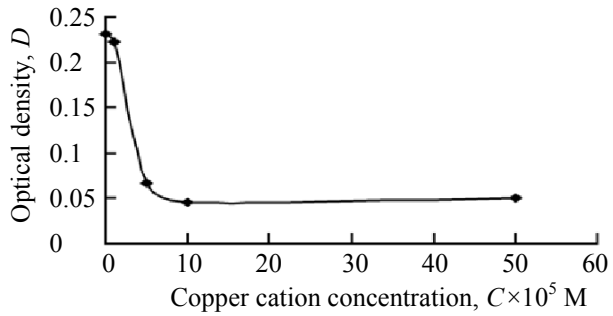

Fig. 1. Dependence of the optical density of the reaction solution on the concentration of copper(II) cation in water. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [EDTA] = 1.2×10^{-6} M, pH = 7.8, τ = 10 min, 20°C, λ = 540 nm.

Ethylenediaminetetraacetate (EDTA) and gelatin protein were tested as nitrogen-containing chelating agents. The ligands were chosen because EDTA is known to be one of the most effective chelating agents [23] and while gelatin is a natural high-molecular ligand simulating the interaction of *d*-metal cations with nitrogen-containing biopolymers *in vivo*.

The feasibility of coordination bonds formation for the molecule of gelatin is provided by the presence of side chains of arginine, lysine as well as nitrogen atoms of the imidazolyl fragment of hystidine.

The abundance of these aminoacids residues in gelatin molecule is illustrated by the table [24]. The side chains of arginine, lysine, hydroxylysine, and hystidine are the most probable centres of chelation.

Using copper(II) cations as an example, both these chelating agents (EDTA and gelatin) were shown not to change the main features of the influence of metal cations upon the reduction of blue nitrotetrazolium to formazane. Earlier Cu^{+2} cation was shown to slow down the process of blue nitrotetrazolium reduction to formazane [22]. When EDTA is added to the reaction mixture, the formazane generation is retarded (Fig. 1).

Gelatin as an alternative chelating agent also does not materially change the influence of copper cation upon the reaction of tetrazole reduction to formazane (Fig. 2).

A simultaneous introduction of EDTA and gelatin into the reaction mixture also does not alter the influence of Cu^{+2} cation on the reaction of tetrazolium reduction to formazane (Fig. 3).

So both in the presence and in the absence of chelating agents copper(II) cation slows down the process of tetrazolium reduction to formazane. The effect of other investigated metal ions like Cd^{+2}, Ni^{+2}, Co^{+2} cations on the tetrazolium reduction to formazan both in the presence and in the absence of chelating additives is the same as the effect of copper cation.

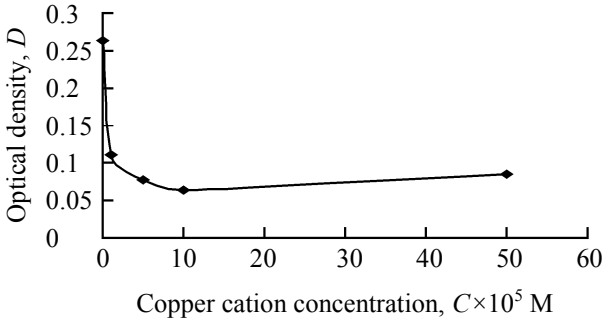

Fig. 2. Dependence of the optical density of the reaction solution on the concentration of copper(II) cation in water. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, pH = 7.8, τ = 10 min, 20°C, λ = 540 nm.

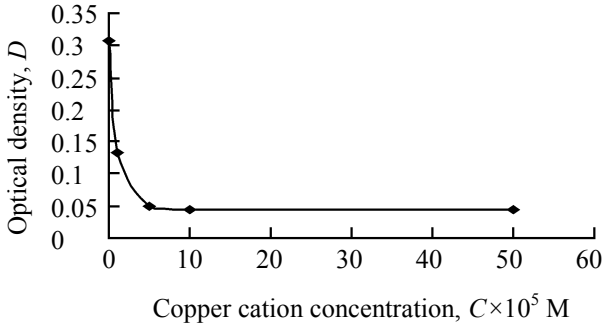

Fig. 3. Dependence of the optical density of the reaction solution on the concentration of copper(II) cations in water. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA = 1.2×10^{-6} M, pH = 7.8, τ = 10 min, 20°C, λ = 540 nm.

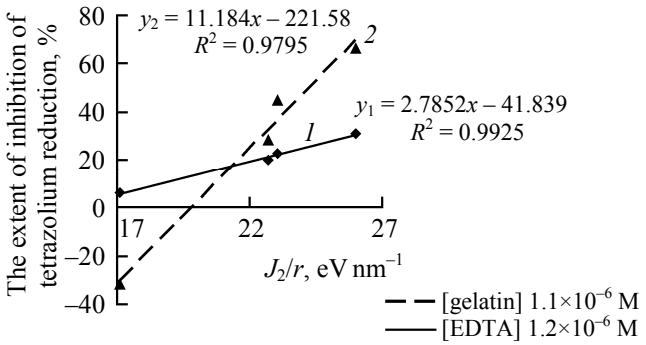

Fig. 4. The influence of different *d*-metals on the process of retardation of nitrotetrazolium blue reduction to formazane. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [Me] = 5×10^{-5} M, pH = 7.8, τ = 10 min, 20°C.

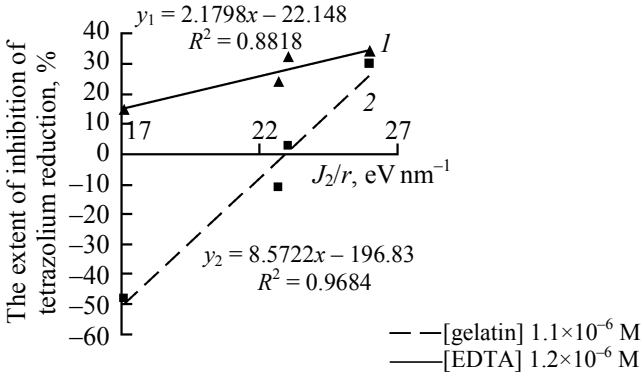

Fig. 5. The influence of different *d*-metals on the process of retardation of nitrotetrazolium blue reduction to formazane. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [Me] = 10^{-4} M, pH = 5.0, τ = 10 min, 20°C.

A linear correlation of the magnitude of the inhibiting effect on the tetrazolium reduction to formazane with the I_2/r parameter is observed, where I_2 is the second ionization potential and r is the cation radius of Me^{+2} (Figs. 4, 5).

The negative value corresponds to the inhibiting effect on the process of reduction of nitrotetrazolium blue to formazane. The linear dependences of the extent of inhibition of the reaction of tetrazole reduction to formazane on I_2/r parameter characterizing the cation are similar in the presence of different chelating additives. The slopes of the dependences however significantly differ which may be rationalized in terms of the different nature of the introduced nitrogen- containing additives.

In the presence of the protein in the reaction mixture the dependence of the inhibiting effect on I_2/r parameter is 4 times stronger. It should be mentioned that the slopes for the dependence of the inhibiting effect on I_2/r parameter characterizing the cation are the same at different pH values of the media (Figs. 4, 5). This fact supports our suggestions on the exclusive influence of the cation nature on the effect being studied.

In the case of Cd^{+2} cation the inversion of the metal ion effect takes place (Fig. 4), viz., at the high cation concentrations the acceleration of tetrazolium reduction to formazane is observed. The magnitude of the accelerating effect at high concentrations of Cd^{+2} significantly depends on the additive nature. In the presence of gelatin in the solution the effect of acceleration increases many times (about 7.6 times) in comparison with the EDTA additive (Fig. 6).

Though both in the presence and in the absence of nitrogen-containing chelating agents the copper cation

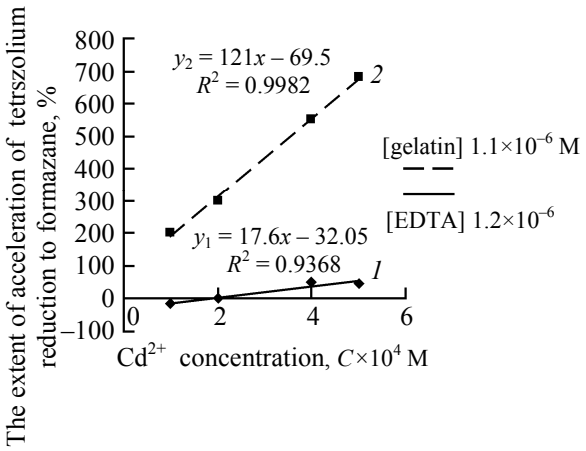

Fig. 6. Acceleration of the reaction of nitrotetrazolium blue reduction to formazane under the influence of cadmium cation in the presence of different additives. [Tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, pH = 5.0, τ = 10 min, 20°C.

retards the process of tetrazolium reduction to formazane the inhibiting effect considerably varies for the given cation in the presence of a chelating additive. Thus the values of the inhibiting effect (relative to the blanc, %) under the effect of copper cation in the absence and in the presence of chelating additives are equal to 49% (without additives), 31% (in the presence of EDTA), and 66% (in the presence of gelatin).

EDTA known to form chelate complex through the carboxy moieties neutralizing the charge of the metal cation reduces the inhibiting effect of the copper cation in the reaction of tetrazole reduction to formazane. This effect may be due to diminishing the effective charge of the cation and lowering its reactivity in the oxidation process:

$$O_2^{\cdot -} + Cu^{+2} \rightarrow Cu^{+1} + O_2. \quad (1)$$

The introduction of a protein, gelatin, into the reaction medium, on the contrary, somewhat increases the inhibiting effect. Such kind of influence of this ligand on the process of nitrotetrazolium blue reduction to formazane is apparently connected with negligible changes of the copper cation charge. The presence of a large number of easily polarized fragments in the protein structure facilitates the distribution of additional electron density during electron transfer from the superoxide radical ion.

The principal difference in the nature of ligands from the point of view of the theory of hard and soft acids and bases (HSAB) should be mentioned. EDTA belongs to the pronounced hard bases group whereas gelatin is a representative of highly polarized soft bases.

In the range of cations Cu^{+2}, Co^{+2}, Ni^{+2} as Lewis acids the hardness falls successively from Cu^{+2} to Ni^{+2} [25]. When a soft base, gelatin, is introduced into the reaction medium the largest effect is observed for Cu^{+2} cation as the softest Lewis acid (the growth of the retardation effect amounts to 43%) and the minimal effect was shown by Ni^{+2} which is known as a hard Lewis acid (the growth of the retardation effect equals 12%).

The influence of EDTA as a hard Lewis base on the retardation of the process of tetrazolium reduction to formazane is less pronounced and does not show any evident relation to the hardness of the cation as a Lewis acid.

Not only the polarizability of chelated cations but also their tendency to the oxidative–reductive transformations characterized by the value of the ionization potential should exert a pronounced influence on the binding of the ligand and consequently on the process of tetrazolium reduction to formazane as it is the case for free cations.

Special attention should be paid to the influence of chelating additives on the process of blue nitrotetrazolium reduction to formazane in the presence of cadmium cation. The introduction of gelatin to the reaction medium results in the reversal of the observed effect: so, the cadmium cation in the presence of gelatin considerably (by 32%) accelerates the process of tetrazolium reduction to formazane. The amplification of the effect in the presence of gelatin is probably connected with its more pronounced ability to form complexes due to the much higher number of centers of coordination in its molecule.

As it was already mentioned, the formation of coordination complexes with *d*-metal cations usually takes place with the participation of nitrogen and oxygen atoms of the ligand. Changes in the close surrounding of the atoms taking part in the coordination strongly influence the structure of the forming chelate and consequently its oxidative–reductive properties that may lead even to the changes of the initial valence of the metal [23].

The comparative affinity of these complex-forming agents towards *d*-metals is unknown, so the investigation of the simultaneous influence of these

additives on the process of blue nitrotetrazolium reduction to formazane was of great interest. In the process of our study it was found that lowering the parameter I_2/r in the case of simultaneous presence of gelatin and EDTA in the reaction medium like the same in the case of introduction of only one chelating compound results in the lowering of the inhibiting effect on the tetrazolium reduction to formazane. The dependence of the inhibiting effect upon the nature of the cation in the nitrotetrazolium blue reduction to formazane in the case of simalteneous presence of EDTA and gelatin in the reaction mixture is given below: [tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA] = 1.2×10^{-6} M, [Me] = 5×10^{-5} M, pH = 7.8, τ = 10 min, 20°C.

Cation	Cu^{+2}	Co^{+2}	Ni^{+2}	Cd^{+2}
Percent of retardation, %	48.5	33.7	29.5	8.5
I_2/r, eV/nm [29, 30]	26.013	23.040	22.681	17.078

In this case the effect of retardation of the tetrazolium blue reduction to formazane under the influence of *d*-metal cations (Me^{+2}) (parameter *y*) in the presence of the mixture of chelating additives is proportional to the value I_2/r (parameter *x*) characterizing the redox properties of the cation, and it may be described with the help of the equation below under the following conditions:

$y = 4.40x - 67.65$; $R^2 = 0.9867$; : [tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA] = 1.2×10^{-6} M, [Me] = 5×10^{-5} M, pH = 7.8, τ = 10 min, 20°C.

The slope of the dependence of the retardation effect on the value of I_2/r however, significantly differs from the values observed in the case of the presence of only one chelating agent. The slope for the dependence tan $\alpha = f(I_2/r)$ in the presence of different chelating agents is given below: [gelatin] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA] = 1.2×10^{-6} M, [Me] = 5×10^{-5} M, τ = 10 min, 20°C, pH 7.8 (a), 5.0 (b).

Conditions	without additives	[EDTA] = 1.2×10^{-6} M	[gelatin] = 1.1×10^{-6} M	[gelatin] = 1.1×10^{-6} M [EDTA] = 1.2×10^{-6} M
tan α (a)	4.985	2.785	11.184	4.400
tan α (b)	2.428	1.226	4.248	1.420

It should be mentioned that the effect exhibited by various chelating agents significantly depends on pH value of the reaction medium.

Indeed, at pH 5.0 the effect of retardation of the tetrzolium reduction to formazane turned out to be considerably greater in comparison with the effect of retardation in the neutral medium, though the influence of oxidative–reductive characteristics of the cation was negligible. The influence of the oxidative–reductive properties of the cation on the effect of retardation of the reaction of tetrazolium reduction to formazane under the conditions of the simultaneous presence of EDTA and gelatin is shown below: [tetrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA] = 1.2×10^{-6} M, [Me] = 5×10^{-5} M, pH = 5.0, τ = 10 min, 20°C.

Cation	Cu^{+2}	Co^{+2}	Ni^{+2}	Cd^{+2}
Percent of retardation, %	70	66.7	67.8	57.7
I_2/r, eV/nm [29, 30]	26.013	23.040	22.681	17.078

The weak effect of the cation nature on the process of reduction of nitrotetrazolium blue to formazane in the presence of chelating additives in low acidic medium (pH 5.0) may be demonstrated by means of the correlation equation relating the inhibiting effect in the tetrazolium reduction to formazane to the redox characteristics of the cation (I_2/r) which has a very small slope (1.42) under the given below conditions: $y = 1.42x + 34{,}02$; $R^2 = 0.9576$; [tatrazolium] = 4.2×10^{-4} M, [FMS] = 2.8×10^{-6} M, [$NADH_2$] = 1.16×10^{-4} M, [gelatin] = 1.1×10^{-6} M, [EDTA] = 1.2×10^{-6} M, [Me] = 5×10^{-5} M, pH = 5.0, τ = 10 min, 20°C.

The influence of pH of the reaction medium on the inhibiting effect is not unexpected. Evidently the variation of the effect is connected with the changes in the charge of the chelating additive.

At pH 5.0 the guanidine fragment is completely protonated (pK_a 12.5) [26], the imidazole fragments of hystidine are mainly neutral (pK_a 6.0) and the considerable fraction of carbonyl groups of glutamic and aspartic acids are ionized (pK_a values are equal to 4.3 and 3.9 [26], respectively). At pH 5.0 the acetic fragments of EDTA are electroneutral.

Under such conditions the complexation between metal cations and the ligands such as proteins and EDTA in protonated forms is hampered because of the electrostatic repulsion between metal cations and protonated ligands.

In this connection the influence of the chelating additives on the rate of the nitrotetrazolium blue reduction to formazane in acidic media in practice is not observed. In neutral and low alkaline media at pH 7.8 the influence of chelating additives on the process of nitrotetrazolium blue reduction to formazane is highly pronounced that is supported by the changes in the slope of dependence tan $\alpha = f(I_2/r)$ in the presence of EDTA, gelatin, and their mixtures.

The maximum value of the slope is observed in the presence of gelatin which is the additive with distinct chelating properties. In the presence of EDTA the slope of the dependence tan $\alpha = f(I_2/r)$ is minimal. Therefore the EDTA as a chelating additive when coordinating with *d*-metal cations, totally levels off the effect of the cation nature on the process of tetrazolium reduction to formazane. Simultaneous presence of EDTA and gelatin lowers the value of the slope of the dependence tan $\alpha = f(I_2/r)$ in comparison with the same in the presence of gelatin as a sole additive.

Consequently gelatin may be treated as a differentiating additive, contrary to EDTA as a leveling additive relative to the influence of d-cations on the process of reduction of tetrazolium to formazane. Coordination with soft ligands increases the influence of the *d*-metal cations on the nitrotetrazolium blue reduction to formazane. So it may be expected that in biosystems the influence of *d*-metals on the processes of free radical oxidation involving AOS will grow and the introduction of soft, readily polarized ligands will enhance this effect.

EXPERIMENTAL

The following reactants were used in this work: a solution of nitrotetrazolium blue, Fluka, PFA grade, 2.8×10^{-3} M; a solution of phenazine metasulfate (PMS) Fluka, PFA grade, 9.2×10^{-5} M; a solution of nicotinamide phosphate ($NADH_2$), Fluka, PFA grade, 1.9×10^{-3} M, in tris-EDTA buffer system; a solution of food-stuff gelatin, a solution of dimethyl sulfoxide, "chemically pure" grade, 13.2 M; the solution of ethylenediaminitatraacetate, "chemically pure" grade, 2.0×10^{-5} M and a phosphate buffer solution, pH 7.8.

The experiments were carried out in the following way. The blanc and experimental samples were prepared simultaneously and contained 2.0 ml of buffer, 0.5 ml of tetrazolium solution, 0.1 ml of PMS solution, 0.2 ml of distilled water, 0.2 ml of $NADH_2$ solution. These reagents were added to the control sample in the consecutive order. The stop-watch was immediately started and the samples were kept in darkness at a constant temperature. In 10 min. period the optical density was measured at 540 nm against the buffer solution.

The experimental sample was treated in the same way but 0.05–0.2 ml of the solution of investigated metal salt was added instead of distilled water. The free volume was filled with distilled water.

The chelating additives were added to the reaction mixture before the introduction of the metal salt. The total volume of the solutions of metal salt and chelating additive did not exceed 0.2 ml. When the total volume of these two reagents was less than 0.2 ml, the free volume was filled with distilled water.

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